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Fungi with Cellulolytic Potential: Screening, Inoculum, and Methodology for Isolation

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Abstract**—***The selection of microorganisms with the greatest potential of composer enables to increase efficiency and reduce the time of the composting process. The isolation of fungi has received special attention because they are the main responsible for the degradation of substances of difficult decomposition during the composting process. This review presents a survey of fungi with cellulolytic enzymatic potential, the methods of isolation and screening of these fungi and the methods for determining the potential enzymatic assay. The fungi that decompose rich cellulose residues are the main decomposing agents of ruminal residue, have a diversity of enzymes with degradation capacity of complex organic compounds, such as cellulose, hemicellulose, acids aromatics, and some proteins. Thus, with the indication of fungi that increase the composting efficiency, specific glasses can be developed for the transformation of this type of residue.*
Keywords *— **enzymatic activity, cellulases, cellulose degradation.***

I. INTRODUCTION

Organic residues are considered one of the main habitats for the population of microorganisms. Fungi are found in communities ranging from 10^4 the 10^6 microorganisms per gram, actively participating in biodegradation processes, contributing to nutrient cycling and, consequently, to the maintenance of ecosystems [1].

Agroindustry sectors produce a large amount of waste, that, if not availed adequately, represent losses of high value biomass and nutrients, in addition to elevating the final prices of the products. This is due to the treatment, transport and final disposal of residues that directly influences the cost of the process. Nowadays, concepts of recovery, utilization of by-products and bioconversion of waste are increasingly widespread and necessary to ensure the economic viability and biocompatibility of agroindustry chains, as well as reduce the environmental impact that the accumulation of these wastes causes [2-4].

The large residue availability, such as bovine ruminal, stimulates the search for efficient microorganisms. The transformation of cellulose residues can be performed by cellulolytic fungi, important organisms during the thermophilic phase of the aerobic composting [5]. Composting can be accomplished by the bioconversion of lignocellulosic polymers [6], means of microorganisms and their corresponding enzymes [7, 8]. The process involves the synergistic action of a cellulolytic complex of fungi formed by endoglucanases, exoglucanases, and *β*-D-glucosidases [9, 10].

Cellulolytic fungi include species of the genera *Trichoderma* e *Aspergillus* [11] as *Trichoderma reesei* E. G. Simmons, *T. koningii* Oudem., *T. lignorum* (Tode) Harz, *Aspergillus nigerniger* Tiegh and others like

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Sporotrichum pulverulentum Novobr., *Penicillium funiculosum* Thom, *P. iriensis* Boretti, Garofano, Montec. & Spalla, *Schizophyllum spp., Chaetomium spp. and Humicola spp*. [12]. The degradation of residues by the action of fungi can result in different products of high added value such as biofuels, chemicals, enzymes, biofertilizers, among others [13, 14].

The interest in fungi is due to its ability to decompose different substrates, which can generate products or processes, such as the isolation, purification, characterization, and cloning of enzymes capable of degrading compounds consisting of cellulose, hemicellulose, and lignin [15-18].

II. MATERIALS AND METHODS

The methods used for this study were based on the methodology of the article experimental planning factorial: A brief review de DE OLIVEIRA, et al. (2018) [19]. Therefore, this bibliographic review sought to present the fungi species with cellulolytic potential, to demonstrate the forms of isolation, to determine the enzymatic potential and to perform the screening of fungi with this potential.

III. RESULTS AND DISCUSSION

3.1 CELLULOLYTIC FUNGI

Filamentous fungi are usually found in decaying soils and wood [20]. They have been used as a source of production of many metabolites and enzymes, being commercially exploited as "factories" of enzymes. Most commercial cellulases are produced by fungi, mainly those belonging to the genera *Trichoderma* e *Aspergillus* [14].

Cellulolytic fungi, which decompose cellulose substances, occur in the soil and colonize the vegetables, the roots, and their residues, with an important function of nutrient recycling. The fungal activity depends on the organic matter content in the soil, which determines the occurrence and distribution of these organisms [21]. They are the main decomposing agents of the plant material since they possess an enzymatic arsenal with degradation capacity of complex organic compounds, such as cellulose, hemicellulose, aromatic acids, and some proteins.

The fungi have a number of characteristics that make them interesting for application in waste processing systems. They are able to grow under environmental stress conditions, which limit bacterial growth. Moreover, the way of the growth of fungi, induced chemo statically towards the source of organic carbon through the stretching and branching of hyphae, allows the colonization of large areas [22].

They are considered the most adapted microorganisms for waste transformation because their hyphae can grow on the surface of the particles and penetrate the spaces intraparticle [23]. After the germination of the spores, the fungus hyphae develops in a mycelial tangle, which can be projected, forming air hyphae that penetrate the residue the accumulation of hyphae forms the mycelium, which has the ability to break the cutinized surface of the plant tissue, penetrating the mesophyll, promoting fungal growth [24]. The empty spaces between the air hyphae are often filled by airing, while the empty spaces between the mycelial tangle and particles are filled by water. The metabolic activity occurs mainly near the surface of the substrate and between the pores; however, exposed regions of the mycelium (aerial hyphae) also demonstrate metabolic actions and serve as carriers of substances for the penetrative hyphae [14, 22].

Hydrolytic enzymes are produced by mycelium, diffusing to the solid matrix and catalyze the degradation of macromolecules in smaller units. During this catalysis, oxygen (O_2) is consumed and carbonic gas (CO_2); water (H_2O), Heat and biochemical metabolites are produced. Therefore, gradients develop within biofilms that, for example, force the O_2 to spread to the gaseous phase in deep regions of the biofilm and the CO_2 for gaseous regions [25]. Heat generation causes an increase in temperature to occur rapidly, which is a serious problem during solid state fermentation (FES) and can be removed from the substrate by conduction and evaporation of water. The balance system of the H_2O also includes the H_2O slowly passing through the mycelium. Another important factor is the pH site, which is altered due to the release of carbonic acids and exchange of ammonia [23].

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substrate via water conduction and evaporation. The balance system of the H_2O also includes the H_2O slowly passing through the mycelium.

Another important factor is the local pH, which is altered due to the release of carbonic acids and ammonia exchange in addition to the secretion of enzymes, which are critical to the decomposition of cellulose, the fungal growth is accomplished by the formation of mycelium and have the ability to break down the cutinized surface of the plant, penetrating the mesophyll [24].

Lignocellulolytic fungi are classified as white degradation and brown degradation. White degradation fungi decompose all wood polymers, including lignin, leaving wood with a white and fibrous aspect [26]. They degrade lignin thanks to the ability conferred by extracellular enzymes such as lignin peroxidase, manganese peroxidase, and laccase, defined as phenol oxidases [24]. Among the white degradation, fungi are *Phanerochaete chrysosporium* Burds, *P. carnosa* (Burt) Parmasto, *Ganoderma lucidum* (Curtis) P. Karst; *Irpex lacteus* (Fr.) Fr., *Pleurotus sapidus* Sacc and other [16, 22]

The fungi of brown degradation, these degrade preferentially the cellulose and cause a rapid decrease in the degree of polymerization [27]. The enzymatic hydrolysis of cellulose in glucose involves the synergistic action of at least three different enzymes: Endoglucanase, exoglucanases, and glycosidase [28]. Endoglucanases randomly cleat the amorphous internal sites of the cellulose polysaccharide chain, originating oligosaccharides of various lengths; Exoglucanases act in the reduction or non-reducing terminals of the cellulose chains, releasing glucose or cellobiose; and glucosidase hydrolyzed cellobiose and glucose soluble oligosaccharides [29]. The synergism between these three types of enzymes makes possible the effective degradation of cellulose [10]. As a result of the initial attack of the brown degradation fungus, causing the depolymerization of cellulase, the rigidity of the wood decays rapidly [30].

The microorganism to be considered ideal for enzymatic production process, according to EL-MANSI et al. (2018) and SAHOO et al. (2019) [25, 3] should present the following characteristics: Be safe from the biological point of view, i.e., not be pathogenic; present high capacity for synthesis and excretion of the enzyme; withstand adverse environmental conditions related to osmotic pressure a temperature be tolerant to the presence of toxic substances, which can be generated in the treatment process of the raw material or by the cellular metabolism itself.

Among the most cited brown degradation fungi in cellulase production are *Aspergillus niger* e *Trichoderma reesei* [18, 31, 32]. Most commercial cellulases are produced by fungi, mainly those belonging to the genera Trichoderma e Aspergillus.

3.2 *TRICHODERMA REESEI* E. G. SIMMONS

Many species of this genus are mycoparasites, predominating in the soil microbiology of different ecosystems, such as fields, pastures, forests, and even deserts adapted to survive in various climatic zones. The genre Trichoderma it has species capable of producing enzymes and/or attacking and inhibiting other fungi and attracts great attention in several research areas, such as biological control of plant diseases, enzyme production, as well as studies genetic and manipulation in filamentous fungi are usually found in decomposing soils and woods [20]. Many species of this genus are mycoparasites. Trichoderma predominates in the soil microbiology of different ecosystems, such as fields, pastures, forests and even deserts adapted to survive in various climatic zones.

Trichoderma reesei is one of the main producers of cellulases, and the microorganism whose cellulases system has been more studied [17, 18]. It produces at least six endoglucanases and two active cellobiohydrolases in different sources of cellulose, crystalline or amorphous [33].

Of the most well studied cellulosic systems, cellobiohydrolases, particularly CBH I, have been considered as essential enzymes for the efficient saccharification of cellulose. Experimental evidence indicates that the removal of the gene encoding for the production CBH I reduces in 70% the activity on crystalline cellulose. In addition, cellobiohydrolases have great synergistic interaction with other cellulases, especially those of known endoglucanase activity [34].

3.3 *ASPERGILLUS NIGER* TIEGH

The genre Aspergillus is highly aerobic and comprises several hundred species found worldwide [35]. Regarding the influence of temperature and water activity, they are able to grow in the temperature range of 6 ◦C the 47 ◦C, with an optimum temperature between 35 – 37 ◦C [36]. *Aspergillus nigeris* a fungus widely used in the production of citric acid and also in the production of enzymes. The ability of these fungi to produce enzymes is related to the ability to use a wide variety of substrates thanks to its well developed enzymatic system [37]. They secrete large amounts of cellulolytic enzymes and, along with the *Trichoderma reesei*, have been extensively studied for the industrial production of these enzymes [37, 38]

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3.4 PENICILLIUM ECHINULATUM E. DALE

The genre Penicillium is among the microorganisms with great potential for the production of cellulases [40]. The mutant strains used in these studies are from the wild lineage called 2HH [41].

The importance of the enzymatic complex of *Penicillium echinulatum* it is also due to the fact that it presents a balanced proportion of activities of FPA (total cell activity) e *β*-glycosidase, a fact relevant to the hydrolysis of cellulose. Additionally, FPA e *β*-glycosidases of *P. echinulatum* are important for cellulose hydrolysis because the cellulases produced have thermal stability between 50 ºC and 55 ºC, respectively [41, 42]

Schneider et al. (2014) [43] studied the effect of six different carbon sources on the morphogenesis and enzymatic production of the lineage S1M29 of *P. echinulatum*. Among these sources, pulp and sugar cane bagasse were the most suitable for FPA, endoglucanases, xygenase, and *β*-glucosidases. With regard to the growth morphology of *P. echinulatum*, higher enzymatic activities were observed when the mycelium grew in a dispersed form, a possibly explained correlation due to greater interaction between the substrate and hyphae.

3.5 GANODERMA SPP.

It is a fungus that is present in natural habitats all over the world. More than 250 species have been reported [44]. Regarding the production of lignocellulolytic enzymes, they found that the proteins produced by the fungus *Ganoderma lucidum* (Curtis) P. Karst with 24% correspond to cellulases and 5% to hemicellulases, in addition to 24% of lignin degradative enzymes, indicating this fungus as a producer of lignocellulolytic enzymes. Others of the same genus are also but in smaller quantities. In addition, they verified that the species produces the complete set of cellulases.

3.6 CELLULOLYTIC FUNGI AS A SOURCE OF INOCULUM

The composting process can occur naturally with the involvement of microorganisms present in organic residues. However, insufficient quantity or low biodegradability of the native microbial community may lead to low composting efficiency and undesirable quality of the compound [45]. In order to achieve a reduction in waste transformation time, inoculation is indicated as an efficient technique, as it introduces a population of microorganisms to initiate and accelerate the decomposition process of the organic residue [46]. The inoculum would, therefore, be a way to increase in number and diversity the microbial community of the composting windrows, besides being able to direct to the degradation of a specific residue and/or to ensure more complete degradation of the components of composting [3, 47].

Inoculation consists of the addition of microorganisms from pure culture, or mass inoculation consisting of a large number of organisms, usually related to the material to which the inoculum will be applied [48]. Composting can be accelerated by the addition of inoculum both in the mesophilic phase and in the thermophilic phase; however, studies have indicated that the inoculation of exogenous and natural microbial populations throughout the whole process is more efficient than the inoculum in just one of the phases [48, 49].

3.7 FUNGAL ISOLATION WITH CELLULOLYTIC POTENTIAL

The isolation methodology is proposed by CLARK (1965) [50] with few 112 modifications. Consist of add 10 g of organic compost in 90 ml of saline 113 sterile peptone (0.85% (w/v) sodium chloride and 0.1% (w/v)) of bacteriological peptone [51]. The first dilution is shaken in "Shaker", for 15 minutes 115 to 127 rpm. Subsequently, successive serial dilutions are performed and 100 μL of sample is spread on the surface of Petri dishes with culture medium. The means used for growth are: Yeast extract peptone glucose (YEPG), composed of (g L^{-1}): Yeast extract, 10; bacteriological peptone, 20; glucose, 20; bacteriological agar, 35.5, plus chloramphenicol antibiotic 0.1% for yeast growth; Nutrient agar (AN) (g L^{-1}): Yeast extract, 3; Bacteriological Peptone, 5; Sodium chloride, 3; Bacteriological Agar, 13, plus nystatin antibiotic (1 ml for 250 ml of culture medium) for bacterial growth; Agar potato dextrose (BDA) (g L^{-1}) prepared according to the medium used for growth is the potato dextrose agar (BDA) (in G L^{-1}) prepared according to the manufacturer plus antibiotic chloramphenicol 0.1% for fungal growth [52]. The inoculated plates are incubated at 28±2 ºC and evaluated daily for up to 5 days. The colonies are separated based on the aspect of the mycelium, spore color and characteristics of the obverse and reverse of the colonies. The colonies are transferred to the plates containing potato dextrose agar culture medium (BDA) for 5 days, 28±2 ºC. These colonies were reinoculated in selection medium until pure crops were obtained. Pure fungi colonies were preserved following the Castellani method [53].

The bacteria are purified by the technique of exhaustion to obtain isolated colonies. This technique consists of making stretch marks, with the aid of a platinum strap, in a solid medium, where, by exhaustion, isolated colonies are obtained at the end of the stretch

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marks. As for the purification of the fungi, after the colonies appear, they are separated based on the aspect of the mycelium, spore color and other characteristics of the obverse and reverse of the colonies. These colonies are reinoculated, through stretch marks, in selection medium until the attainment of pure crops [54]. After the incubation period, the total count of the morphotypes found is performed and characterized according to their morphology, including size, shape, color, edge, appearance, brightness and elevation according to the methodology of YARROW, (2008) [55]. Pure fungi colonies are preserved following the Castellani method [53]. All tubes/plates are stored in a refrigerator, the temperature of approximately 4 °C.

3.8 SCREENING OF CELLULOLYTIC FUNGI

The isolated fungi are evaluated for their cellulolytic activities in plaques containing agar and Vogel salts [56], containing (g L^{-1}): $Na_3C_6H_5O_7.5H_2O$, 150; KH_2PO_4, 250; NH_4NO_3, 100; $MgSO_4.7H_2O$, 10; $CaCl_2.2H_2O$, 5, biotin solution (0,1 mg ml^{-1}) 5; Chloroform 0.2 ml, solution of trace elements (g L^{-1}), 5 ml [$C_6H_8O_7.H_2O$, 50; $ZnSO_4.7H_2O$, 50; $Fe(NH_4)_2(SO_4)_2.6H_2O$,10; $CuSO_4.5H_2O$, 2.5; $MnSO_4.H_2O$, 0.05; H_3BO_3, 0.05; $Na_2MoO_4.2H_2O$, 0.05], diluted 50 times in distilled water, supplemented with 0.2% of yeast extract, supplemented with 1% (m/v) of the carbon source, with carboxymethylcellulose (CMC), and 1.5% (m/v) agar. The isolates are previously activated in BDA medium [agar potato dextrose (in g L^{-1}) 42 g] plus chloramphenicol antibiotic 0.1% and inoculated in the center of the petri dish incubated at a temperature of 30 °C.

3.9 EVALUATION OF CELLULOLYTIC ACTIVITY

The revelation for the determination of cellulolytic activity is performed by the addition of the solution of Congo red 0.25% (m/v) in Tris-HCl buffer 0.1 M pH 8.0, according to the method proposed by HANKIN and ANAGNOSTAKIS, (1975) [57]. The washing of the middle surface is conducted with a 1M NaCl solution in the same buffer and observed transparent halo in contrast to opaque medium.

3.10 CALCULATION OF THE ENZYMATIC INDEX

The crops in which it is possible to observe the formation of halo around the colony has its activity evaluated through the enzymatic index (IE), that is, the diameter of the halo divided by the diameter of the colony [58].

3.11 DETERMINATION OF ENZYMATIC POTENTIAL

The industrial cultivation of fungi with the purpose of obtaining enzymes, resulting from its metabolism, is biotechnological activity in wide expansion. The two main strategies for determining the potential of enzymes are the processes of submerged fermentation and solid state fermentation [59].

Submerged fermentation is the technique mostly used in industrial processes due to the ease of growth of fungi in controlled conditions of pH and temperature, in addition to making it easy to recover the enzymes extracellular [18]. This process uses fermentative medium, where the sources of nutrients used are soluble and the development of the microorganism occurs in the presence of free water. The water content in this process is greater than 95% [60]. It is a system capable of generating a variety of metabolites in which filamentous fungi are of great importance [61]. In relation to solid state fermentation, submerged cultivation has as its advantage the possibility of having better rationalization and standardization of the process, which is fundamental for the industry [23] in addition to allowing a homogeneous culture system [62].

Regarding solid state fermentation (FES), the culture medium is composed of solid substrates, acting as a carbon source, without free water, which makes this condition of growth try to approach the natural habitat. It is an alternative cultivation system for the production of value added products from fungi, especially enzymes [63]. Products or by-products from agroindustry, in the form of raw waste, are employed in FES as substrates to serve as solid matrix and provide carbon for the growth of the fungus, in addition to presenting low cost [60]. FES is suitable for filamentous fungi, as these can develop in environments that have low levels of relative humidity. In addition, filamentous fungi have favorable air hyphae for the colonization of solid substrates [65].

Second Paranthaman et al. (2008) [63] FES has advantages over submerged fermentation, such as lower energy requirements, lower reactor volume, and high productivity, low capital investment, low waste of water, a higher concentration of metabolites obtained and lower processing cost. However, FES also has some limitations, such as the difficulty of heat dissipation generated by microbial metabolism, limited oxygen transfer depends on substrate granulometry; difficulty in temperature control and beyond these variables, there is a greater difficulty in collecting representative samples during the process, due to the non-homogeneity of the mass in fermentation [63].

3.12 EVALUATION OF ENZYMATIC ACTIVITY IN SUBMERGED FERMENTATION

The submerged fermentation is prepared in

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Erlenmeyer vials of 125 mL, containing 30 mL of Vogel's liquid medium [56], supplemented with 1% of the carbon source (e.g.: Wheat bran) and sterilized at 121 ºC for 15 minutes for the production of FPase enzymes, endoglucanase, exoglyanase, and xylanase. The crops are inoculated with 1 mL of a suspension of 107 spores mL^{-1} and are kept for 5 days at 100 rpm, 30 ºC. Fermented broths are filtered in an ice bath and frozen for evaluation of enzymatic activity [66].

3.13 EVALUATION OF ENZYMATIC ACTIVITY BY SOLID STATE FERMENTATION

The fermentation on solid substrates can be carried out in polypropylene or Erlenmeyer plastic bags containing the raw material plus distilled water and then autoclaved to 121 ºC during 20 minutes. The inoculation of the substrate is performed with 1 mL of spore suspension standardized at 10^6 spores mL^{-1}, properly homogenized. In mixed crops, inoculation is performed with 1 mL of spore suspension standardized at 10^6 spores mL^{-1} for each fungus used. The crops are kept at 30 ºC for 5 days.

The extraction of the enzymes can be accomplished by adding 100 mL of chilled distilled water to the fermented medium to which, subsequently, it is subjected to orbital agitation at 8000 rpm, for 20 minutes. The medium is then filtered by vacuum, an ice bath and the extract wrapped in test tubes in the freezer subsequently used to evaluate the enzymatic activity [66].

3.14 TOTAL CELLULASES ACTIVITY (FPASE)

The activity of total cellulases (FPase) is determined according to the recommendations of the IUPAC [52] using filter paper (Whatmann Nº1) measuring 1.0 x 6.0 cm, equivalent to 50 mg, as substrate [67]. The reaction medium consists of a mixture of sodium citrate buffer 0.05 M pH 4.8 and a strip of filter paper. The reaction is initiated with the addition of the enzyme extract, remaining for 60 minutes and 50 ºC. The reaction is interrupted by the addition of 3 mL of DNS and maintained at 100ºC for 5 minutes. After cooling the samples are added 20 mL of distilled water. The readings are performed in a spectrophotometer at 540 nm. The release of reducing sugars is determined according to MILLER (1959) [68], using glucose as standard, from the method of dinitrosalicylic acid (DNS). An enzyme activity unit is defined as the amount of enzyme required to release 1 mmol glucose per minute per mL [9].

3.15 ENDOGLUCANASE ACTIVITY, EXOGLYCINASE, AND XYLANASE

The activities of endoglucanase, exoglucanase, and xylanase are determined using 1% carboxymethylcellulose solution (CMC), Avicel® (microcrystalline cellulose) and xylina, respectively, in sodium acetate buffer 0.05 M, pH 5.5. The reactional medium consists of 400 mL of the substrate and 400 µL of the enzyme extract, kept at 60°C. After time intervals 0 (control), 5 and 10 minutes, aliquots of 200 μL are removed from the reaction medium and the response interrupted by the addition of 200 μL of DNS and boiled for 5 minutes. The samples are cooled, and 2 mL of distilled water is added. The readings are performed in a spectrophotometer at 540 nm. The release of reducing sugars is determined according to the MILLER (1959) [68], using glucose or xylose as standard (molar extinction coefficient (ε) glucose 80.54 m^{-1} cm^{-1} or xylose 74.27 m^{-1} cm^{-1}), from the acid method dinitrosalicylic acid (DNS). An enzyme unit is defined with the amount of enzyme required to release 1 μmoL of glucose or xylose per minute per milliliter [69].

3.16 CELLULOLYTIC MICROORGANISMS AS A SOURCE OF INOCULUM

The composting process can occur naturally with the involvement of microorganisms present in organic residues. However, insufficient quantity or low biodegradability of the native microbial community may lead to low composting efficiency and undesirable quality of the compound [45]. In order to achieve a reduction in waste transformation time, inoculation is indicated as an efficient technique, as it introduces a population of microorganisms to initiate and accelerate the decomposition process of the organic residue [46]. The inoculum would, therefore, be a way to increase in number and diversity the microbial community of the composting windrows, besides being able to direct to the degradation of a specific residue and/or to ensure more complete degradation of the components of composting [47].

Inoculation consists of the addition of microorganisms from pure culture, or mass inoculation consisting of a large number of organisms, usually related to the material to which the inoculum will be applied [48]. Composting can be accelerated by the addition of inoculum both in the mesophilic phase and in the thermophilic phase, however, studies have indicated that the inoculation of exogenous and natural microbial populations throughout the whole process is more efficient than the inoculum in only one of the phases [48, 49].

IV. CONCLUSION

Fungi with cellulolytic potential are important in the process of transforming agricultural residues in products of higher added value, such as organic compost, enzymes and developing glasses.

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