UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(ÁREA: MICROBIOLOGIA APLICADA)

TÁSSIO BRITO DE OLIVEIRA

FUNGOS NA COMPOSTAGEM DA TORTA DE FILTRO: DIVERSIDADE, GENÔMICA E POTENCIAL BIOTECNOLÓGICO

Tese apresentada ao Instituto de Biociências, do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Área: Microbiologia Aplicada).

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Orientador: Prof. Dr. André Rodrigues

Co-orientadora: Profª. Drª. Eleni Gomes

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Dezembro – 2016

589.2 Oliveira, Tássio Brito de
O48f Fungos na compostagem da torta de filtro : diversidade, genômica e potencial biotecnológico / Tássio Brito de Oliveira. - Rio Claro, 2016
127 f. : il., figs., gráfs., tabs., fots.

Tese (doutorado) - Universidade Estadual Paulista, Instituto de Biociências de Rio Claro
Orientador: André Rodrigues
Coorientadora: Eleni Gomes

1. Fungos. 2. Pirosequenciamento. 3. Cana-de-açúcar. 4. Enzimas lignocelulolíticas. 5. Tratamento de resíduo. 6. Fungos termofílicos. I. Título.

Ficha Catalográfica elaborada pela STATI - Biblioteca da UNESP
Campus de Rio Claro/SP

unesp

UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Rio Claro

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: FUNGOS NA COMPOSTAGEM DA TORTA DE FILTRO: DIVERSIDADE, GENÔMICA E POTENCIAL BIOTECNOLÓGICO

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Rio Claro, 18 de novembro de 2016

AGRADECIMENTOS

Ao meu orientador, Dr. André Rodrigues, quem muito admiro pela competência profissional e genialidade, pela oportunidade, dedicação e incentivo à pesquisa durante sua orientação, que me abriu caminhos e diversas colaborações, permitindo meu crescimento profissional e pessoal.

À minha co-orientadora, Drª. Eleni Gomes, pela contribuição na idealização do projeto, e disponibilização do seu laboratório, desde infraestrutura até material necessário para realização de experimentos.

À minha supervisora no exterior, Drª. Nina Gunde-Cimerman, por abrir as portas do seu laboratório e por todo suporte oferecido, desde infraestrutura, até contato e todo material necessário para a realização da pesquisa.

À UNESP e ao Programa de Pós-Graduação em Ciências Biológicas (Área: Microbiologia Aplicada) pela oportunidade de estudo e à FAPESP pela bolsa regular de doutorado e a Bolsa Estágio de Pesquisa no Exterior (BEPE) concedidas (processos FAPESP #2012/14594-7 e #2015/25252-8, respectivamente).

Ao engenheiro agrônomo Haroldo Castilho pelos ensinamentos sobre compostagem e à Usina Santa Lúcia (Araras, SP) por conceder a torta de filtro para a montagem das leiras.

Ao Dr. Adrian Tsang, pela disponibilização dos genomas de fungos sequenciados em seu projeto *Genozymes for Bioproducts and Bioprocesses Development*.

À toda a equipe do Labortório de Ecologia e Sistemática de Fungos, aos novos e aos antigos membros, que de forma direta ou indireta contribuíram com o meu trabalho. Foram quatro anos de muitas conversas, discussões, ajudas, aprendizado, diversão, experiências compartilhadas, tanto dentro quanto fora do laboratório.

À toda equipe do Laboratório de Biologia de Micro-organismos da Universidade de Ljubljana, Eslovênia. Agradeço à Polona Zalar e Cene Gostinčar, pelo apoio direto no desenvolvimento da pesquisa. Em especial, à Monika Novak Babič e Monika Kos, minhas companheiras de escritório, por toda amizade, carinho e os momentos de diversão que tivemos.

À minha família por todo apoio, incentivo, por sempre acreditarem em mim e que mesmo estando distante não pouparam esforços para realização dos meus sonhos. Em especial à minha Mãe que é um exemplo de força, honra, coragem, companheirismo e determinação que tento sempre seguir.

Aos meus amigos de república Ana Maria, Rafael Rodrigues e Gustavo Pedroso. Agradeço pela amizade, cumplicidade, por todo carinho e por não me deixarem desanimar em nenhum instante.

Aos que colocaram obstáculos no caminho, pois despertaram em mim a força e a determinação que ainda não conhecia, me fizeram crescer para ser capaz de ultrapassá-los. Sem vocês eu não seria o que eu sou hoje.

À todos aqueles, mesmo que o nome não esteja aqui, que de forma direta ou indireta colaboraram para a realização desse trabalho.

Muito obrigado!

RESUMO

A torta de filtro é gerada após o processo de filtração do caldo de cana (cerca de 26 a 40 kg por t de cana) e geralmente é utilizada como fertilizante nas lavouras sem qualquer tratamento prévio. A diversidade de fungos presentes na torta de filtro fresca e no processo de compostagem desse substrato foi acessada utilizando sequenciamento em larga escala. Além disso, fungos tolerantes ao calor foram isolados e avaliados quanto à capacidade de produzir enzimas de degradação da biomassa (celulase, xilanase, lacase e poligalactoronase). Considerando que esse grupo de fungos carece de uma revisão taxonômica atual, aproveitamos as recentes mudanças proporcionadas pelo Código de Nomenclatura para Algas, Fungos e Plantas para gerar uma revisão taxonômica do grupo. Uma gama de patógenos oportunistas foi encontrada entre os taxa mais abundantes na torta de filtro fresca, como *Lomentospora prolificans* (43,13%), *Trichosporon* sp. (10,07%), *Candida tropicalis* (7,91%) e *Hormographiella aspergillata* (8,19%). Isso indica que a torta de filtro pode ser uma potencial fonte de fungos patogênicos, apresentando riscos para a saúde humana se aplicado como fertilizante sem qualquer tratamento. No entanto, o processo de compostagem reduz efetivamente a carga desses fungos. Além disso, cria um ambiente interessante para fungos capazes de produzir enzimas com potencial aplicação biotecnológica, uma vez que todos os 110 isolados avaliados foram capazes de produzir, pelo menos, uma das enzimas avaliadas. Além disso, a análise comparativa de genes codificantes para peptidases presentes nos genomas de fungos termofílicos (encontrados em sistemas de compostagem) e mesofílicos mostrou que a termofilia levou à várias adaptações para a termoestablidade enzimática.

Palavras-chave: Pirosequenciamento. Cana-de-açúcar. Enzimas lignocelulolíticas. Tratamento de resíduo. Fungos termofílicos. Fungos termotolerantes.

ABSTRACT

Pressmud is derived from sugarcane juice filtrate (around 26 to 40 kg per ton of sugarcane) and it is mainly used as fertilizer in crops without prior treatment. Here, the fungal diversity present in both fresh and composting pressmud was revealed by 454 pyrosequencing. In addition, heat-tolerant fungi were isolated and surveyed for their repertoire of biomass-degrading enzymes (cellulase, xylanase, laccase and polygalactoronase). The fact that the taxonomy of such organisms is still obscure, we revised their taxonomy in the light of the recent changes adopted in the Code of Nomenclature for Algae, Fungi and Plants. A wide range of opportunistic pathogens was found among the most abundant taxa in fresh pressmud, such as *Lomentospora prolificans* (43.13%), *Trichosporon* sp. (10.07%), *Candida tropicalis* (7.91%), and *Hormographiella aspergillata* (8.19%). This indicates that fresh pressmud may be a source of human pathogenic fungi, presenting a potential threat to human health if applied as fertilizer without treatment. Composting of the pressmud effectively reduces the load of such fungi. Furthermore, the composting system creates an interesting environment for fungi able to produce enzymes with biotechnological applications, since all the 110 isolates screened were able to produce at least one of the tested enzymes.Furthermore, comparative analysis of peptidases genes encoded by thermophilic (generally found in composting systems) and mesophilic fungi showed that thermophyly selected for thermostable enzymes.

Keywords: Pyrosequencing. Sugarcane. Lignocellulolytic enzymes. Residue treatment. Thermophilic fungi. Thermotolerant fungi.

SUMÁRIO

1 INTRODUÇÃO

O Brasil é o maior produtor de cana-de-açúcar no mundo e consequentemente, o maior produtor de resíduos tais como bagaço, vinhaça e torta de filtro. A última, foco deste estudo, é produzida durante a fase de clarificação do caldo de cana e, como alternativa para o manejo desse resíduo, é empregado na lavoura de cana para enriquecimento do solo. No entanto, poucas usinas tratam o resíduo antes de sua aplicação no campo (p. ex. compostagem). Embora essa prática seja comum, nada se sabe a respeito da comunidade microbiana presente nesse resíduo, a qual pode incluir espécies patogênicas e oportunistas.

Nesse contexto, o presente trabalho teve como foco caracterizar as comunidades de fungos presentes na torta de filtro fresca e avaliar o efeito da compostagem sobre essa comunidade. Para isso, foi empregado método independente de cultivo (sequenciamento em larga escala) nas três principais fases da compostagem: fase mesofílica (ou torta de filtro fresca), termofílica e de maturação.

Além disso, a compostagem, em sua fase termofílica, atinge temperaturas elevadas, tornando-se um ambiente proprício para o crescimento de fungos termofílicos e termotolerantes. Tais fungos são interessantes do ponto de vista biotecnológico por serem decompositores naturais da matéria orgânica, além de produzirem enzimas com maior estabilidade térmica, quando comparados aos fungos mesofílicos.

Com isso, os fungos termofílicos e termotolerantes foram isolados ao longo do processo de compostagem. Posteriormente, esses fungos foram avaliados quanto ao potencial biotecnológico para a produção de enzimas de interesse na hidrólise de material lignocelulósico (xilanase, celulase, pectinase e lacase).

Com o aumento no número de genomas disponíveis em bancos de dados e a busca incessante pelo setor de P&D por enzimas termoestáveis, realizamos a mineração

do genoma de fungos termofílicos para buscar genes codificadores de enzimas de interesse industrial, como as peptidases, além de examinar padrões que indiquem adaptações dessas enzimas à termoestabilidade.

Embora haja um grande interesse biotecnológico acerca desses fungos resistentes a altas temperaturas, a taxonomia e classificação desse grupo não tem recebido a mesma atenção, o que estimulou a realização de uma revisão acerca desta temática contextualizando com as recentes mudanças na taxonomia de fungos.

Os resultados deste trabalho estão divididos em três capítulos na forma de manuscritos, a saber:

Capítulo 1: Tendo em vista os recentes avanços na taxonomia e nomenclatura de fungos (proporcionada pelo lançamento do novo código de nomenclatura em 2012), foi identificado que existe uma lacuna na aplicação dos novos conceitos taxonômicos para fungos, incluindo os termofílicos, especialmente para a área de micologia ambiental e aplicada (voltada à indústria e biotecnologia). Nesse sentido, o artigo de revisão teve como objetivo inserir a taxonomia dos fungos termofílicos no contexto moderno da nomenclatura de fungos, proporcionado pelo movimento "Um Fungo = Um Nome". Como resultado, foi gerada uma lista compreensiva de nomes de fungos utilizados comumente na indústria e a situação desses nomes quanto a espécimes tipos depositados em coleções de cultura e as respectivas sequências de DNA *barcodes* depositadas nas bases de dados *online* (p. ex. NCBI-GenBank).

Capítulo 2: Estimulado por estudos anteriores que mostram a presença de patógenos oportunistas em resíduos utilizados em compostagem e o efeito desta sobre esses micro-organismos, foi avaliada a diversidade de fungos presentes na torta de filtro fresca e em

fases subsequentes da compostagem. A comunidade de fungo na torta de filtro fresca é composta, predominantemente, por patógenos oportunistas como *Lomentospora prolificans, Trichosporon* sp., *Candida tropicalis* e *Hormographiella aspergillata*, indicando a necessidade de cuidados no manuseio desse resíduo. No entanto, a compostagem mostrou-se eficiente na redução da carga de fungos patogênicos. Além disso, a compostagem é um ambiente propíco para a prospecção de fungos de interesse biotecnológico, como os resistentes a altas temperaturas, os quais apresentaram atividade enzimática positiva para uma ou mais enzimas avaliadas (xilanase, celulase, poligalacturonase e lacase).

Capítulo 3: Com o aumento no número de genomas disponíveis, novas estratégias são aplicadas para a prospecção de fungos produtores de enzimas de interesse biotecnológico, tais como a mineração genômica. Como resultado dessa prospecção *in silico*, nesse trabalho foi gerado um catálogo de genes codificadores de peptidases encontradas nos genomas de espécies resitentes a temperaturas elevadas e espécies mesofílicas relacionadas filogeneticamente. Como esperado, observamos que a redução no tamanho do genoma de fungos termofílicos afeta o número de cópias de genes codificadores de proteases, mas ainda mantém um grande número de peptidases que podem ser exploradas para fins biotecnológicos. Além disso, foram observadas diferenças nas sequências das peptidases de fungos termofílicos, em relação às dos mesofílicos, incluindo o aumento de Ala, Asp, Glu, Gly, Pro e Arg; com consequente redução de Cys, Phe, His, Ile, Lys, Leu, Met, Asn, Gln e Thr. Adicionalmente, foi observado o aumento de resíduos carregados e hidrofóbicos e uma redução de resíduos polares nessas proteínas. Em termos estruturais, foi observada uma redução na quantidade de cavidades nas peptidases aspárticas. Todas essas mudanças possivelmente

conferem a termoestabilidade das enzimas dos fungos termofílicos comparada com às dos mesofílicos. Com isso, a mineração genômica e o estudo comparado de espécies mesofílicas e termofílicas, além de acelerarem as buscas de fungos produtores de enzimas específicas, permite a busca de padrões que aumentam a termoestabilidade destas enzimas, direcionando estudos futuros de engenharia bioquímica para a expressão heteróloga de enzimas termoestáveis.

2 CHAPTER I

Thermophilic fungi in the new age of fungal taxonomy

Status: Published

Citation:

OLIVEIRA, T. B.; GOMES, E.; RODRIGUES, A. Thermophilic fungi in the new age of fungal taxonomy. **Extremophiles** v. 19, n. 1, p. 31-37, 2015. doi: 10.1007/s00792-014-0707-0

Thermophilic fungi in the new age of fungal taxonomy

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Running title: Updates on the taxonomy of thermophilic fungi

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Abstract

Thermophilic fungi have wide applied interest due to their potential to produce heat tolerant enzymes for biotechnological processes. However, the taxonomy of such organisms remains obscure, especially since the new events in the nomenclature of fungi. Here, we examined the taxonomy of the most commonly used thermophilic fungi in industry in the light of the recent taxonomic changes after the adoption of the Code of Nomenclature for Algae, Fungi and Plants in Melbourne and based on the movement One Fungus = One Name. Despite the widespread use of these fungi in applied research, several thermotolerant fungi still remain classified as thermophiles. Furthermore, we found that some thermophilic fungi had their genome sequenced, however many taxa still do not have barcode sequences of reference strains available in public databases. This lack of basic information is a limiting factor for species identification of thermophilic fungi and for the new metagenomic studies in this area. Based on next-generation sequencing, such studies generate high amounts of data, which may reveal species of thermophilic fungi not yet described in different substrates (composting systems, geothermal areas, piles of plant material). As discussed in this study, there are intrinsic problems associated with this method, which may interfere with the process of ecological discovery from this group of organisms. To overcome such difficulties, taxonomy of this group should move towards standardizing the names of species commonly used in industry and to assess the possibility of including new systems for describing species based on environmental sequences.

Keywords: thermophiles, classification, name changes, nomenclature

Introduction

Each ecosystem presents a number of biotic and abiotic factors that makes it unique. Some have particular characteristics from those found in most environments, such as high salt concentrations, extremes of pH and temperature, or a combination of such factors. In response, some organisms developed adaptations that allow them to colonize and survive under harsh conditions, considered harmful for most organisms.

The thermophilic organisms are found in domains *Bacteria*, *Archaea* and *Eukarya*. However, in *Eukarya* the tolerance to high temperatures is narrower than to the prokaryotes, and species that tolerate temperatures above 61 °C have not been observed. Among the eukaryotes, some fungi have the ability to remain active and propagate in environments with high temperatures. These heat-tolerant fungi can be classified as thermophilic or thermotolerant, basically due to the cardinal growth temperatures. Here, we use the definition by Cooney and Emerson (1964) who consider as thermophilic fungi those with maximum growth temperature of 50 °C or above and minimum growth temperature at 20 °C or above; on the other hand, thermotolerant fungi are those that grow at maximum temperatures up to 50 °C, with a minimum growth temperature below 20 °C. Although this classification is quite practical, it is not applicable for some fungi. For example, *Aspergillus fumigatus* is able to grow at temperatures above 50 °C and below 20 °C (Mouchacca 2000a). Alternatively, Maheshwari et al. (2000) proposed a simpler classification (and widely used in applied mycology and industries), in which thermophilic fungi, are those species that have optimum growth temperature of 45 °C or above.

If on one hand heat-tolerant fungi draw attention due to their potential in the production of heat resistant enzymes; on the other the proper classification regarding

their tolerance to high temperatures and the correct identification and use of current taxonomy has been neglected in most applied studies. This work presents a perspective on this group of fungi, and brings basic aspects of their biology and classification in the light of the recent changes in fungal taxonomy (One fungus = One name movement). Particularly, we draw attention to the importance of correct classification of heat-tolerant fungi. Also we trace the current profile of access to references strain (i.e. type species) deposited in culture collections and their barcode sequences deposited in online public databases. Finally, we emphasize that the rapid progress towards the use of fungi in applied research, as well as in molecular ecological studies, is not accompanied by taxonomic advances.

Origin and ecology

It is speculated that thermophily in the Kingdom Fungi arose as an adaptation to seasonal changes and high daytime temperatures and not as an adaptation for occupation of new niches, where the temperature is kept constantly high (Powell et al. 2012). Some authors suggest that thermophilic fungi descended from mesophilic ancestors associated in bird nests, as those found in Australia (Megapodiidae) (Cooney and Emerson 1964; Rajasekaran and Maheshwari 1993). These birds thermoregulate their nests with decaying plant material functioning as a natural composting system. In this scenario, fungi are the major players in the decomposition of this substrate, raising the nest temperature around 45 °C (Seymour and Bradford 1992).

Although comprising an ecological defined group, the thermophilic fungi are distributed in different taxonomic groups: representatives can be found within the phylum "Zygomycota" (which is now regarded as complex of several non-related

subphyla sensu Hibbett et al. 2007) and the phylum Ascomycota (Salar and Aneja 2007); also, one Basidiomycota species is known so far (Tabela S1). Phylogenetic studies by Morgenstern et al. (2012) suggest that the heat-tolerant fungi form a paraphyletic group, found in different phylogenetic lineages in the fungal tree of life, indicating that thermophily had multiple independent origins in the Kingdom Fungi. On the other hand, the most parsimonious scenario in the family *Chaetomiaceae* (which comprises many species of heat-tolerant Ascomycetes fungi) is that thermophily originated from a common ancestor with multiple losses within the family (Morgenstern et al. 2012; Van Noort et al. 2013).

According to reports available in the literature, thermophilic fungi have been found in several culture-depend studies in a variety of environments, including different soil types (Pan et al. 2010; Powell et al. 2012; Redman et al. 1999; Salar and Aneja 2006), power plant cooling systems, pond sediments (Ellis 1980), piles of plant material (Tansey 1971) and composting systems (Kane and Mullins 1973; Klamer and Søchting 1998; Straatsma et al. 1994) where temperature, humidity and atmosphere make such substrates favorable for their development (Salar and Aneja 2007).

Although having a wide distribution, such fungi are not active in certain environments, probably occurring only as propagules, which are generally transported by air (Thakur 1977; Rajasekaran and Maheshwari 1993; Le Goff et al. 2010). This dispersion occurs mainly from composting systems, due the production of aerosols carrying spores when revolving the piles (Le Goff et al. 2010). In fact, little is known about the ecology of these organisms in nature and studies involving the direct sampling of substrates in the environment may reveal more information about these fungi.

Fungal adaptations to thermophily

Among all factors affecting the cell stability, temperature influences the function of biomolecules and the maintenance of biological structures and most organisms can only grow within a narrow temperature range. However, the existence of geothermal stable environments allows selection or persistence of microorganisms that not only resist, but also require high temperatures to survive.

As a result of this adaptive process, peculiar features are found in thermophilic fungi, for example, the genome size reduction when compared to closest mesophilic species (Van Noort et al. 2013). This process involves the loss of protein-coding genes and transposable elements, and the presence of reduced introns and intergenic regions. In contrast to the genome reductionist tendency, duplication of intriguing genes may give insights on the evolution of thermophily, such as the gene responsible for hyphal melanization, which would be involved in resistance to high temperatures, desiccation and UV radiation.

The adaptation of a particular microorganism to survive and grow at elevated temperatures involves crucial aspects such as modifications of the cytoplasmic membrane, proteins and DNA at temperatures above the mesophilic range. The adaptation of the thermophilic microorganisms membranes corresponds to a process called homeoviscous, which consists of the replacement of unsaturated fatty acids for saturated fatty acids, so that the membrane acquires a balance between density and fluidity, necessary for the maintenance of their physical and functional integrity at elevated temperatures. This adaptation occurs in the domains *Bacteria* and *Eukarya*, whilst the latter found only in the Kingdom Fungi (Adams 1993).

Extracellular proteins of filamentous fungi are mainly depolymerizing enzymes

that in thermophilic environments need to present dynamic and kinetic thermostability for the continuity of the activities. Some differences in sequence, structure, function, dynamics and thermodynamic properties can be observed between psychrophilic, mesophilic and thermophilic enzymes (Niehaus et al. 1999). Two evolutionary strategies seem to define the thermostability: (i) intrinsic factors or directly associated with the structure of the molecule, leading to stiffness and folding and (ii) extrinsic factors, that help to stabilize the proteins in a given environment, as solutes, binders, molecular chaperones and the substrate itself (Bruins et al. 2001). Up to date, there is no fungi with growth above 61 °C, fact that may be related to greater thermolability of their membrane systems than the thermostability of enzymes or other cellular structures.

Taxonomy and name changes

While a few fungal species are considered true thermophilic according to the concept of Cooney e Emerson (1964), many were identified under different names (Mouchacca 1997; Mouchacca 2000b). Ambiguities in the taxonomy of these fungi resulted in conflicts and miss identification overtime. Furthermore, in applied research many thermotolerant fungi are classified as thermophilic mostly due to the existence of several concepts that define fungi as thermophilic or thermotolerant (Mouchacca 2000a). The use of cardinal and optimum growth temperatures as a baseline to delimit thermophilic from thermotolerant fungi is conflicting due to the existence of several fungi that do not fit in these concepts. Perhaps, an alternative to distinguish between thermophilic and thermotolerant strains is to classify fungi with optimum growth ranging from 40 to 50 °C and do not grow below 20 °C as thermophilic; on the other hand, fungi with optimum growth ranging from 40 to 50 °C but are able to grow below

20 °C should be classified as thermotolerant. To correct classify fungi as thermophilic or thermotolerant, in addition to use current nomenclature of fungi, is of great academic and economic interest since many of these fungi are involved in biotechnological processes and acquisition of patents (Mouchacca 2000a; 2000b).

Few studies have been conducted regarding the diversity of heat-resistant fungi and on the applied side of thermophilic fungi various synonyms are used to the same fungus (Table S1). In this sense, the current state of the taxonomy of thermophilic fungi creates a certain difficulty when one wants to compare between studies (due to the different nomenclarure used by the authors). An interesting tool that help to standardize fungal nomenclature is the Index Fungorum (www.indexfungorum.org), a nomenclatural database where one can check the updated names of fungi (i.e. synonyms and basionyms) and some information about classification. Additionally, the MycoBank database (www.mycobank.org) is an important tool where new names are recorded along with illustrations and descriptions of the each taxon. Both databases are the current repositories of fungal names, including the names of fungal novelties, like genera and species.

Recently, an important step towards the stabilization of scientific names in fungal taxonomy was put forward. During a symposium in 19-20 April 2011, a group of mycologists launched "The Amsterdam Declaration on Fungal Nomenclature" with the purpose to end the due nomenclatural system for asexual and sexual fungi. Long deem, such initiative directed to the end of duality existing in the nomenclature of pleomorphic fungi where species described from different phases of the reproductive cycle, asexual (anamorphic) or sexual (teleomorph), received different names (Hawksworth et al. 2011). At the XXIIIth International Botanical Congress in Melbourne in 2011, the nomenclature section adopted the changes proposed by mycologists resulting in the new

International Code of Nomenclature for Algae, Fungi, and Plants (ICN). Such code triggered the movement called "1 fungus = 1 name" which seeks to stabilize fungal nomenclature (Taylor 2011; Wingfield et al. 2012). Although the change has been implemented since 2012, the current state is as "1 fungus = Which name?" because for many described fungi it has not been determined which name to be applied under this new rule. Such names will change based on various criteria, for example, the priority of the oldest name. To widely known species, the current name of the species, anamorph or teleomorph, may be accepted, however, if the name of the anamorph is indicated, it will be necessary to go through the approval of the General Committee (GC) of the ICN, even though this is the oldest species name (Hibbett and Taylor 2013). Such changes in fungal nomenclature had several several impacts on the taxonomy of several fungi, inclunding the thermophilic fungi.

According to the review of Maheshwari et al. (2000), about 50,000 fungi described until 2000, only 17 genera were considered thermophilic (*Canariomyces*, *Chaetomium*, *Coonemeria*, *Corynascus*, *Dactylomyces*, *Malbranchea*, *Melanocarpus*, *Myceliophthora*, *Myriococcum*, *Paecilomyces*, *Rhizomucor*, *Scytalidium*, *Stilbella*, *Talaromyces*, *Thermoascus*, *Thermomyces* and *Thielavia*) comprising a total of 30 species. Since then, many names of fungi have undergone taxonomic changes. For example, *Stilbella thermophila* was reclassified to *Remersonia thermophila* (Seifert et al. 1997). Some other fungi have also suffered nomenclatural changes as the representatives of the genus *Coonemeria* and *Dactylomyces*, currently classified in *Thermoascus* (Table S1).

More recently, changes in the genus *Talaromyces* have occurred. Reviewing this genus, Houbraken et al. (2012) found that *Talaromyce*s do not have thermophilic species, according to phylogenetic analyses considering multiple molecular markers.

The authors observed that the species *Talaromyces dupontii* was phylogenetically close-related to the type species of *Thermomyces lanuginosus* indicating the need to revise the taxonomy of this group. Subsequently, this fact was confirmed and the species was reclassified as *Thermomyces thermophilus* (Houbraken et al. 2014; Table 1). In addition to this, three other species previously classified as *Talaromyces*, two species of *Geosmithia* and a new species, *Rasamsonia brevistipitata*, have been transferred to the new genus *Rasamsonia*, which includes thermophilic and thermotolerant representatives (Houbraken et al. 2012). Thus, the authors concluded that, within the order Eurotiales, thermophily is restricted to species of the genus *Thermomyces, Thermoascus* and *Rasamsonia.*

The species of *Corynascus*, some recognized to be thermophilic, were reclassified to the genus *Myceliophthora* (Table 1). Recently, a new species of *Myceliophthora* was isolated from soil in China, *Myceliophthora guttulata*. Therefore, this genus comprises five truly thermophilic species, namely: *M. fergusii*, *M. fusca*, *M. guttulata*, *M. heterothallica*, *M. hinnulea*, *M. sulphurea* and *M. thermophila* (van den Brink et al. 2012; Zhang et al. 2014, Table S1).

Classification based on environmental sequences

Following the changes in the classification of fungi, several windows of discussions were broadened regarding the classification of new taxa from environmental sequences. Such data are usually obtained from metagenomic studies, mostly next-generation sequencing data (Hibbett and Taylor 2013). Considering the limited number of thermophilic species known so far (Table S1), the use of new sequencing

technologies to assess the community of fungi in substrates with high temperatures will certainly lead to discovery of new species.

If the classification of thermophilic fungi using morphology in culture have generated unclear data, the classification based on environmental sequences, as proposed by Hibbett and Taylor (2013), is still a goal to be reached. For many valid thermophilic species, barcode sequences do not exist (Table S1). It means we do not have intraspecific variation data on the ITS marker to help in the discovering of molecular species. In fact, sequences from putative "new" species obtained directly from substrates could be from fungal species that have been already described for which molecular data are not available. The study by Nagy et al. (2011) clearly showed this situation. The authors demonstrated that generating ITS sequences from type material of several species of *Mortierella* (such sequences were not available in the database NCBI – GenBank), it was possible to identify the majority of sequences from the database that had no taxonomic affiliation (for example, sequences annotated as "uncultured *Mortierella*").

In order to move towards a classification system based on environment sequences some actions are necessary by the mycological community, as the deposit of sequences from thermophilic fungi in public database (such as NCBI-GenBank), including sequences from type material (or other reference material) or of fungi obtained in culture-dependent studies. Such efforts should be coupled with the deposit of specimens in well-known and easily accessible culture collections. Recently, an important step this goal was the choice of the ITS region (*Internal Transcribed Spacer*) as the universal barcode marker for fungi (Schoch et al. 2012). This tool assists the identification of thermophilic fungi, since the corresponding databases (for example: BOLD Systems, www.boldsystems.org) are structured with reference sequences

(sequences from type strains or strains identified by leading taxonomists). In this sense, taxonomic and phylogenetic studies using this molecular marker associated with morphological characters constitute an important strategy for identification and classification of this group of fungi.

Studies using large-scale sequencing technologies demonstrated the potential of thermophilic environments, such as compost heaps, to harbor putative new species to science. Langarica-Fuentes et al. (2014a) applied a dual approach using culture-based and molecular techniques to evaluate the diversity of fungi present in two compost samples. The authors noted that the diversity of fungi recovered by 454-pyrosequencing is greater than that observed by applying culture-depend methods (175 Operational Taxonomic Units – OTUs – obtained, against 8 morphospecies). Of the two compost tested, labeled A and B, 64 and 43 OTUs, respectively, were classified only to the rank of family or above. In addition, the authors found that culture-dependent studies can bias the diversity index and overestimate the importance of certain heat-tolerant species in the composting process (for example, *A. fumigatus*) or underestimated (e.g., *Scytalidium thermophilum*, *Myriococcum thermophilum*, *T. thermophilus* and *Myceliophthora verrucosus*). Langarica-Fuentes et al. (2014b) studying the succession of fungi in an in-vessel composting system found 251 OTUs; of these, 76 were classified only to family level or above or classified as uncultured fungus or unidentified fungus. Many of these non-cultivated fungi may have biotechnological potential of great interest. In addition, some of the obtained sequences without taxonomical affiliation may in fact belong to described species which barcode data do not exist.

Amid the taxonomic disorder, why the thermophilic fungi are interesting?

Filamentous fungi are organisms with high capacity to secrete enzymes with variety mechanisms of action and substrates specificity. The thermophilic fungi have received significant attention in recent years due to production of thermostable enzymes with various biotechnological applications. The search for enzymes as amylases, cellulases, xylanases, lipases and proteases has shown to be promising to achieve thermostable enzymes with optimum activity at high temperatures and with higher hydrolysis rate (Johri et al. 1999; Bhat 2000). Due to act at high temperatures, enzymes have the advantage of accelerating the reaction time and decrease the viscosity of the material and reduce the contamination by mesophilic microorganisms (Fernandes et al. 2008).

For instance, *T. lanuginosus*, a thermophilic fungus commonly found in compost systems, has been reported as the largest producer of xylanase already described in the literature. Recently, this fungus had its genome sequenced in order to obtain additional information for a better assessment in the industry (Mchunu et al. 2013). Exploring the genome of the thermophilic *Rhizomucor mihei*, Zhou et al. (2014) reported the existence of a large number of genes coding for proteolytic, amylolytic and lipolytic enzymes demonstrating the potential of this fungus to produce thermostable enzymes on an industrial scale.

In recent years, this group of fungi has been prospected for targeting enzymes to hydrolyze the plant biomass (Li et al. 2011) because they are natural decomposers of plant biomass and have the genetic make up to grow at high temperatures. In this present decade much effort has been directed towards the use of residual plant biomass for obtainment of fermentable sugars with several technological purpose but mainly for

the production of second generation ethanol. The application of thermophilic fungi or their enzymes on saccharifying the plant biomass in industrial scale provides benefits such as a reduction in energy costs, because there is no need for cooling after treatment of the bagasse by steam, a step proposed as promising method for separating the main constituents of plant biomass and increases their susceptibility to bioconversion (Dashtban et al. 2009).

Thermophilic fungi such as *Myceliophthora thermophila* (Moretti et al. 2012) and *Myceliophthora heterothallica* (Van Den Brink et al. 2013) were described as bioconverters of lignocellulosic residues into sugars. The authors point out the potential of these fungi to be used on an industrial scale in the process saccharification of sugarcane bagasse, consequently in the production of second-generation bioethanol. McClendon et al. (2012) demonstrated that *Thermoascus aurantiacus* is another promising fungus in producing thermophilic enzymes for biomass deconstruction. Further, the first study of thermophilic fungi genome suggests that both species studied, *M. thermophila* and *Thielavia terrestris* are capable of hydrolyzing all major polysaccharides present in the biomass (Berka et al. 2011). These data reinforce and encourage the continued search for heat-tolerant fungi and their thermostable enzymes for application in the chain of bioethanol production. Many thermophilic fungi are considered opportunistic pathogens, which makes another reason for the correct identification of the species being studied.

Conclusions

Considering the new taxonomic advances in fungal nomenclature and classification, the thermophilic fungi comprehend a group of 20 genera and

approximately 44 species. The current situation of the nomenclature of thermophilic species will stabilize when the "1 Fungus - 1 Name" be effective under the ICN. Such changes will certainly facilitate the naming of thermophilic fungi. In addition, the massive amount of data generated from next-generation sequencing studies focusing on harsh environments, will result in the discovering of putative new thermophilic species. In order to create a basis for such studies the description of novel thermophilic taxa from cultures must be accompanied by a deposit of barcode sequences (ITS sequences). Also, the provision of barcode sequences for valid publish names is urgently needed to fill the gap in the databases. Tied to this, is need to develop a nomenclatural system for species discovered only by environmental sequences (according Hibbett et al. 2011).

The increase search of heat-tolerant fungi for biotechnological applications requires great attention on the taxonomic question, an old problem that remains poorly crafted. Joint projects involving applied researchers and taxonomists are important aspects for minimizing this problem. Moreover, as advances in the use of thermophilic fungi as source of industrially-relevant enzymes, structural and evolutionary aspects of these fungi also remain poorly understood and studies regarding this purpose could provide key information about the thermal stability of molecular components of these organisms.

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SUPPLEMENTARY MATERIAL

Thermophilic fungi in the new age of fungal taxonomy

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Table S1. Classification of thermophilic fungi according to Index Fungorum with references to DNA barcode sequences (internal transcribed spacer - ITS) available in NCBI-GenBank database.

Phylum	Genus	Species	Synonymys	Reference strain	ITS accession #	References	Complete genome
"Zygomycota"	*Rhizomucor*	*Rhizomucor miehei* (Cooney & Emers.) Schipper (1978)	*Mucor miehei* Cooney & Emers. (1964); *Mucor miehei* var. *miehei* Cooney & Emers. (1964); *Rhizomucor nainitalensis* Joshi (1982); *Mucor miehei* var. *minor* Subrahm. & Gopalkr. (1984)	CBS 182.67^{T}	DQ118995	[14]	-
					HM999961	[17]	-
					HM999961	[18]	-
				CGMCC 4967	-	-	[20]
		Rhizomucor pusillus (Lindt) Schipper (1978)	*Mucor pusillus* Lindt (1886); *Mucor septatus* Bezold (1889); *Rhizomucor septatus* (Bezold) Lucet & Costantin (1901); *Mucor parasiticus* Lucet & Costantin (1899); *Rhizomucor parasiticus* (Lucet & Costantin) Lucet & Costantin (1900); *Rhizopus parasiticus* (Lucet & Costantin) Lendn (1908); *Rhizomucor pakistanicus* Qureshi & Mirza (1979); *Mucor muriperda* Sacc. & Sinig (1913); *Tieghemella muriperda* (Sacc. & Sinig.) Naumov (1935); *Mucor buntingii* Lendn (1930); *Mucor hagemii* Naumov (1935)	CBS 354.68^{ET}	JN206312	[18]	-
	Thermomucor	*Thermomucor indicae-seudaticae* Subraham., Mehrotra & Thirum. (1977)	-	CBS 104.75^{T}	HM999963	[17]	-
					JN206300	[18]	-
				CBS 446.78	JN206302	[18]	-
				CBS 447.78	JN206301	[18]	-
Ascomycota	*Acremonium*	*Acremonium thermophilum* Gams &	-	CBS 734.71^{T}	-	-	-

	Lacey (1972)					
Arthrinium	*Arthrinium pterospermum* (Cooke & Massee) Arx (1981)	*Coniosporium pterospermum* Cooke & Massee (1891); *Pteroconium pterospermum* (Cooke & Massee) Grove (1914)	CBS 134000^{ET} CBS 123185	NR_121561 KF144913 KF144912	[3] [3] [3]	- - -
Canariomyces	*Canariomyces thermophilus* Guarro & Samson (1988)*	-		-	-	-
Chaetomidium	*Chaetomidium pingtungium* (Chen & Chen) Mouch. (1999)	*Thielavia pingtungia* Chen & Chen (1996)	8608-5^{T} (TAI-Mycology)	-	-	-
Chaetomium	*Chaetomium britannicum* Ames (1963)*	-	BPI 581378 ^{T}	-	-	-
	Chaetomium mesopotamicum Abdullah & Zora (1993)	-	BSRA 10245^{T}	-	-	-
	Chaetomium senegalense Ames (1963)*	-	CBS 728.84^{T}	-	-	-
	Chaetomium thermophilum La Touche (1950)	*Chaetomium thermophilum* var. *coprophilum* Cooney & Emers. (1964); *Chaetomium thermophilum* var. *dissitum* Cooney & Emers. (1964); *Chaetomium thermophilum* La Touche (1950) var. *thermophilum*	CBS 144.50^{T} CBS 143.50	- GQ922527	- [5]	[1] -
	Chaetomium virginicum Ames (1963)	-	BPI 1101414^{T}	-	-	-
Humicola	*Humicola hyalothermophila* Moub., Mazen & Abdel-Hafez (1979)	-	CBS 454.80^{T}	-	-	-
Malbranchea	*Malbranchea cinnamomea* (Lib.) Oorschot & de Hoog (1984)	*Geotrichum cinnamomeum* (Lib.) Sacc. (1882) *Malbranchea pulchella* var. *sulfurea* (Miehe) Cooney & Emers. (1964); *Malbranchea sulfurea* (Miehe) Sigler & Carmich. (1976); *Malbranchea sulfurea* (Miehe) Pidopl. [as 'sulphurea'] (1953);	CBS 343.55 CBS 960.72	JF412018 JF922020	[10] [12]	- -

		Thermoidium sulphureum Miehe [as 'sulfureum'] (1907); *Trichothecium cinnamomeum* Lib. (1857)				
Melanocarpus	*Melanocarpus albomyces* (Cooney & Emers.) Arx (1975)	*Myriococcum albomyces* Cooney & Emers. (1964); *Thielavia albomyces* (Cooney & Emers.) Malloch & Cain (1972)	CBS 638.94^{T}	JF412014	[10]	-
	Melanocarpus thermophilus (Abdullah & Al-Bader) Guarro, Abdullah & Al-Bader (1996)	*Thielavia minuta* var. *thermophila* Abdullah & Al-Bader (1992)	CBS 886.97	AJ271586	[15]	-
Myceliophthora	*Myceliophthora fergusii* (Klopotek) Oorschot (1977)	*Chaetomidium thermophilum* (Fergus & Sinden) Lodha (1978), *Chrysosporium fergusii* Klopotek (1974), *Corynascus thermophilus* (Fergus & Sinden) Klopotek (1974), *Thielavia thermophila* Fergus & Sinden (1969)	CBS 405.69^{IT}	HQ871793	[16]	-
				JF412005	[10]	-
			CBS 174.70	HQ871792	[16]	-
			CBS 406.69^{T}	HQ871794	[16]	-
	Myceliophthora fusca Doyer (1927)	-	CBS 190.25^{T}	-	-	-
	Myceliophthora guttulata Zhang & Cai (2013)	-	CGMCC 3.15185^{T}	KC352943	[19]	-
	Myceliophthora heterothallica (Klopotek) van den Brink & Samson (2012)	*Corynascus heterothallicus* (Klopotek) Arx (1984); *Thielavia heterothallica* Klopotek (1976)	CBS 131.65	HQ871770	[16]	-
			CBS 202.75	HQ871771	[16]	-
			CBS 203.75	HQ871772	[16]	-
			CBS 375.69	HQ871773	[16]	-
			CBS 663.74	HQ871774	[16]	-
	Myceliophthora hinnulea Awao & Udagawa (1983)	-	CBS 597.83^{T}	HQ871791	[16]	-
			CBS 539.82	HQ871786	[16]	-
			CBS 540.82	HQ871787	[16]	-
			CBS 541.82	HQ871788	[16]	-
			CBS 542.82	HQ871789	[16]	-
			CBS 544.82	HQ871790	[16]	-
	Myceliophthora sulphurea Goddard	-	-	-	-	-

	(1913)					
	Myceliophthora thermophila (Apinis) Oorschot (1977)	*Chrysosporium thermophilum* (Apinis) Klopotek (1974); *Sporotrichum thermophilum* Apinis [as 'thermophile'] (1963)	CBS 117.65^{T} CBS 173.70 CBS 381.97 CBS 669.85 ATCC 42464	HQ871764 HQ871765 HQ871766 HQ871767 HQ871769	[16] [16] [16] [16] [16]	- - - - [2]
Myriococcum	*Myriococcum thermophilum* (Fergus) Aa (1973)	*Papulaspora thermophila* Fergus (1971)	CBS 389.93^{T}	JF412008	[10]	-
Rasamsonia	*Rasamsonia emersonii* (Stolk) Houbraken & Frisvad (2011)	*Talaromyces emersonii* Stolk (1965)	CBS 393.64^{T} CBS 396.64	JF417478 JF417479	[7] [7]	- -
	Rasamsonia byssochlamydoides (Stolk & Samson) Houbraken & Frisvad (2011)	*Talaromyces byssochlamydoides* Stolk & Samson (1972)	CBS 413.71^{T} CBS 533.71	JF417476 JF417477	[7] [7]	- -
Remersonia	*Remersonia thermophila* (Fergus) Seifert & Samson (1997)	*Stilbella thermophila* Fergus (1964)	ATCC 22073^{T}	JF412016	[10]	-
Scytalidium	*Scytalidium indonesiacum* Hegder, Samson, Basuki (1982)	-	CBS 259.81	-	-	-
	Scytalidium thermophilum (Cooney & Emers.) Austwick (1976)	*Phaeoscopulariopsis paisii* (Pollacci) Ota (1928); *Scopulariopsis paisii* (Pollacci) Nann. (1932); *Torula paisii* Pollacci [as 'pais'] (1921); *Torula thermophila* Cooney & Emers. (1964)	CBS 625.91^{T} CBS 621.91 CBS 622.91	JF412007 KF971714 KF971715	[10] - -	- - -
Sordaria	*Sordaria thermophila* Fields (1968)*	-	-	-	-	-
Thermoascus	*Thermoascus aegyptiacus* Ueda & Udagawa (1983)	*Coonemeria aegyptiaca* (Ueda & Udagawa) Mouch. (1997)		-	-	-
	Thermoascus aurantiacus Miehe (1907)	*Thermoascus aurantiacus* Miehe (1907) var. *aurantiacus*; *Thermoascus aurantiacus* var.	NRRL 5861	EU021617	[11]	-

		levisporus Upadhyay, Farmelo, Goetz & Melan (1984)				
	Thermoascus crustaceus (Apinis & Chesters) Stolk (1965)	*Coonemeria crustacea* (Apinis & Chesters) Mouch. (1997); *Coonemeria verrucosa* (Yaguchi, Someya & Udagawa) Mouch. (1997); *Dactylomyces crustaceus* Apinis & Chesters (1964) *Paecilomyces crustaceus* (Apinis & Chesters) Yaguchi, Someya & Udagawa (1995); *Thermoascus crustaceus* (Apinis & Chesters) Stolk (1965) var. *crustaceus*; *Thermoascus crustaceus* var. *verrucosus* Yaguchi, Someya & Udagawa (1995)	CBS 181.67^{T}	JF922031 JF412002 FJ389925	[11] [10] [13]	- - -
	Thermoascus taitungiacus Chen & Chen (1996)	-	8709-2^{T} (TAI-Mycology)	-	-	-
	Thermoascus thermophilus (Sopp) Arx (1970)	*Dactylomyces thermophilus* Sopp (1912); *Penicillium thermophilum* (Sopp) Sacc. (1931)	CBS 528.71^{NT}	-	-	-
Thermomyces	*Thermomyces ibadanensis* Apinis & Eggins (1966)	-	CBS 281.67^{T}	-	-	-
	Thermomyces lanuginosus Tsikl. (1899)	*Humicola brevis* var. *thermoidea* Subrahm. (1975); *Humicola brevispora* Subrahm. & Thirum. (1999); *Humicola grisea* var. *indica* Subrahm. (1980); *Humicola grisea* var. *thermoidea* Cooney & Emers. (1964); *Humicola insolens* var. *thermoidea* (Cooney & Emers.) Ellis (1982); *Humicola lanuginosa* (Tsikl.) Bunce [as 'lanuginosus'] (1961); *Humicola lanuginosa* var. *catenulata* Morinaga (1986); *Humicola lanuginosa* (Griffon & Maubl.) Bunce (1961) var. *lanuginosa*; *Monotospora lanuginosa* (Tsikl.)	CBS 632.91^{NT}	AY706335	[6]	-

			Mason (1933); *Sepedonium lanuginosum* (Tsikl.) Griffon & Maubl. (1911)				
		Thermomyces stellatus (Bunce) Apinis (1963)	*Humicola stellata* Bunce [as 'stellatus'] (1961); *Humicola stellata* var. *gigantea* Khanna [as 'giganteus'] (1963); *Humicola stellata* Bunce (1961) var. *stellata*	CBS 272.61^{T} CBS 241.62	- -	- -	- -
		Thermomyces thermophilus (Stolk) P.M. Kirk (2014)	*Talaromyces dupontii* (Griffon & Maubl.) Apinis (1963); *Talaromyces thermophilus* Stolk (1965)	CBS 236.58^{T}	JF412001	[8]	-
		Thermomyces verrucosus Pugh, Blakeman & Morgan-Jones (1964)	-	CBS 116.64^{T} CBS 113533	- -	- -	- -
	Thielavia	*Thielavia australiensis* Tansey & Jack (1975)	-	CBS 493.74	AJ271590 JF412017	[15] [10]	- -
		Thielavia terrestris (Apinis) Malloch & Cain (1972)	*Allescheria terrestris* Apinis (1963)	CBS 492.74 NBRC 9732	JX280874 AJ271589	[4] [15]	- -
		Anamorph: *Acremonium alabamense* Morgan-Jones (1974)		NRRL 8126	-	-	[2]
		Thielavia terricola (Gilman & Abbott) Emmons (1930)	*Chaetomium terricola* Gilman & Abbott (1971); *Thielavia minor* (Rayss & Borut) Malloch & Cain (1973); *Thielavia terricola* f. *minor* Rayss & Borut (1958); *Thielavia terricola* (Gilman & Abbott) Emmons (1930) f. *terricola*; *Thielavia terricola* var. *minor* (Rayss & Borut) Booth (1961); *Thielavia terricola* (Gilman & Abbott) Emmons (1930) var. *terricola*	CBS 165.71 CBS 611.74	JX280875 AJ271582	[4] [15]	- -
Basidiomycota							
	Thermophymatospora	*Thermophymatospora fibuligera* Udagawa, Awao & Abdullah (1986)	-	CBS 531.94 CBS 122040	- -	- -	- -

* Uncertainty may exist regarding the thermophilic nature *sensu* Cooney and Emerson (1964). ^{T} type material; ^{ET} ex-epitype; ^{NT} neotype; ^{IT} isotype.

ATCC: American Type Culture Collection (Manassas, Virginia, USA); BPI: US National Fungus Collections (Beltsville, Maryland, USA); BSRA: Herbarium, University of Basrah (Basrah, Iraq); CBS: Fungal Biodiversity Center (Ultrecht, Netherlands); NBRC: NITE Biological Resource Center, National Institute of Technology and Evaluation (Chiba, Japan); NRRL: Microbial Genomics and Bioprocessing Research, National Center for Agricultural Utilization Research, USDA/ARS (Peoria, Illinois, USA); CGMCC: China General Microbiological Culture Collection (Beijing, China).

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3 CHAPTER II

Fungal communities in pressmud composting harbor beneficial and detrimental fungi for human welfare

Status: Published

Citation:

OLIVEIRA, T. B.; LOPES, V. C. P.; BARBOSA, F. N.; FERRO, M.; MEIRELLES, L. A.; SETTE, L. D.; GOMES, E.; RODRIGUES, A. Fungal communities in pressmud composting harbors beneficial and detrimental fungi for human welfare. **Microbiology** v. 162, n. 7, p. 1147-1156, 2016. doi: 10.1099/mic.0.000306

Fungal communities in pressmud composting harbors beneficial and detrimental fungi for human welfare

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Running title: Fungi in pressmud composting

Key words: 454-pyrosequencing; thermophilic fungi; filter cake; lignocellulose; sugarcane

Contents category: Environmental Biology

Summary length: 238 words

Paper length: 4,179 words

Number of figures: 3

Number of Tables: 4

Deposited sequences: GenBank (NCBI) sequence database under accession numbers KU855380-KU855387. The de-multiplexed ITS dataset obtained using pyrosequencing was deposited in MG-RAST under project number 254695 (accession numbers 4689389.3–4689394.3).

ABSTRACT

Pressmud is a substrate derived from sugarcane juice filtrate and around 26 to 40 kg of this residue are produced per ton of sugarcane. It is mainly used as fertilizer in crops, but its application in the field is often made without any prior treatment, but in this research it was alerted for the risk of this practice for humans' health. Stimulated by previous results indicating the presence of opportunistic pathogens in residues used in various composting systems and the extensive use of fresh pressmud in agriculture, here the fungal diversity present in both fresh and composting pressmud was revealed using 454 pyrosequencing. Additionally, heat-tolerant fungi were isolated and surveyed for their enzymatic repertoire of biomass-degrading enzymes (cellulase, xylanase, laccase and polygalacturonase). A wide range of opportunistic pathogens was found among the most abundant taxa in the fresh pressmud, such as *Lomentospora prolificans* (43.13%), *Trichosporon* sp. (10.07%), *Candida tropicalis* (7.91%), and *Hormographiella aspergillata* (8.19%). This indicates that fresh pressmud might be a putative source of human pathogenic fungi, presenting a potential threat to human health if applied as fertilizer without any treatment. In regards to the heat-tolerant fungi found in this substrate, all the 110 isolates screened were able to produce at least one of the tested enzymes. The pressmud composting process not only effectively reduces the load of pathogenic fungi, but also creates an interesting environment for fungi able to produce thermostable hydrolytic and oxidative enzymes with biotechnological applications.

1. INTRODUCTION

Brazil is the largest sugar producer worldwide, with an estimated production of 654.6 million tons of sugarcane in 2015 (CONAB, 2015). Considerable amounts of by-products (i.e., pressmud, bagasse and molasses) are generated during the production process. Pressmud (or filter cake) is derived from sugarcane juice filtrate; it is rich in phosphorus, nitrogen, and organic matter and has high moisture content (Prado et al., 2013). Approximately 26 to 40 kg of this residue are produced after crushing one ton of sugarcane (Bhosale et al., 2012).

Pressmud is largely used as fertilizer in crops and it is often applied without any treatment (as for example, composting) (Balakrishnan & Batra, 2011). Composting is one of the possible treatments and it concerns in a biotransformation process of solid organic matter into a matured and stabilized substrate due to microbial action under aerobic conditions. Usually, three different phases can be recognized during the composting process: (1) mesophilic phase (up to 40 °C), which usually lasts for a couple of days; (2) thermophilic (over 40 °C), which can last from a few days to several days; and (3) mesophilic curing or maturation phase (up to 40 °C) which can last for several months (Mehta et al., 2014).

The potential advantages of composting pressmud include: (i) the production of a marketable product, (ii) reduction of offensive odors caused by rotting, (iii) reduction of environmental pollution by solid waste, (iv) weight reduction of the residue as a result of drying and decomposition of organic matter and (v) an increase in the nutrient concentration in the pressmud due to the removal of water and cellulose (Bernhardt & Notcutt, 1993).

Although several environments have been studied using massive parallel sequencing just a few composting systems were investigated (De Gannes et al., 2013;

Langarica-Fuentes et al., 2014a, 2015). These studies demonstrated a large diversity of fungi including several sequences that could not be assigned to known taxa and may represent novel species. Additionally, opportunistic human pathogens have been found in residues used in composting processes (De Gannes et al., 2013) but their occurrence in fresh pressmud is still unknown. On the other hand, because composting often reaches high temperatures, it may harbor a high diversity of heat-tolerant fungi with relevance for biotechnological purposes (Oliveira et al., 2015).

Here, the diversity and succession of fungi in a pressmud composting system were characterized using high-throughput 454-pyrosequencing of ITS libraries from the fresh pressmud and during the composting process (thermophilic and maturation phases). Furthermore, the occurrence of opportunistic pathogens in the fresh pressmud and throughout the composting process was evaluated. In addition, heat-tolerant fungi were isolated in the fresh pressmud and in the different stages of the composting process to access their enzymatic potential for biomass conversion.

2. MATERIAL AND METHODS

2.1 Experimental heaps

Approximately 1 ton of fresh pressmud was sampled to assemble three composting heaps (A, B and C, see Supplementary Figure 1). The substrate was collected from Usina Santa Lucia (Araras, SP, Brazil) during the sugarcane harvesting season in 2013. Initially, the whole raw material was mixed and moistened. Then, three piles were assembled in well-ventilated and sheltered area using the windrow method, which consists placing the residue in long narrow piles that are generally revolved to improve oxygen content. The piles were placed 1 m apart from one another and each had dimensions of 0.8 meters high, 1 m wide and 1.5 m long.

2.2 Sampling and physico-chemical analysis

After mixing and moistening the fresh pressmud, samples from 5 to 10 cm below the surface were taken at various points from the raw substrate, then combined into a composite sample and stored in polyethylene bags. The same procedure were done for each pile during the thermophilic and maturation phases, except for the thermophilic phase of the pile A which lasted only three days and it did not reach high temperatures.

The organic carbon content of the compost was estimated by the weight loss using the combustion method at 430 °C for 24 h (Nelson & Sommers, 1982), and the total amount of carbon in the sample was determined by dividing the percentage of observed organic matter content in the sample by the correction factor 1.8, based on the assumption that humified organic matter contains around 54% C (Jiménez & García, 1992). Total nitrogen was measured by the Kjeldahl method following digestion in sulfuric acid with catalysts (Bremner & Mulvaney, 1982).

The temperature of all piles was monitored daily over the first month of composting and twice a week during the second month using a digital soil thermometer with a 50 cm rod-shaped sensor (Instrutherm, São Paulo, SP, Brazil).

2.3 Assessing fungal diversity by pyrosequencing

A total of six samples were collected as described in item 2.2 (Fresh pressmud; thermophilic phase of piles B and C; and maturation phase of piles A, B and C) and maintained at -80 °C. DNA extraction was carried out using PowerSoil DNA Isolation Kit (MO-BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol. A total of 0.3 g of substrate from each pile was used for DNA extraction.

DNA concentration and quality were checked in NanoDrop (Thermo Scientific, Waltham, MA, USA) and stored at -20 °C.

ITS amplification, library preparation and sequencing were performed by Macrogen (http://www.macrogen.com, Seoul, Korea). Briefly, ITS region of fungi belonging to each sample was amplified using the ITS1F-ITS4 primer pair. These primers were connected with a barcode sequence, to identify each library, and an adaptor region. Amplicons derived from all samples were pooled together in an equimolar ratio and the sequencing was performed in a 454-pyrosequencing Genome Sequencer FLX Plus (Life Sciences, Branford, CT, USA).

2.4 Analysis of pyrosequencing data

Sequence processing, grouping in Operational Taxonomic Units (OTUs), taxonomic affiliation and diversity metrics were performed in MacQIIME v.1.8.0 (Caporaso et al., 2010). The raw sequences were filtered for quality (Phred >25), length (>400 bp) and homopolymer (length > 6bp). Quality-checked sequences were then submitted to *de novo* chimera detection in UCHIME (Edgar et al., 2011) using the USEARCH v. 5.2.236 (Edgar, 2010) pipeline in MacQIIME.

After processing, sequences with 97% of similarity were clustered in OTUs in USEARCH. A representative sequence of each OTU was selected using *pick_rep_set.py* script in MacQIIME. For taxonomic assignment, it was used the RDP classifier (minimum confidence of 0.5) (Wang et al., 2007) against UNITE+INSDC database (https://unite.ut.ee, Abarenkov et al., 2010). To confirm the taxonomic assignment of the most abundant OTUs, sequences were compared with similar sequences available in NCBI-GenBank database (http://www.ncbi.nlm.nih.gov/) using MEGABLAST algorithm (Morgulis et al., 2008). After reviewing the results of the OTU clustering,

there were still OTUs unassigned to any fungal phyla; BLAST searches of representative sequences of these OTUs revealed that they were likely chimera sequences that passed through to the *de novo* detection; therefore, such OTUs were removed before the diversity analyses.

Fungal diversity in composting heaps was evaluated by alpha diversity indices (Shannon and Simpson), richness estimator (Chao1) and rarefaction curves. Beta-diversity between fresh pressmud and different composing phases was evaluated by similarity indices (Bray-Curtis) and Principal coordinate analysis (PCoA) calculated in MacQIIME. To determine differences in fungal community structure between the samples from the different composting phases, an analysis of similarity (ANOSIM) was used based on the Bray–Curtis distance matrix. Then, differences in the initial and final proportion of pathogenic fungi were assessed by the Chi-square test ($P < 0.05$) in R v. 3.0.1 (R Development Core Team 2013).

The de-multiplexed ITS dataset obtained using pyrosequencing was deposited in MG-RAST under project number 254695 (accession numbers 4689389.3–4689394.3).

2.5 Isolation and identification of thermophilic and thermotolerant fungi

To obtain heat-tolerant fungi with biomass-degrading potential it was performed isolation from the fresh pressmud and in the thermophilic and maturation phases of composting. Samples of 10g from each pile and each composting stage (as described in item 2.2) were subjected to serial dilution. Then, 100 μL from the 10^{-3} to 10^{-5} dilutions were spread onto two standard culture media widely used for thermophilic fungi (Salar & Aneja, 2007) (in g L^{-1}): (1) Yeast Starch Agar (4 g yeast extract, 15 g soluble starch, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$ and 20 g agar); (2) Yeast Glucose Agar (5 g yeast extract, 10 g glucose and 20 g agar) and a third medium (nutrient-balanced medium):

(3) Malt Extract Agar 2% (MA2%) (20 g malt extract and 15 g agar). Both the yeast starch agar and yeast glucose agar were supplemented with 50 mg L^{-1} rose bengal to restrict the development of fast-growing fungi. All media were supplied with 30 mg L^{-1} streptomycin sulfate and penicillin G (Sigma-Aldrich, St Louis, MO, USA) to inhibit bacterial growth. Plates were incubated at 45 °C for five days and monitored daily.

All fungi recovered from the substrates were purified on MA2% and subjected to morphological screening. Representative isolates of each morphospecies were identified using morphological (macro and microscopic characteristics; see APPENDIX in the end of the thesis) and molecular (sequencing of the most appropriated molecular marker, ITS or β-tubulin, as described below) approaches.

For each morphospecies, genomic DNA from representative isolates was extracted by physical lysis with glass beads (425-600 μm diameter) following a combined protocol from Moller *et al.* (1992) and Gerardo *et al.* (2004) using fresh mycelia. After DNA extraction, the ITS region or β-tubulin gene were amplified with primers ITS4 and ITS5 (White et al., 1990) for the ITS region or βT2a and βT2b (Glass & Donaldson, 1995) for β-tubulin. Amplification reactions consisted of 0.2 mM of each dNTP, 5x KCl buffer, 1.5 mM $MgCl_2$, 0.5 μM of each primer and 1U of Taq polymerase (Promega, Madison, WI, USA) and 2 μL of DNA in a final volume of 25 μL. Amplicon purification was performed using the Wizard SV Gel and PCR Clean-up System kit (Promega, Madison, WI, USA), and amplicons were quantified in the NanoDrop (Thermo Scientific, Waltham, MA, USA). The sequencing reaction was performed using the BigDye Terminator Cycle Sequencing kit v.3.1 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol and applied to the ABI 3330 sequencer (Life Technologies, Carlsbad, CA, USA).

Forward and reverse sequences were compiled in contigs using BioEdit v7.1.3. The contigs were compared with homologous sequences deposited in NCBI-GenBank and the database of the Fungal Biodiversity Centre (CBS-KNAW). To confirm the species identification, the sequences were subjected to phylogenetic analysis in MEGA v.5.05 using additional sequences from reference strains (mostly from CBS-KNAW). The sequences were deposited in the GenBank (NCBI) sequence database under accession numbers KU855380-KU855387. The isolate TBO264 was identified only by morphology.

2.6 Enzymatic profiling of heat-tolerant fungi

To test the enzymatic potential of the heat-tolerant strains for biomass conversion it was performed a screening of four different enzymes involved in the process (cellulase, xylanase, laccase and polygalacturonase). A total of 110 fungi isolated from the composting heaps were submitted for enzymatic screening. Isolates identified as *Aspergillus fumigatus* were excluded from the screening because this fungus is considered to be a potential opportunistic pathogen, although it is a good producer of some of the evaluated enzymes. The strains maintained on MA2% slants at 25 °C were subcultured in MA2% and incubated at 45 °C for five days prior to enzymatic screening.

The screening for fungi that produce hemicellulolytic enzymes (cellulase and xylanase) was carried out cultivating fungi in microtubes (2 mL) containing 1.1 mL of specific liquid medium to induce enzyme production. The medium used to induce cellulase production consisted of 0.2%, $NaNO_3$ 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.2% CMC and 0.02% peptone, in 100 mL of distilled water (Kasana et al., 2008). To induce

the production of xylanases the medium consisted of: YNB 0.67%, xylan (beechwood) 1%, in 100 mL of distilled water (Strauss et al., 2001).

The tubes were incubated for eight days at 45 °C under agitation (120 rpm). Then, the tubes were centrifuged at 3000 rpm for 10 min and the supernatant recovered for enzyme extraction. Next, 50 µL of the extracts were added to sterilized plastic cylinders and arranged in plates containing medium specific for each enzyme. The plates were incubated at 45 °C in the dark for 24 hours.

The result was considered positive when a defined halo was observed after washing the plates with specific dyes for each substrate. For xylanases, we used Gram's Iodine for 5 min. After that, the dye was poured and washed with distilled water. For cellulases, we used 0.1% Congo red for 20 min. After this time, the plates were covered with a NaCl 1M solution for 20 min.

The screening for polygalacturonase and laccase-producing fungi was performed in individual plate assays. The medium to induce the polugalacturonase activity consisted of 50.0 mL (pH 5.5) of 2.5% polygalacturonic acid, 0.67% YNB, 0.2% glucose and 0.5% agarose, in 50 mL of 100 mM KH_2PO_4 (pH 5.5) buffer (McKAY, 1988). To detect the production of laccase, the medium consisted of 1% glucose, 0.02% peptone, 0.1% yeast extract, 2% agar, 4 mM guaiacol in 100 mL of distilled water (D'Souza et al., 2006). To detect the poligalacturonase acitivity, we used a solution of 0.1% rutenio red for 6-10 min. For laccase, the production of a brown halo around the colony was indicative of a positive result.

3. RESULTS

3.1 Physico-chemical analysis of pressmud composting

The composting heaps quickly reached the thermophilic phase. Pile C reached the thermophilic phase (temperature above 40 °C) on the second day of composting, while piles A and B reached the thermophilic phase on the third day (Figure 1). Pile A remained in the thermophilic phase from the 3rd to the 5th day and reached 46 °C (Figure 1). Pile B remained in the thermophilic phase from the 3rd to the 14th day of composting and reached a temperature peak of 52 °C. Pile C remained in the thermophilic phase from the 2nd to the 13th day and reached the highest temperature peak of 55 °C (Figure 1). Because pile A reached a maximum temperature of 46 °C followed by the maturation stage, no sampling was performed during the thermophilic phase for this heap.

The C/N ratio of the initial compost heaps was 29.10 (Table 1). This ratio decreased during the maturation stage for the three piles, mostly due to the increased N content compared to the beginning of the process. Pile A showed the lowest change in the C/N ratio (Table 1).

3.2 Massive parallel sequencing of pressmud composting system

A total of 148,643 sequences were obtained of the six samples analyzed. After processing, the final dataset consisted in 85,605 sequences, with an average of 14,267.33 ± 1,712.38 sequences per library. All sequences were clustered in 175 OTUs (see Supplementary Table 1).

Fungi of the phylum Ascomycota were predominant throughout the composting process (70.3% of the total sequences), followed by Basidomycota (17.7%). It was also found representatives of the phylum Chytridiomycota (fungi that need water to

reproduce), however in low amounts (lower than 0.01%). The remaining sequences (12%) were classified as unidentified fungi.

Regarding the taxa comprising the phylum Ascomycota, the most abundant order was Microascales (24.6% of the sequences), followed by Eurotiales and Sordariales (16.8% both, Figure 2). Considering the phylum Basidiomycota the most abundant order was Agaricales (6.9%, Figure 2). The most abundant OTUs (>1% of the total sequences) found in the composting piles are summarized in the Table 2 (the full list is available in supplementary Table 2).

A marked predominance of *Lomentospora prolificans* (Microascales, 43.13% of the sequences) was observed in the fresh pressmud. In addition, it was found in the same substrate *Thermomyces lanuginosus* (Eurotiales, 10.44%), a thermophilic fungus often associated with composting systems, and *Trichosporon* sp2 (Trichosporonales, 10.06%, Table 2).

In the thermophlic phase, *L. prolificans* was found as the most abundant OTU in pile B (43.65%), however, in pile C, which achieved the highest temperature (55 °C), there was a drastic reduction in the abundance of this fungus (14.91%). In contrast, the abundance of *T. lanuginosus* during this phase was reduced in the pile B but increased in pile C (7.47 and 21.57%, respectively). Furthermore, *Mycothermus thermophilum* (Leotiomycetidae), also considered a thermophilic fungus, and *Zopfiella* species (Sordariales) were the most abundant in pile C (11.35 and 13.17%, respectively).

During the maturation phase of the compost, *L. prolificans* abundance was reduced in piles B and C (5.67 and 8.97%, respectively), but the same did not occur in pile A (19.42%), which did not reach high temperature (maximum of 46 °C) and the thermophilic phase lasted for only three days. Furthermore, *T. lanuginosus* became the most abundant in piles B and C during the maturation phase. However, in pile A there

was a higher growth of Basidiomycetes fungi such as *Coprinus cordisporus* (Agaricales), *Trichosporon* sp1, and *Coprinus* sp. (13.39, 11.09 and 6.25%, respectively) when compared to the other piles.

It is worth mentioning the presence of a wide range of fungi considered opportunistic pathogens among the most abundant taxa in the fresh pressmud, such as *L. prolificans* (43.13%), *Trichosporon* sp. (10.07%), *Candida tropicalis* (Saccharomycetales, 7.91%), *Hormographiella aspergillata* (Agaricales, 8.19%). However, all these fungi had a remarkably reduced load ($P < 0.05$) after the thermophilic stage, especially in piles B and C, where temperatures were higher.

Along the pressmud composting process, there was a slightly tendency to increase the number of species (see Supplementary Table 3). Fresh pressmud showed 83 OTUs; 80 and 89 OTUs were observed during the thermophilic phase of piles B and C, respectively. Lastly, the highest number of OTUs was observed in the maturation phase in the three composting piles (mean of 116.7 ± 6.1 OTUs, see Supplementary Table 2).

The species richness estimator Chao1 showed that the number of observed taxa was close to the expected in almost all samples (see Supplementary Table 2), corroborating the rarefaction analysis of OTUs at 97% of similarity (see Supplementary Figure 2); this indicates that sampling successfully assessed a comprehensive part of the fungal diversity present in the pressmud composting. Alpha diversity indices (Shannon and Simpson) followed a similar pattern as OTUs richness, showing a tendency to increase throughout the composting process (see Supplementary Table 2). Furthermore, Bray-Curtis similarity index showed that different piles share an average of 58.5 ± 9.3% of OTUs, even considering the same stages of composting.

The ANOSIM test confirmed that the observed community composition were not significantly different among the different stages (fresh pressmud, thermophilic and

maturation phases) (R = 0.545, $P > 0.1$). However, as the diversity increased in the piles, fungal diversity became more distinct, indicating succession over the composing process; the greatest differences were found mainly when comparing the thermophilic (T2) to the maturation phase (T3) (see Supplementary Table 2). PCoA confirms that the fungal community structure in the end of the composing process (maturation phase) trend to be different from the initial and thermophilic phases (Figure 3). However, PCoA also confirmed distinguishable microbial communities when comparing different piles at the same stage, possible because piles reached likely due to the different temperatures reached in the termophilic phase (e.g., piles B and C ended up with a similar fungal community in the maturation phase, different from pile A, which did not reach high temperatures; Figure 3). The change in dominance of the opportunistic pathogen *L. prolificans* seems to be the mainly influencing factor in this variance. PCoA corroborates this fact, since very high percentages were found in the fresh pressmud sample and in the thermophilic phase for pile B, but lower values were found for the other samples, especially for the mesophilic phase in piles B and C (Figures 2 and 3).

3.3 Assessment of heat-tolerant fungi and their biomass-degrading enzymes

A total of 332 heat-tolerant fungi were isolated from the six data points (fresh pressmud, thermophilic stage of piles B and C and maturation stage of piles A, B and C, Table 3). The temperature of the fresh pressmud was 25 °C and eight fungal species were obtained from this substrate. During the thermophilic phase, seven and six fungal species were recovered from piles B (52 °C) and C (55 °C), respectively. During the maturation phase, the temperature of pile A was 27 °C and the temperature of piles B

and C were 32 °C. Three, five and eight fungal species were obtained from these piles, respectively (Table 3).

Among all the isolates obtained, five species belonged to the phylum Ascomycota (91.6% of the isolates) and four to the subphylum Mucoromycotina (8.4%). Fungi in the phylum Ascomycota comprised the orders Eurotiales (*Aspergillus fumigatus* and *T. lanuginosus*), Onygenales (*Myceliophthora fergusii* and *M. thermophila*) and Helotiales (*S. thermophila*). All representatives of Mucoromycotina belonged to the order Mucorales (*Lichtheimia ramosa, Rhizomucor mihei, Rhizopus microsporus* and *Thermomucor indicae-seudaticae*).

A. fumigatus was the most abundant species isolated in culture (66.3% of the isolates), followed by *T. lanuginosus* (16%). Both fungi were prevalent and consistently isolated in all samples. Other species were also observed in the different stages of composting, although they were not common to all stages of each pile (i.e., *M. fergusii*, *M. thermophila*, *R. pusillus* and *T. indicae-seudaticae*, Table 3). The thermotolerant species *L. ramosa* was found only in the fresh pressmud. *M. thermophilum* was present in the fresh pressmud but was not observed during the thermophilic phase. Later in the maturation stage, this species reappeared in piles B and C. *Rhizopus microsporus* appeared during the thermophilic phase and remained during the maturation phase.

Regarding to the enzymatic potential of the fungi, all isolates were positive at least for one of the tested enzymes (Table 4). Strains belonging to the same species presented the same enzymatic profile. The majority of fungi was positive for xylanase (60.9% of the total number of isolates), followed by cellulase and laccase (44.5% for both) and polygalacturonase (30.9%).

The production of laccase was observed only for *T. lanuginosus* isolates, which were negative for cellulase. The fungal genera *Lichtheimia, Rhizomucor, Rhizopus* and

Thermomucor produced polygalacturonase and xylanase. Furthermore, *T. indicae-seudaticae* was the only one able to produce three different enzymes (Table 4). Fungi in the genus *Myceliophthora* are most likely involved in the production of cellulolytic enzymes and were the only fungi that did not show the production of xylanase.

4. DISCUSSION

The massive ITS sequencing revealed the dynamics of the fungal communities present in fresh pressmud and their change over the composting process, raising new information about the species present in the system. An increase of fungal diversity from the fresh pressmud to the maturation phase was observed. In contrast, other studies with non-sugarcane material, which also used culture-independent techniques to assess fungal diversity, showed an opposite trend, with reduction of fungal diversity after the thermophilic phase (Tiquia, 2005; Bonito et al., 2010; Hultman et al., 2010). However, De Gannes *et al*. (2013) evaluated three different composting systems (rice straw, sugarcane bagasse and coffee hulls) and observed the sugarcane bagasse composting follows the same pattern found in pressmud composting, indicating that the increasing of fungal diversity might be a characteristic of sugarcane residues and its composting process. Furthermore, Ascomycota is often the prevalent phylum in composting systems as observed in pressmud composting, generally comprising more than 60% of the sequences (Langarica-Fuentes et al. 2014a; 2015) or in some cases reaching up to 93% (De Gannes et al., 2013).

Ghazifard et al. (2001) reported that the increase in temperature caused a modification of the microbiota, reducing the diversity of mesophilic fungi and leading to an increase in the diversity of heat-tolerant fungi. In pressmud composting, the increase in temperature most likely led to an increase in the microbial load of some

species (manly heat-tolerant species) over the reduction of others (mesophilic and pathogens), which did not withstand the temperature reached during the thermophilic phase, instead of alter species composition.

Fresh pressmud is often applied directly in sugarcane crops, without any prior treatment (Balakrishnan & Batra, 2011). It is noteworthy that, *L. prolificans* along with *C. tropicalis* accounted for more than 50% of the sequences in the fresh pressmud and both are classified as Biosafety Level 2 microorganisms by the American Biological Safety Association (ABSA).

Considering specifically *L. prolificans*, it is an emerging opportunistic pathogen with high levels of resistance to most antifungal drugs and infections caused by this fungus are often fatal (Berenguer et al., 1997; Song et al., 2011). However, basic aspects of its biology, such as the natural reservoir, are still poorly known; clarifying these issues is necessary to prevent exposure of immune-compromised individuals to this fungus (Thornton et al., 2015). Previously, the prevalence of *L. prolificans* was also observed in the mesophilic phase of sugarcane bagasse composting (De Gannes et al., 2013) suggesting that sugarcane, among other environments, might be a natural reservoir of this fungus.

Langarica-Fuentes *et al.* (2014b) evaluated the diversity of fungi in two commercial compounds in room temperature and after exposure to heating (50 °C). One of the compounds at room temperature, the authors observed the presence of *L. prolificans*, although in low abundance (1.6%). However, this fungus was not present among the most abundant taxa (>0.3%) after heating both compounds up to 50 °C.

Supported by indirect results from De Gannes *et al.* (2013) and Langarica-Fuentes *et al.* (2014b), the notably reduction of *L. prolificans* at the end of the pressmud composting process highlights the composting as a promissing treatment for pressmud

residues before their direct application in agriculture. Specifically, the reduction in *L. prolificans* is possibly due to the natural community succession since this fungus is not adapted to high temperatures. Therefore, the heat treatment derived from pressmud composing systems could potentially reduce the chances of handlers to be exposed to high amount of the emerging pathogen during application of this substrate in the field. This is also valid for *C. tropicalis* and the other pathogenic fungi present in the pressmud, which were also decreased in abundance over the composting process.

Studies on the diversity of fungi in composting systems employing culture-dependent tools commonly report *Aspergillus fumigatus* as prevalent (Ghazifard et al., 2001; Dehghani et al., 2012). *A. fumigatus* was also found as the prevalent species isolated in piles of pressmud composting (66.3% of the total isolates). However, here this fungus was not present among the most abundant when using a culture-independent method, suggesting that this fungus does not play an important role in the system but it has the advantage to grow rapidly in the culture media (i.e. a copiotrophic fungus). On the other hand, *T. lanuginosus* was found among the most abundant when it was assessed with both culture-dependent and independent methods. This fungus is thermophilic and often associated with various composting systems in which it is found among the most abundant taxa (Mchunu et al., 2013; Langarica-Fuentes et al., 2014a, b). During the isolation survey *L. prolificans* was not obtained, possibly because it was performed at high temperatures (45 ºC), targeting isolation of thermophilic fungi.

Although fresh pressmud is rich in pathogenic fungi, it composting process contains a variety of fungi with biotechnological potential, as for example the production of enzymes for biomass conversion. *T. lanuginosus* has been reported to be the largest producer of cellulase-free xylanases, which has prompted the use of genome sequencing to assess the genetic potential of this species for industrial applications

(Mchunu et al., 2013). Zhou *et al.* (2014) exploited the genome of *Rhizomucor mihei* and reported the existence of a large number of genes encoding proteolytic, amylolytic and lipolytic enzymes including xylanase and β-glucanase, revealing the potential of this fungus for the degradation of organic matter.

Several studies have suggested that *M. thermophila* (Moretti et al., 2012; Pereira et al., 2015) and *M. heterothallica* (Van Den Brink et al., 2013) are interesting bioconverters of lignocellulosic residues to simple sugars. Furthermore, the genomic study of *M. thermophila* and *Thielavia terrestris* suggested that they were capable of hydrolyzing all of the major polysaccharides present in the plant biomass (Berka et al., 2011). *M. thermophila* has the largest number of hemicellulolytic enzymes and accessory enzymes observed to date; it contained eight genes encoding endoglucanases, seven cellobiohydrolases, nine β-glucosidases, 25 lytic polysaccharide monooxygenases (LPMOs), and other enzymes of the group including xylanase, arabinases, mannanase, pectinases and esterases (Karnaouri et al., 2014).

Therefore, we alert for the use of fresh pressmud, because it is a putative source of human pathogenic fungi and it presents a potential threat if applied as fertilizer without any treatment. However, composting with thermophilic phase minimizes the amount of opportunistic pathogenic fungi in this substrate. At the same time, the pressmud composting process creates an interesting environment to survey fungi able to produce thermostable hydrolytic and oxidative enzymes. Such fungi have the potential to be used as a starter culture consortium to accelerate the composting process of pressmud, other plant wastes or for industrial purpose.

ACKNOWLEDGEMENTS

The authors would like to thank FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) for providing financial support to AR (Young Research Award # 2011/16765-0) and for a scholarship to TBO (# 2012/14594-7). We are also grateful to all members of our research group for providing helpful comments on this manuscript. This work was performed under permit #010554/2014-9 issued by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico).

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TABLES

Table 1. Chemical characterization of pressmud composting of three piles during different phases.

Sample	Nitrogen (%)	Organic Matter (%)	Carbon (%)	C/N
Fresh pressmud*	1.41	73.81	41.10	29.10
T2B	1.84	77.65	43.14	23.46
T2C	1.77	74.33	41.29	23.29
T3A	1.70	79.87	44.37	26.12
T3B	1.94	69.39	38.55	19.86
T3C	1.98	65.70	36.15	18.27

* Fresh Pressmud: mesophilic phase. T2: thermophilic phase, T3: maturation phase, A, B and C indicate the different composting piles.

Table 2. Relative abundance (%) of the most abundant taxa (OTUs) in different stages of pressmud composting*.

Fungal taxa	Sample					
	Fresh Pressmud**	**T2B**	**T2C**	**T3A**	**T3B**	**T3C**
Lomentospora prolificans	43.13	43.65	14.91	19.42	5.67	8.97
Thermomyces lanuginosus	10.44	7.47	21.57	5.59	22.26	26.54
Mycothermus thermophilum	0.35	2.22	20.99	3.87	4.0	11.35
Trichosporon sp1	0.01	16.58	0.26	11.09	0.3	0.12
Trichosporon sp2	10.06	0.52	14.10	2.05	1.17	1.06
Zygopleurage zygospora	3.61	1.3	0.13	5.88	11.46	1.33
Coprinopsis sp.	0.04	9.4	10.82	0.49	0.19	0.28
Unidentified Fungus	4.23	0.72	0.65	3.67	10.42	1.92
Coprinus cordisporus	0.02	1.44	1.32	13.39	1.57	3.28
Zopfiella sp.	0.16	0.36	0.36	2.3	4.33	13.17
Coprinus sp.	-	3.5	3.82	6.25	1.49	1.15
Cercophora coronata	1.59	1.61	0.05	0.7	9.69	1.39
Candida tropicalis	7.91	1.8	0.65	0.3	0.64	0.29
Sordariales	0.04	0.63	0.53	2.4	2.11	6.64
Hormographiella aspergillata	8.19	1.3	1.08	0.03	0.02	0.04
Podospora communis	0.16	1.59	0.01	2.63	3.37	1.14
Cercophora sp.	0.12	0.82	0.42	0.35	5.17	0.43

* Considering the total number of sequences representing more than 1% of the sequences in each library.

** Fresh Pressmud: mesophilic phase. T2: thermophilic phase, T3: maturation phase, A, B and C indicate the different composting piles.

Table 3. Abundance of thermophilic and thermotolerant fungi during composting of pressmud

Fungal species	Mesophilic	Thermophilic		Maturing			Total	%
	Fresh pressmud	A*	B	C	A	B	C		
Aspergillus fumigatus	60		26	44	26	34	30	220	66.3
Lichtheimia ramosa	1							1	0.3
Myceliophthora fergusii	3		4	6			6	19	5.7
Myceliophthora thermophila	1		3	1	1		2	8	2.4
Rhizomucor pusillus	4		2	2			1	9	2.7
Rhizopus microsporus			3	1		5	2	11	3.3
Mycothermus thermophilum	1					2	1	4	1.2
Thermomucor indicae-seudaticae	1		2			2	2	7	2.1
Thermomyces lanuginosus	4		11	7	13	12	6	53	16
Total	75		51	61	40	55	50	332	100

* Fresh pressmud: mesophilic phase. T2: thermophilic phase, T3: maturation phase, A, B and C indicate the different composting piles. Because pile A did not reach >50 ºC, it was not sampled during the thermophilic phase (see methods)

Table 4. Enzymatic profile of thermophilic and thermotolerant fungi isolated from a pressmud composting system.

Fungal species	**Enzymes**			
	Cellulase	**Xylanase**	**Polygalacturonase**	**Laccase**
Lichtheimia ramosa		+	+	
Myceliophthora fergusii	+			
Myceliophthora thermophila	+			
Rhizomucor pusillus		+	+	
Rhizopus microsporus		+	+	
Mycothermus thermophilum	+	+		
Thermomucor indicae-seudaticae	+	+	+	
Thermomyces lanuginosus		+		+

FIGURE CAPTIONS

Figure 1. Temperature monitoring during composting of pressmud in three heaps (A, B and C). Composting stages are denoted in the figure.

Figure 2. Mycota (ranked by order) of the fresh pressmud and of the different composting stages. The different fungal orders are represented by different colors as well as by symbols. T1: fresh pressmud (mesophilic phase), T2: thermophilic phase, T3: maturation phase. A, B and C indicate the different piles.

Figure 3. Principal coordinate analysis (PCoA) of the fungal communities in a pressmud composting system. Fresh Pressmud (red), thermophilic phase (orange), maturation phase (blue).

FIGURES

Figure 1.

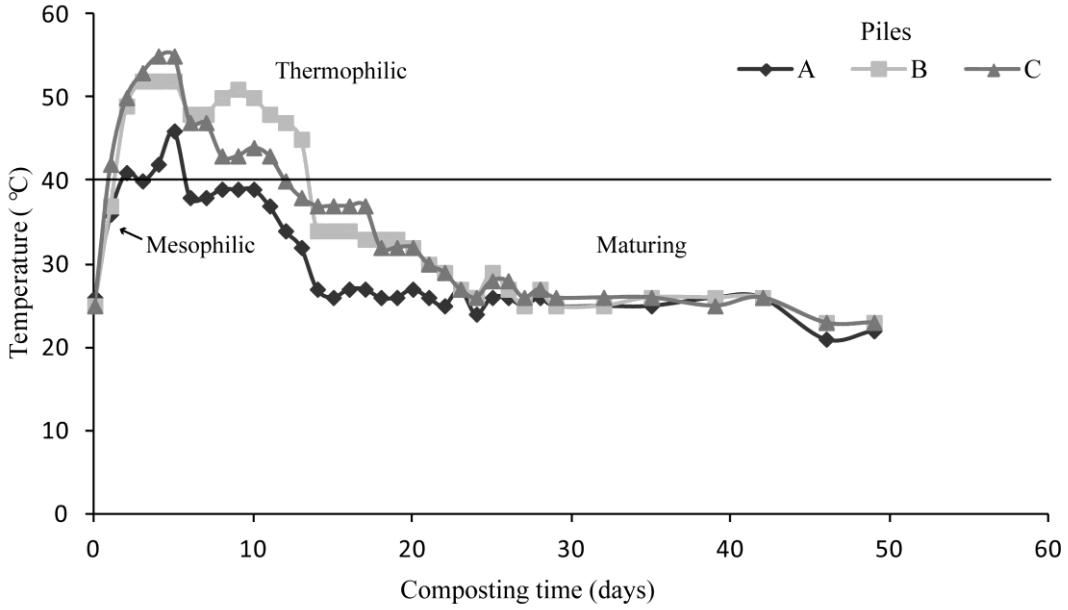
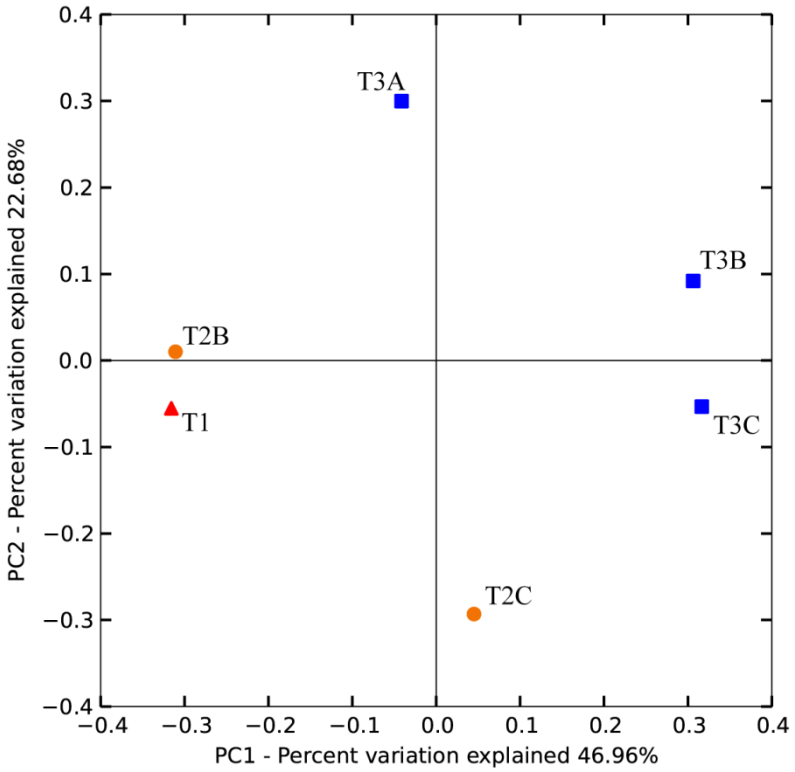


Figure 2.


Fresh Pressmud
T2B
T2C
T3A
T3B
T3C
MAIN TAXA DETECTED
Microascales
Eurotiales
Sordariales
Unidentified Fungi
Saccharomycetales
Other Basidiomycota
Agaricales
Other Tremellomycetes
Unidentified Ascomycota

Figure 3.


T3A
T3B
T2B
T1
T3C
T2C
PC2 - Percent variation explained 22.68%
PC1 - Percent variation explained 46.96%
0.4
0.3
0.2
0.1
0.0
−0.1
−0.2
−0.3
−0.4
−0.4
−0.3
−0.2
−0.1
0.0
0.1
0.2
0.3
0.4

SUPPLEMENTARY MATERIAL

Fungal communities in pressmud composting harbors beneficial and detrimental fungi for human welfare

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Figure S1. Assembling of composting piles using residues from sugarcane milling (Pressmud). Mixing (A), watering (B) and separation of the three composting heaps examined in this study (C).

Figure S2. Rarefaction analysis of the species observed during the composting process of the sugarcane pressmud. T1: Fresh Pressmud (mesophilic phase). T2: thermophilic phase; T3: maturation phase; A, B and C indicate the different piles.

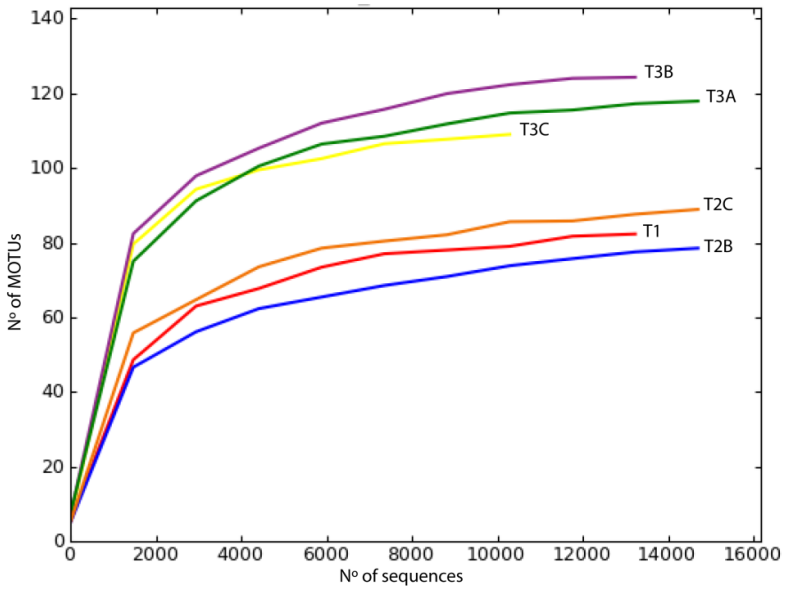

Table S1. Characteristics of the amplicon library before and after processing in QIIME (quality filter, sequence length and chimera).

Data	**Samples (# of sequences)**						**Total**
	Fresh Pressmud*	T2B	T2C	T3A	T3B	T3C	
Raw	20,835	26,912	24,534	27,596	28,406	20,360	148,643
QIIME	13,677	16,443	15,165	14,790	14,624	10,905	85,605
OTUs	83	80	89	118	122	110	175

* T1: Fresh Pressmud (mesophilic phase). T2: thermophilic phase, T3: maturation phase, A, B and C indicate the different composting piles.

Table S2. Taxonomic classification of the OTUs obtained by 454-pyrosequencing of the fresh pressmud and throughout the composting process (using the UNITE+INSDC* in MacQIIME).

#OTU ID	Fresh Pressmud**	T2B	T2C	T3A	T3B	T3C	Taxonomy
0	5884	7177	2260	2868	829	977	k__Fungi; p__Ascomycota
1	1424	1229	3271	826	3252	2891	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales
2	48	365	3183	572	584	1237	k__Fungi; p__Ascomycota; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedScytalidium
3	1	2727	39	1637	44	13	k__Fungi; p__Basidiomycota; c__Tremellomycetes
4	1373	86	2138	302	255	127	k__Fungi; p__Basidiomycota
5	492	214	20	868	1674	145	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Lasiosphaeriaceae
7	5	1546	1641	72	28	30	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified
6	577	118	98	542	1523	209	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified
10	3	237	200	1978	230	357	k__Fungi; p__Basidiomycota; c__Agaricomycetes; o__Agaricales; f__Coprinaceae; g__Coprinus; s__Coprinus_cordisporus
11	22	60	55	339	632	1435	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Lasiosphaeriaceae; g__Zopfiella
9	0	575	579	923	217	125	k__Fungi; p__Basidiomycota; c__Agaricomycetes; o__Agaricales; f__Coprinaceae; g__Coprinus; s__Coprinus_cordisporus
103	217	265	8	103	1415	151	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Lasiosphaeriaceae; g__Cercophora; s__Cercophora_coronata
12	1079	296	99	44	94	32	k__Fungi; p__Ascomycota; c__Saccharomycetes; o__Saccharomycetales; f__Incertae_sedis; g__Candida; s__Candida_tropicalis
14	6	103	81	354	308	723	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__unidentified; g__unidentified
15	1117	213	164	4	3	4	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified
16	22	261	2	389	493	124	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus
8	17	135	64	52	756	47	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Lasiosphaeriaceae; g__Cercophora; s__Cercophora_coronata
138	96	198	116	172	139	167	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Microascales; f__Microascaceae; g__Lomentospora; s__Lomentospora_prolificans
17	2	19	23	453	99	180	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Lasiosphaeriaceae; g__Zopfiella
19	47	3	6	574	4	19	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Sordariaceae
119	44	53	7	117	164	122	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Lasiosphaeriaceae
20	219	39	36	32	19	102	k__Fungi; p__Basidiomycota; c__Tremellomycetes; o__Tremellales
102	1	12	161	159	47	30	k__Fungi; p__Ascomycota
22	0	0	0	13	296	8	k__Fungi; p__Basidiomycota; c__Agaricomycetes; o__Agaricales; f__Psathyrellaceae; g__Coprinopsis

130	0	5	23	7	10	261	k__Fungi; p__Ascomycota; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedScytalidium
24	1	0	8	7	218	37	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Lasiosphaeriaceae; g__Cercophora
271	0	1	0	70	63	101	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Lasiosphaeriaceae
85	0	106	92	19	1	8	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified
25	54	18	14	56	21	57	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Hypocreales; f__Nectriaceae; g__Fusarium
21	130	24	0	16	31	1	k__Fungi; p__Ascomycota; c__Saccharomycetes; o__Saccharomycetales; f__Dipodascaceae; g__Galactomyces; s__Galactomyces_geotrichum
137	98	2	0	75	20	0	k__Fungi; p__Ascomycota; c__Saccharomycetes; o__Saccharomycetales; f__Dipodascaceae; g__Galactomyces; s__Galactomyces_geotrichum
234	22	14	42	15	42	28	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales
26	30	0	15	57	16	35	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales; f__Trichocomaceae; g__Paecilomyces
39	0	0	90	4	26	30	k__Fungi; p__Ascomycota
27	10	0	0	93	39	4	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Lasiosphaeriaceae; g__Cercophora
260	0	10	62	23	28	18	k__Fungi; p__Ascomycota; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedScytalidium
29	0	3	0	18	98	14	k__Fungi; p__Ascomycota; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedAscomycota
30	1	10	0	55	56	5	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus
276	7	14	26	12	38	27	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales; f__Incertae_sedis; g__Thermomyces; s__Thermomyces_lanuginosus
255	0	4	49	21	12	37	k__Fungi; p__Ascomycota; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedScytalidium
31	83	1	5	5	24	2	k__Fungi; p__Basidiomycota; c__Agaricomycetes; o__Polyporales; f__Phanerochaetaceae; g__Phanerochaete; s__Phanerochaete_chrysosporium
28	5	3	0	0	1	110	k__Fungi; p__Basidiomycota
33	0	0	6	3	35	75	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus
36	0	0	0	57	45	5	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus
37	0	1	30	33	25	12	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales; f__Trichocomaceae; g__Aspergillus; s__Aspergillus_fumigatus
40	8	0	0	64	1	23	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified
141	6	22	22	7	19	15	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales; f__Incertae_sedis; g__Thermomyces; s__Thermomyces_lanuginosus
41	53	21	4	6	4	0	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Hypocreales; f__Nectriaceae; g__Fusarium
93	0	24	35	17	6	4	k__Fungi; p__Basidiomycota; c__Agaricomycetes; o__Agaricales; f__Coprinaceae; g__Coprinus; s__Coprinus_cordisporus
42	0	0	32	0	52	1	k__Fungi; p__Ascomycota; c__Dothideomycetes; o__Pleosporales; f__unidentified; g__unidentified; s__unculturedPleosporales
43	0	0	0	6	0	76	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Microascales; f__Microascaceae
248	0	1	1	26	26	26	k__Fungi; p__Ascomycota; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedScytalidium

46	0	1	2	48	11	17	k__Fungi; p__Basidiomycota; c__Agaricomycetes; o__Agaricales
44	0	0	2	5	6	66	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus
45	5	0	0	48	16	6	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus
48	41	13	2	13	4	0	k__Fungi; p__Basidiomycota; c__Tremellomycetes
71	0	2	1	6	34	30	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Lasiosphaeriaceae; g__Zopfiella
262	2	14	12	11	19	15	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales; f__Incertae_sedis; g__Thermomyces; s__Thermomyces_lanuginosus
131	0	20	29	4	13	2	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified
51	0	1	15	1	7	41	k__Fungi; p__Ascomycota
50	0	0	6	9	0	48	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus
53	41	0	0	17	4	0	k__Fungi; p__Ascomycota; c__Saccharomycetes; o__Saccharomycetales; f__Dipodascaceae
215	1	0	0	43	5	11	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Lasiosphaeriaceae; g__Zopfiella
55	37	1	10	4	6	2	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Hypocreales; f__Hypocreaceae; g__unidentified
83	0	0	0	19	31	6	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus
52	0	0	0	0	0	55	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Lasiosphaeriaceae
84	0	3	9	19	14	8	k__Fungi; p__Ascomycota; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedScytalidium
269	0	1	3	36	6	6	k__Fungi; p__Basidiomycota; c__Agaricomycetes; o__Agaricales; f__Coprinaceae; g__Coprinus; s__Coprinus_cordisporus
220	0	3	32	4	2	11	k__Fungi; p__Ascomycota; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedScytalidium
154	41	4	3	0	0	0	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified
140	14	10	14	6	1	0	k__Fungi; p__Ascomycota; c__Saccharomycetes; o__Saccharomycetales; f__Incertae_sedis; g__Candida; s__Candida_tropicalis
56	23	0	0	0	4	15	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Hypocreales; f__Nectriaceae; g__Fusarium
88	0	0	0	11	5	26	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales
67	0	0	7	9	0	26	k__Fungi; p__Ascomycota; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedScytalidium
136	0	16	0	22	1	0	k__Fungi; p__Basidiomycota
266	6	5	9	6	6	6	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales; f__Incertae_sedis; g__Thermomyces; s__Thermomyces_lanuginosus
58	2	9	21	3	2	1	k__Fungi; p__Basidiomycota; c__Tremellomycetes; o__Filobasidiales; f__Filobasidiaceae; g__Cryptococcus; s__Cryptococcus_laurentii
81	15	5	3	0	4	10	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales
68	0	2	0	6	28	1	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus
235	0	19	3	10	1	4	k__Fungi; p__Basidiomycota; c__Agaricomycetes; o__Agaricales; f__Coprinaceae; g__Coprinus; s__Coprinus_cordisporus

76	9	1	11	14	0	0	k__Fungi; p__Ascomycota; c__Saccharomycetes; o__Saccharomycetales; f__Dipodascaceae; g__Galactomyces; s__Galactomyces_geotrichum
97	14	2	6	1	1	10	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Hypocreales; f__Hypocreaceae; g__Trichoderma
61	0	0	24	1	8	0	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales
60	0	0	1	18	13	0	k__Fungi; p__Chytridiomycota; c__Chytridiomycetes; o__Spizellomycetales; f__Spizellomycetaceae; g__Gaertneriomyces; s__Gaertneriomyces_semiglobifer
258	6	2	6	3	9	6	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales; f__Incertae_sedis; g__Thermomyces; s__Thermomyces_lanuginosus
63	0	0	1	1	0	29	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Hypocreales; f__unidentified; g__unidentified; s__unculturedHypocreales
62	0	0	0	27	0	4	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus
64	0	0	0	29	0	0	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedsoil_fungus
216	0	8	1	4	5	10	k__Fungi; p__Basidiomycota
202	0	9	9	7	2	0	k__Fungi; p__Basidiomycota; c__Agaricomycetes; o__Agaricales; f__Psathyrellaceae; g__Coprinopsis; s__Coprinopsis_sp_YM71
123	0	1	8	3	8	6	k__Fungi; p__Ascomycota; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedScytalidium
264	0	0	0	3	11	11	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Lasiosphaeriaceae; g__Zopfiella
161	0	0	13	8	3	1	k__Fungi; p__Ascomycota
205	0	10	2	12	1	0	k__Fungi; p__Basidiomycota; c__Agaricomycetes; o__Agaricales; f__Coprinaceae; g__Coprinus; s__Coprinus_cordisporus
75	25	0	0	0	0	0	k__Fungi; p__Ascomycota; c__Saccharomycetes; o__Saccharomycetales; f__Saccharomycetaceae; g__Debaryomyces; s__Debaryomyces_hansenii
73	0	17	0	0	3	5	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus
78	0	0	0	0	25	0	k__Fungi; p__Basidiomycota; c__Agaricomycetes; o__Cantharellales; f__Ceratobasidiaceae; g__Thanatephorus; s__Rhizoctonia_solani
86	0	0	0	5	14	5	k__Fungi; p__Ascomycota; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedScytalidium
79	5	0	5	0	2	12	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified
87	21	0	2	0	0	0	k__Fungi; p__Ascomycota
177	0	10	12	1	0	0	k__Fungi; p__Basidiomycota; c__Agaricomycetes; o__Agaricales
178	0	0	0	2	18	1	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Lasiosphaeriaceae; g__Zygopleurage; s__Zygopleurage_zygospora
89	21	0	0	0	0	0	k__Fungi; p__Ascomycota; c__Saccharomycetes; o__Saccharomycetales
149	0	0	0	0	15	6	k__Fungi; p__Ascomycota
129	0	2	6	0	10	2	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales; f__Incertae_sedis; g__Thermomyces; s__Thermomyces_lanuginosus
95	17	3	0	0	0	0	k__Fungi; p__Ascomycota; c__Saccharomycetes; o__Saccharomycetales; f__Dipodascaceae; g__Galactomyces; s__Galactomyces_geotrichum
232	1	1	5	1	8	4	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales; f__Incertae_sedis; g__Thermomyces; s__Thermomyces_lanuginosus

125	1	7	3	1	8	0	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales; f__Trichocomaceae
121	0	0	1	16	1	1	k__Fungi; p__Ascomycota; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedScytalidium
99	0	0	17	1	1	0	k__Fungi; p__Ascomycota; c__Orbiliomycetes; o__Orbiliales; f__Orbiliaceae; g__Dactylellina; s__Dactylellina_haptospora
101	0	0	1	0	10	7	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Hypocreales; f__Bionectriaceae; g__Mycoarachis; s__Mycoarachis_inversa
111	16	1	0	0	1	0	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Lasiosphaeriaceae; g__Zygopleurage; s__Zygopleurage_zygospora
112	0	0	0	0	16	2	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales; f__Incertae_sedis; g__Thermomyces; s__Thermomyces_lanuginosus
106	9	0	1	0	2	5	k__Fungi
105	0	0	0	8	4	5	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Lasiosphaeriaceae; g__Zopfiella
90	5	0	8	3	1	0	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales; f__Trichocomaceae; g__Penicillium
115	3	0	0	4	0	8	k__Fungi; p__Ascomycota; c__Orbiliomycetes; o__Orbiliales; f__Orbiliaceae; g__Arthrobotrys; s__Arthrobotrys_amerospora
113	5	0	10	0	0	0	k__Fungi; p__Ascomycota; c__Dothideomycetes; o__Pleosporales; f__Pleosporaceae; g__Cochliobolus
109	0	0	0	0	1	14	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales
261	0	0	0	0	6	8	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales
142	1	0	0	1	12	0	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified
120	12	1	0	0	1	0	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Hypocreales; f__Incertae_sedis; g__Acremonium
124	1	1	0	5	3	3	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Trichosphaeriales; f__Incertae_sedis
132	0	0	0	12	0	0	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales
257	1	4	5	0	2	0	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales; f__Trichocomaceae; g__Penicillium
148	0	0	0	0	0	12	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus
139	6	0	0	5	0	0	k__Fungi; p__Ascomycota; c__Dothideomycetes; o__Pleosporales; f__Sporormiaceae
193	0	0	0	11	0	0	k__Fungi; p__Ascomycota; c__Dothideomycetes; o__Pleosporales; f__Sporormiaceae; g__Preussia
143	5	0	1	0	4	1	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales; f__Trichocomaceae; g__Penicillium; s__Penicillium_commune
145	1	0	0	9	0	0	k__Fungi; p__Ascomycota; c__Dothideomycetes; o__Capnodiales; f__Mycosphaerellaceae; g__Passalora
153	0	10	0	0	0	0	k__Fungi
152	0	0	0	0	10	0	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales
165	9	0	0	0	0	0	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Hypocreales; f__Incertae_sedis; g__Myrothecium
170	0	0	0	0	0	9	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified
162	0	0	0	0	9	0	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus

183	0	0	0	0	4	5	k__Fungi; p__Ascomycota; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__Ascomycota_sp_AR_2010
168	3	0	0	1	5	0	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus
169	0	0	0	3	4	2	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Lasiosphaeriaceae; g__Cladorrhinum
158	0	0	0	0	9	0	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus
135	0	0	0	4	0	4	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus
179	0	8	0	0	0	0	k__Fungi; p__Basidiomycota; c__Tremellomycetes
176	5	0	0	3	0	0	k__Fungi; p__Ascomycota; c__Incertae_sedis; o__Incertae_sedis; f__Incertae_sedis; g__Dokmaia
182	0	0	0	0	0	8	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Hypocreales
190	0	0	0	0	8	0	k__Fungi; p__Ascomycota; c__Orbiliomycetes; o__Orbiliales; f__Orbiliaceae; g__Arthrobotrys; s__Arthrobotrys_musiformis
212	6	0	0	0	1	0	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Hypocreales; f__Incertae_sedis; g__Acremonium; s__Acremonium_sp_ATT126
213	0	1	0	0	0	6	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Hypocreales
198	0	0	7	0	0	0	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales; f__Trichocomaceae; g__Talaromyces; s__Talaromyces_byssochlamydoides
195	0	0	0	0	7	0	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__unidentified; g__unidentified
194	0	0	0	0	7	0	k__Fungi; p__Ascomycota; c__unidentified; o__unidentified; f__unidentified; g__unidentified
192	0	0	6	0	0	1	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus
196	0	0	0	7	0	0	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales; f__Trichocomaceae; g__Talaromyces; s__Talaromyces_emersonii
204	4	0	0	2	0	1	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Hypocreales
201	0	0	0	0	7	0	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified
210	0	0	0	6	0	0	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales
218	0	0	0	0	6	0	k__Fungi; p__Ascomycota; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedAscomycota
221	0	0	0	0	6	0	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Hypocreales
224	0	0	0	0	6	0	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales; f__Trichocomaceae; g__Penicillium; s__Penicillium_commune
226	2	0	0	3	0	0	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Xylariales; f__Amphisphaeriaceae; g__Pestalotiopsis
241	0	0	0	0	5	0	k__Fungi; p__Basidiomycota; c__Agaricomycetes; o__Agaricales
240	5	0	0	0	0	0	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Cephalothecaceae; g__Phialemonium
242	0	0	1	0	4	0	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales
250	0	0	0	2	0	3	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Lasiosphaeriaceae; g__Schizothecium
229	3	0	2	0	0	0	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales; f__Trichocomaceae; g__Penicillium

228	5	0	0	0	0	0	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified; s__unculturedfungus
238	5	0	0	0	0	0	k__Fungi; p__Ascomycota; c__Saccharomycetes; o__Saccharomycetales; f__Incertae_sedis; g__Candida; s__Candida_sp_AUMC_7768
239	5	0	0	0	0	0	k__Fungi; p__Ascomycota; c__Saccharomycetes; o__Saccharomycetales; f__Metschnikowiaceae; g__Clavispora; s__Clavispora_lusitaniae
230	0	0	0	0	0	5	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Lasiosphaeriaceae; g__Schizothecium
231	4	0	0	0	1	0	k__Fungi; p__unidentified; c__unidentified; o__unidentified; f__unidentified; g__unidentified
249	0	0	0	5	0	0	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales; f__Incertae_sedis; g__Thermomyces; s__Thermomyces_lanuginosus
166	0	0	0	4	0	0	k__Fungi; p__Ascomycota; c__Saccharomycetes; o__Saccharomycetales; f__Dipodascaceae
263	0	0	0	4	0	0	k__Fungi; p__Ascomycota; c__unidentified; o__unidentified; f__unidentified; g__unidentified
268	0	0	0	4	0	0	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Chaetomiaceae; g__Chaetomium
259	0	0	0	1	0	3	k__Fungi; p__Ascomycota; c__Eurotiomycetes; o__Eurotiales; f__Trichocomaceae; g__Paecilomyces
253	0	0	0	0	4	0	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Sordariales; f__Lasiosphaeriaceae; g__Zygopleurage; s__Zygopleurage_zygospora
272	4	0	0	0	0	0	k__Fungi; p__Ascomycota; c__Sordariomycetes; o__Ophiostomatales; f__Ophiostomataceae; g__Sporothrix; s__Sporothrix_sp_KUC4053

* INSDC: International Nucleotide Sequence Database Collaboration.

** T1: Fresh Pressmud (mesophilic phase). T2: thermophilic phase, T3: maturation phase, A, B and C indicate the different composting piles.

Table S3. OTUs richness, species richness estimator and diversity indices of OTUs in the fresh pressmud and throughout the composting process.

Sample	Richness	Richness estimator	Diversity indices	
	S	**Chao 1**	**Shannon**	**Simpson**
Fresh Pressmud*	83	99.5	3.15	0.77
T2B	80	91	3.06	0.76
T2C	89	94.6	3.40	0.85
T3A	118	131.75	4.35	0.91
T3B	122	137.6	4.26	0.90
T3C	110	114.5	4.15	0.88

* T1: Fresh Pressmud (mesophilic phase). T2: thermophilic phase, T3: maturation phase, A, B and C indicate the different composting piles.

4 CHAPTER III

Genome mining for peptidases in heat-tolerant and mesophilic fungi: exploring adaptations for thermostability

Status: In Prep

ABSTRACT

Genome mining has been applied as an alternative to select target and new enzymes on several organisms, including fungi. In the present study, we profiled the genomes of heat-tolerant fungi and phylogenetic related mesophilic species for genes encoding for peptidases. We generated an extensive catalogue of these enzymes ranging from 241 to 652 peptidases genes in the genomes of 19 fungi. Heat-tolerant species presented the smallest number of peptidases as a consequence of genome reduction. Regarding the catalytic types (Serine, Aspartic, Metallo, Threonine, Cysteine, Glutamic and Asparagine), no fungal genome encoded all of them. We also explored differences between peptidases in the genomes from mesophilic and thermophilic species, which showed significant changes of single and groups of amino acid residues. In particular, peptidases from thermophilic species in relation to the mesophilic ones, had an increased number of Ala, Asp, Glu, Gly, Pro and Arg residues and a decreased number of Cys, Phe, His, Ile, Lys, Leu, Met, Asn, Gln and Thr residues. No differences were observed for Ser, Val and Tyr residues. Moreover, an increase in proportion of hydrophobic and charged amino acids and a decrease in polar amino acids were observed. In regards to the protein structure, an increase in the number of cavities was observed in the peptidases of thermophilic species. Therefore, although thermophilic fungi present less genes enconding for peptidases, these have adaptations that improve thermal resistance.

Keywords: enzyme; protease; modeling; evolution; thermophilic fungi

INTRODUCTION

Isolation and screening of microorganisms has been applied as a strategy to obtain strains able to produce industrially-relevant enzymes. Considering the increased number of available genomes, new rational approaches, as genome mining, are applied as an alternative to find target and new enzymes (Littlechild 2015; Vorapreedra et al. 2015). This is also an interesting alternative to target the prospection of enzymes in fungi deposited in culture collections. This approach has been applied for lipases (Vorapreedra et al. 2015), lignocellulosic-degrading enzymes (Busk et al. 2014; Karnaouri et al. 2014) and peptidases, particularly in *Aspergillus* species (Budak et al. 2014).

Peptidases (EC 3.4) comprehend a large group of hydrolytic enzymes that catalyze the hydrolysis of proteins by cleavage of the peptide bonds between amino acid residues (Shankar et al. 2011). Microbial peptidases have been applied in industrial process such as detergent, textile, leather, dairy and pharmaceutical due to the technical and economic advantages. Peptidases is one of the most important group of industrial enzymes representing one of the largest produced, accounting for approximately 65% of the total enzymes production worldwide (Sundararajan et al. 2011; Annamalai et al. 2014).

The conditions employed in industrial processes are different from those found in the natural environment, which represents a problem in their application for industrial biotransformation (Littlechild 2015). In this context, explore enzymes from thermophilic fungi are not only of industrial interest but also allows the prediction of thermostability patterns to engineering thermostable enzymes. Heat-tolerant fungi, often

found in composting systems, have been reported as producer of thermostable enzymes with industrial applications (Maheshwari et al. 2000).

Here, we investigated *in silico*, the diversity of peptidases in the genome of heat-tolerant fungi and phylogenetic related mesophilic counterparts creating an extended catalogue of peptidases for those fungi. Furthermore, the enzymatic profile and amino acid composition of peptidases encoded in the genomes and the structural patterns of the proteins, using representatives from the A1 family aspartic peptidase, were evaluated to predict enzymatic thermostability.

METHODS

Fungal genomes retrieval and identification of putative peptidases

The annotated genome of thermophilic (*sensu* Oliveira et al. 2015), termotolerant and mesophilic species were retrieved from public databases, including the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/), DOE Joint Genome Institute (JGI, http://genome.jgi.doe.gov/) and Genozyme (http://genome.fungalgenomics.ca/). The latter was authorized by Dr. Adrian Tsang, coordinator of the project Genozymes for Bioproducts and Bioprocesses Development Project (see Table 1). The proteome of the fungi were subjected to BLAST in the peptidase database MEROPS (http://merops.sanger.ac.uk/). The putative peptidases were classified according to their catalytic site and families.

Thermomucor indicae-seudaticae genome retrieval and annotation

Few annotated fungal genomes of the order Mucorales were present in the databases. Thus, in the present study we annotated the genome for *T. indicae-seudeticae*, a mucoralean fungus largely applied in industrial processes. The pipeline MAKER was used for a draft annotation of the genes in the unannotated genome of *T. indicae-seudeticae* (available in GenBank, accession number JSYX01.1). The annotation was carried out in four rounds, since the transcriptome of this species is not available. Additional data were used as evidence to support the annotation in the adopted pipeline such as: (i) all proteins contained in the MEROPS protease database (downloaded 15. 7. 2016); (ii) all proteins of the Swissprot database (downloaded 15. 7. 2016); and (iii) the transcriptome of *Lichtheimia ramosa* (GenBank GCA_000945115.1), a closely related species that belong to the same order (Mucorales).

Statistical analysis of enzymatic profile in fungi and search for putative thermal adaptation

An UPGMA cluster analysis was performed using the total number of putative peptidases of each catalytic type (Serine, Aspartic, Metallo, Threonine, Cysteine, Glutamic, Asparagine) using Bray-Curtis distance matrix. An analysis of similarity (ANOSIM) was performed to check for differences in the catalytic type composition between mesophilic and thermophilic species and the Percentage of Similarity analysis (SIMPER) was performed to identify which catalytic type contributes the most for the differences in the enzymatic profile. The same analyses were conducted to evaluate the difference in composition of peptidases families.

The percentage ratio of each type of amino acids and the percent ratio of charged, polar and hydrophobic amino acids were calculated using PEPSTATS (EMBOSS) (http://www.bi.up.ac.za/cgi-bin/emboss.pl?_action=input&_app=pepstats). A paired *t* test was carried out to determine if single amino acids residues or groups contributed to significant differences between the set of peptidases from thermophilic and mesophilic species (121,695 and 54,047 peptidases, respectively); thermotolerant species were not included in the analysis. All analysis were performed in Past v. 2.17c (Hammer et al. 2001). All results are considering the changes in the direction from mesophilic to thermophilic species.

Selection of functional homologs and representative proteins from the subfamily A1A aspartic peptidase (AP)

The dataset was scrutinized for the presence of typical AP hallmarks defined as D[TS]G, Y, XXG, D [TS]G, and XXG (where X is any of the hydrophobic residues AFILMV). Sequences lacking any of the hallmarks were considered nonfunctional homologs and excluded from further analysis. The first alignment was made manually by the catalytic motif D[TS]G site as described in Revuelta et al. (2014). The second alignment was performed on ClustalW (http://www.genome.jp/tools/clustalw/).

Phylogenetic analysis was performed to select a cluster of functional sequences. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al. 1992). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood values.

From the initial tree a cluster with 12 amino acid sequences was selected. This cluster was composed by proteins from *Aspergillus fumigatus*, *A. niger*, *Chaetomium globosum*, *C. thermophilum*, *Myceliophthora fergusii*, *M. sepedonium*, *M. thermophila*, *Myriococcum thermophilum*, *Rasamsonia byssochlamydoides*, *Thermoascus crustaceus*, *Thielavia terrestris* and *T. australiensis*. Phylogenetic analyses were conducted in MEGA7 (Kumar et al. 2015).

Construction of three-dimensional model by homology

Homologous proteins of the family A1 AP were selected as described above. The target amino acids sequences were used to build a model using SWISS-MODEL (https://swissmodel.expasy.org/). The SWISS-MODEL template library (SMTL version 2016-09-07, PDB release 2016-09-02) was screened with Blast (Altschul et al. 1997) and HHBlits (Remmert, et al. 2011) for evolutionary related structures matching the target sequence. Accuracy of predicted model and its stereo chemical properties were evaluated by PROCHECK (Vriend, 1990). The model was selected on the basis of various factors such as overall G-factor, number of residues in core allowed, generously allowed and disallowed regions in Ramachandran plot. The model was further analyzed by QMEAN (Benkert et al. 2009), Lgscore in ProQ (Wallner and Elofsson, 2003) and Z-score in ProSA (Wiedertein and Sippl, 2007).

The sequences were submitted to a bidimensional eletrophosresis *in silico* using the JVirGel 2.0 (Hiller et al., 2006) to predict the theoretical pI (isoelectric point) and Mw (molecular weight). It was also evaluated the number of α-helix, β-strand and β-sheet structures, number of cavities, superficial area and volume using Swiss-PdbViewer (http://www.expasy.org/spdbv/). All those data were combined in a set of

proteins from mesophilic and thermophilic species and tested for significance using ANOVA (for continuous data) and Kruskal-Wallis (for counting data).

RESULTS

Peptidases found in fungal genomes

The total number of putative genes encoding for peptidases in the genomes of the fungi explored in this study ranged from 241 to 652 (Table 2). Regarding the catalytic types (Serine, Aspartic, Metallo, Threonine, Cysteine, Glutamic and Asparagine), no fungal genome had all of them. *Rhizopus microsporus* showed the largest number of putative peptidases (total of 652) followed by *Myceliophthora sepedonium* (494), *Chaetomium globosum* (469), *Rhizopus delamar* (464) and *Aspergillus niger* (437). These fungi are classified as mesophilic (Table 2). Both species of the thermophilic genus *Thermomyces, T. dupontii* and *T. lanuginosus*, showed the smallest number of putative peptidases (241 and 246, respectively).

Asparagine and Glutamic peptidases are not widely distributed among the genomes of fungi explored in the present study, for example *Thermomyces stellatus* is the only fungus that presented Asparagine peptidases while Glutamic peptidases are absent in the genome of fungi in the Mucorales order. The cluster analysis showed that thermophilic and mesophilic species have distinct peptidases profiles (Fig. 1). Most interesting, even considering phylogenetically-related species, such as *M. sepedonium*, *M. fergusii* and *M. thermophila*, differences between thermophilic and mesophilic species still retain (Fig. 1).

The ANOSIM showed that peptidase profiles of mesophilic and thermophilic species are significantly different mainly by reduction in the number of proteins ($p<$

0.05, R= 0.9315). According to the SIMPER analysis the overall peptidases profile between thermophilic and mesophilic species differed in 25.23%. The contribution of each catalytic type is shown in Table 3.

The total number of peptidases families and the number of homologous peptidases are shown in table 4. From the 118 families of peptidases found, eight are Aspartic peptidases, 29 are Cysteine, one is Glutamic, 46 are Metallo, two are mixed, one is Asparagine, 26 are Serine and five are Threonine. Some families are rarely present in the evaluated genomes: 16 in only one of the species (A1B, C33, C40, C51, C69, C28A, M6, M48X, P2B, N9, S1C, S1E, S13, A1X and A2A), eight in two species (A1X, A2A, C67, C78, C86, M10B, P1 and S3) and two in three species (M54 and M81). Considering the enzyme families, 11 families contributed to almost 50% of the total difference between thermophilic and mesophilic species (Table 4).

Putative thermal adaptations

The comparison between peptidases in the datasets from mesophilic and thermophilic species showed significant changes of single and groups of amino acids residues ($p<0.05$, Table 6) considering changes in the direction from mesophilic to thermophilic species. We observed an increase in single amino acids, Ala, Asp, Glu, Gly, Pro and Arg; while we observed a decrease in Cys, Phe, His, Ile, Lys, Leu, Met, Asn, Gln and Thr. No differences were found to Ser, Val and Tyr. The peptidases from thermophilic species also showed an increase in charged and hydrophobic residues and a decrease in polar residues.

The three-dimensional models evaluated via Procheck, QMEAN, ProSa (Z-score) and ProQ (Lgscore) values support the models shown in figure 2. The stereo-

chemical quality of the model structures showed that the majority of amino acids are in the most favored and additionally allowed favored regions (Table 7).

No significant differences were found in the number of α-helix, β-strand and β-sheet, superficial area, volume, molecular weight and isoeletric point. On the other hand, the number of cavities decreased ($p<0.05$, Table 8). Although the proteins presented a few differences in amino acid composition, the conformational structure are the same, maintaining the basic structure of the family A1A (Fig. 1).

DISCUSSION

Thermophilic fungi have experienced genomic reduction and consequently loss of many genes over the evolutionary time (Van Noort et al. 2013), among them peptidases coding-genes, as shown in this study. Apparently, this adaptive process affected peptidases, reducing mostly those with higher number of copies and maintaining those with lower or single copies. This result is in contrast of those observed for cellulolytic enzymes, which are extended against the reductionist trends in thermophilic fungal genomes in comparison with their mesophilic relatives (Van Noort et al. 2013).

Thermophilic fungi are recognized as interesting sources of hydrolytic enzymes with industrial application, for example amylases, cellulases, hemicellulases, lipases and peptidases (Maheshwari et al. 2000). Despite the reduction in the number of copies of peptidases-coding genes, a large catalogue of these enzymes was reported in our study, demonstranting the potential of heat-tolerant fungi to be explored for their production and industrial applications.

Thermostable peptidases acting in high temperatures (65-85 °C) have successfully been applied in baking, brewing, detergent and the leather industries (Haki and Rakshit, 2003). Peptidases from thermophilic fungi have been evaluated in relation to their biochemical properties (e.g. thermal stability) and industrial applications, for instance, *Thermoascus aurantiacus* and its hydrolytic activity on bovine casein (Merheb et al. 2007), *Thermomucor indicae-seudaticea* and *Rhizomucor miehei* in milk clotting activity (Silva et al. 2014; da Silva et al. 2016).

A promising strategy to improve thermostability in proteins is the site-directed mutagenesis (de Souza et al. 2016). However, there is no consensus about the relationship of amino acid composition and its implication in thermal adaptation. It is often reported the increase in charged or hydrophobic residues, or both, but whether the extent of these changes effectively contribute to thermostability still remains elusive (Zeldovich et al. 2007).

Although commonly reported changes in amino acid composition were observed in the literature (e.g. increased hydrophobic and charged residues, decrease in Arg and increase in Lys), our study found differences in comparison with previous works on thermal adaptation. These differences include reports of increased in Trp (Szilágyi and Závodszky 2000; Van Noort et al. 2013), lower frequency of Asp in eukaryotic proteins (Van Noort et al. 2013), an increase in Tyr and Ile and less Glu and Arg in M4 peptidases in prokaryotes species (Khan and Sylte 2009). These observations suggest that amino acid substitutions have some general patterns but specific trends can differ between archaea, bacteria and eukaryotic organisms and also for different proteins, warning for the need of evaluate each group of proteins separately.

We found an increase in Ala, Asp, Glu, Gly, Pro and Arg residues. Some of these amino acids are known to increase thermostability of proteins. They can improve the thermal stability by (i) forming a large number of electrostatic interactions (e.g. hydrogen bond and salt bridges), such as Glu and Arg (Sokalingam et al. 2012; Wang et al. 2014), (ii) structural characteristics of the amino acids that improves the rigidty, such as the presence of cyclic structure in the side chain of Pro (Wang et al. 2014), (iii) maintaining hydrophobic pockets, such as Ala (Borgi et al. 2009) or (iv) increasing the number of weak interections, such as Gly (Yi et al. 2011). On the other hand, other amino acids are known to reduce thermal stability, as Met and Asn, by the chemical instability of these residues at high temperatures (Szilágyi and Závodszky 2000).

Although protein from thermophilic species generally present more thermostability than their homologous from mesophilic species, the particular structure of the family are maintained. However, we showed that the number of cavities had a marked decrease in peptidases from thermophilic fungi. This was also observed by Szilágyi and Závodszky (2000) for enzymes of hiperthermophilic prokaryotes but not for moderate thermophilic enzymes, such as those of thermophilic fungi. The authors suggest it is an adaptation for protein thermostability.

A combined strategy of both genome mining and exploring the patterns that improve thermal stability in specific proteins, can accelerate the process of finding species able to produce target enzymes. Moreover, this approach can better elucidate further site-directed mutagenesis for the production of enzymes adapted to high temperatures by engineering and hetelogous expression.

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TABLES

Table 1. List of fungal genomes mined for peptidase encoding genes.

Order	Species	Classification	Reference
Eurotiales	*Aspergillus fumigatus*	Thermotolerant	JGI
	Aspergillus niger	Mesophilic	Genozyme
	Rasamsonia byssochlamydoides	Thermophilic	Genozyme
	Thermoascus crustaceus	Thermophilic	Genozyme
	Thermomyces dupontii	Thermophilic	Genozyme
	Thermomyces lanuginosus	Thermophilic	McHunu et al. (2013)
	Thermomyces stellatus	Thermophilic	Genozyme
Onygenales	*Myceliophthora thermophila*	Thermophilic	Berka et al. (2011)
	Myceliophthora fergusii	Thermophilic	Genozyme
	Myceliophthora sepedonium	Mesophilic	Genozyme
Sordariales	*Thielavia terrestris*	Thermophilic	Berka et al. (2011)
	Thielavia australiensis	Thermophilic	Genozyme
	Chaetomium globosum	Mesophilic	JGI
	Chaetomium thermophilum	Thermophilic	Van Noort et al. (2011)
Mucorales	*Rhizomucor pusillus*	Thermophilic	Genozyme
	Rhizopus delamar	Mesophilic	Ma et al. (2009)
	Rhizopus microsporus	Thermotolerant	JGI
	Thermomucor indicae-seudaticae	Thermophilic	Busk et al. (2014)
Incertae sedis	*Myriococcum thermophilum*	Thermophilic	Genozyme

Table 2. Putative peptidases from heat-tolerant and mesophilic fungal species.

Fungi	Putative peptidases	Catalytic type							
		Serine	Aspartic	Metallo	Threonine	Cysteine	Glutamic	Asparagine	Mixed
Aspergillus fumigatus[1]	349	137	9	101	22	77	2	0	1
Aspergillus niger[2]	437	204	15	110	24	79	5	0	0
Chaetomium globosum[2]	469	177	29	123	26	110	4	0	0
Chaetomium thermophilum[3]	277	85	22	74	26	66	4	0	0
Myceliophthora fergusii[3]	281	85	18	81	23	71	3	0	0
Myceliophthora sepedonium[2]	494	194	29	129	31	106	4	0	1
Myceliophthora thermophila[3]	320	108	23	86	26	73	4	0	0
Myriococcum thermophilum[3]	318	106	23	82	28	76	3	0	0
Rasamsonia byssochlamydoides[3]	311	115	16	84	24	70	2	0	0
Rhizomucor pusillus[3]	347	106	35	109	18	79	0	0	0
Rhizopus delamar[2]	464	122	75	126	34	107	0	0	0
Rhizopus microsporus[1]	652	181	53	212	39	167	0	0	0
Thermoascus crustaceus[3]	326	122	21	87	23	72	1	0	0
Thermomucor indicae-seudaticae[3]	297	78	29	101	22	67	0	0	0
Thermomyces dupontii[3]	241	66	11	71	22	68	4	0	0
Thermomyces lanuginosus[3]	246	63	14	72	22	70	5	0	0
Thermomyces stellatus[3]	307	104	14	83	28	76	0	2	0
Thielavia australiensis[3]	293	89	22	78	26	76	1	0	1
Thielavia terrestris[3]	340	110	31	84	26	75	5	0	0

1- Thermotolerant; 2- Mesophilic; 3- Thermophilic

Table 3. Percentage of Similarity Analysis (SIMPER) results showing the contribution of the seven catalytic types for the differences between thermophilic and mesophilic species.

Catalytic type	Contrib. %	Cumulative %
Serine	38.96	38.96
Metallo	25.83	64.79
Cysteine	19.34	84.13
Aspartic	11.07	95.2
Threonine	3.49	98.69
Glutamic	1.10	99.79
Mixed	0.14	99.93
Asparagine	0.07	100

Table 4. Percentage of Similarity Analysis (SIMPER) results showing the peptidase families that most contribute (>1% of contribution) to the differences between thermophilic and mesophilic fungi.

Family	Enzyme type	Contrib. %	Cumulative %
S9X	prolyl oligopeptidase	11.14	11.14
S33	prolyl aminopeptidase	9.24	20.38
S8A	Subtilisin	5.76	26.14
A1A	pepsin A	5.53	31.67
C19	ubiquitin-specific peptidase	3.98	35.65
S9C	acylaminoacyl-peptidase	2.97	38.63
S12	D-Ala-D-Ala carboxypeptidase B	2.65	41.27
A28A	DNA-damage inducible protein 1	2.57	43.84
M38	isoaspartyl dipeptidase	2.12	45.97
S10	carboxypeptidase Y	2.04	48.00
C14B	metacaspase Yca1	1.87	49.87

Table 5. Catalogue of peptidases in thermophilic, thermotolerant and mesophilic fungal genomes.

Peptidase Family/ Subfamily	Enzyme type	Fungal species																		
		Aspergillus niger	*Myceliophthora sepedonium*	*Rhizopus delamar*	*Chaetomium globosum*	*Aspergillus fumigatus*	*Rhizopus microsporus*	*Chaetomium thermophilum*	*Myceliophthora fergusii*	*Myceliophthora thermophila*	*Myriococcum thermophilum*	*Rasamsonia byssochlamydoides*	*Rhizomucor pusillus*	*Thermoascus crustaceus*	*Thermomyces dupontii*	*Thermomyces lanuginosus*	*Thermomyces stellatus*	*Thielavia australiensis*	*Thielavia terrestris*	*Thermomucor indicae-seudaticae*
A1A	pepsin A	12	26	44	22	7	40	19	15	20	20	11	31	16	7	7	11	18	27	25
A1B	Nepenthesin	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
A1	not assigned to subfamily	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
A2A	HIV-1 retropepsin	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
A11A	Copia transposon peptidase	0	0	0	2	0	0	0	0	0	0	1	0	0	1	4	0	0	0	0
A22B	impas 1 peptidase	2	2	1	4	1	3	2	2	2	2	2	1	2	2	2	2	2	3	1
A22A	presenilin 1	0	0	1	0	0	3	0	0	0	0	0	2	0	0	0	0	0	0	2
A28A	DNA-damage inducible protein 1	1	1	24	1	1	7	1	1	0	1	1	1	3	1	1	1	1	1	1
C1A	Papain	0	0	1	1	1	1	0	0	3	0	0	0	0	0	1	1	0	1	0
C2A	calpain-2	3	8	1	6	3	2	5	4	4	6	3	1	3	2	3	5	6	6	2
C1B	bleomycin hydrolase	1	2	1	0	0	2	0	1	1	2	1	1	1	1	1	1	1	0	1
C110	kyphoscoliosis peptidase	2	2	0	2	2	0	2	2	2	2	2	0	2	2	2	2	2	2	0
C12	ubiquitinyl hydrolase-L1	4	7	5	8	4	10	3	2	4	5	3	5	3	3	3	4	5	5	3
C13	Legumain	1	1	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	2
C14B	metacaspase Yca1	5	4	10	8	2	6	2	3	2	4	2	2	3	2	2	3	2	2	3
C15	pyroglutamyl-peptidase I	0	2	0	2	1	0	1	2	1	1	1	0	0	0	1	1	1	1	0

C19	ubiquitin-specific peptidase 14	26	36	40	33	27	75	22	22	23	23	27	27	25	29	28	28	24	23	22
C26	gamma-glutamyl hydrolase	13	16	15	12	12	19	11	13	12	12	11	14	12	11	10	11	13	11	13
C33	equine arteritis virus Nsp2-type cysteine peptidase	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C40	dipeptidyl-peptidase VI	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C44	amidophosphoribosyltransferase precursor	8	8	8	9	4	7	5	6	5	7	5	6	6	4	5	5	6	5	6
C45	acyl-coenzyme A:6-aminopenicillanic acid acyl-transferase precursor	1	0	0	0	1	0	0	0	0	0	1	0	2	0	0	1	0	1	0
C48	Ulp1 peptidase	3	4	8	7	4	6	4	4	4	4	3	5	4	3	3	3	3	6	3
C50	Separasse	0	4	1	2	2	2	1	2	1	0	2	1	2	1	1	1	3	2	0
C51	D-alanyl-glycyl peptidase	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
C54	autophagin-1	1	1	2	2	1	4	1	1	1	1	1	1	1	1	1	1	1	1	2
C56	PfpI peptidase	5	4	3	9	6	7	3	2	4	2	2	3	2	2	2	3	3	3	2
C65	otubain-1	1	1	0	2	1	4	1	1	1	1	1	1	1	1	1	1	1	1	0
C67	CylD peptidase	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
C69	dipeptidase A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
C78	UfSP1 peptidase	0	0	0	0	0	1	0	0	0	0	0	2	0	0	0	0	0	0	0
C82A	L,D-transpeptidase	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
C83	gamma-glutamylcysteine dipeptidyltranspeptidase	0	0	3	0	0	4	0	0	0	0	0	2	0	0	0	0	0	0	2
C85A	OTLD1 deubiquitinylating enzyme	2	2	1	0	2	7	1	1	1	1	2	2	2	2	2	1	1	1	2
C85B	OTU1 peptidase	1	1	1	2	1	2	1	1	1	1	1	1	1	0	1	0	1	1	1
C86	ataxin-3	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
C97	DeSI-1 peptidase	1	3	4	4	1	5	1	2	2	2	1	2	1	1	1	2	2	2	2
G1	scytalidoglutamic peptidase	5	4	0	4	2	0	4	3	4	3	2	0	1	4	5	0	1	5	0
M1	aminopeptidase N	4	5	6	9	4	11	3	3	4	4	4	5	4	4	4	3	4	4	3
M3A	thimet oligopeptidase	6	12	3	6	3	6	3	5	5	4	5	3	3	3	3	5	3	5	3
M4	Thermolysin	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0
M6	immune inhibitor A peptidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
M10B	Serralysin	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0

M12B	Adamalysin	2	1	3	0	3	2	1	1	1	1	1	3	1	0	1	0	1	1	2
M12	not assigned to subfamily	0	0	1	0	1	3	0	0	0	0	0	0	0	1	0	2	0	0	0
M13	Neprilysin	0	1	9	0	1	13	0	0	0	0	1	5	1	0	0	1	0	2	5
M14A	carboxypeptidase A1	2	5	0	5	1	0	4	3	3	3	1	0	1	1	1	2	3	2	0
M16A	Pitrilysin	8	6	7	8	4	10	3	3	3	3	4	6	5	4	4	3	3	3	3
M16B	mitochondrial processing peptidase beta-subunit	5	9	10	9	5	13	6	5	5	5	5	9	5	5	5	3	5	5	5
M16C	Eupitrilysin	2	3	3	4	2	8	2	2	2	2	2	4	2	2	2	2	2	2	4
M17	leucine aminopeptidase 3	0	0	3	0	0	11	0	0	0	0	0	2	0	0	0	0	0	0	3
M18	aminopeptidase I	2	3	3	4	2	5	2	2	3	2	2	1	2	2	2	2	3	2	2
M19	membrane dipeptidase	2	2	1	0	2	4	1	2	2	2	4	1	3	2	2	3	1	2	1
M20A	glutamate carboxypeptidase	4	2	0	1	5	0	1	1	2	2	3	0	2	2	2	5	2	1	0
M20D	carboxypeptidase Ss1	7	4	6	3	4	7	1	1	3	3	1	6	2	0	1	1	2	2	8
M20F	carnosine dipeptidase II	2	3	5	2	3	10	2	2	2	2	2	3	2	2	3	2	2	2	3
M20	not assigned to subfamily	2	0	0	0	2	0	0	0	0	0	1	0	1	1	1	1	0	0	0
M24A	methionyl aminopeptidase 1	4	3	5	2	7	9	2	3	3	3	4	4	3	2	2	3	3	3	4
M24B	aminopeptidase P	6	5	9	9	4	17	5	4	5	5	5	7	5	5	5	7	5	5	4
M24	not assigned to subfamily	3	5	4	6	3	6	2	2	2	1	3	4	3	3	3	2	3	3	3
M28A	aminopeptidase S	2	4	1	2	3	0	4	3	3	4	0	1	3	0	0	5	3	0	2
M28B	glutamate carboxypeptidase II	2	2	4	3	2	5	2	2	2	2	2	3	2	2	2	3	2	2	4
M28E	aminopeptidase Ap1	2	3	2	2	1	2	2	2	2	2	1	1	1	1	1	1	1	2	1
M28	not assigned to subfamily	1	6	3	5	0	9	4	5	4	2	2	4	1	2	2	1	3	3	4
M35	Deuterolysin	0	0	0	0	3	0	0	0	0	0	0	0	1	0	0	0	1	0	0
M36	Fungalysin	1	0	2	0	1	3	0	0	0	0	0	1	0	0	0	1	0	0	1
M38	isoaspartyl dipeptidase	17	16	9	10	9	15	6	10	11	11	12	9	14	6	6	5	8	11	11
M41	FtsH peptidase	2	2	4	3	2	6	2	2	2	2	2	5	2	2	2	2	2	2	5
M42	glutamyl aminopeptidase	0	1	0	2	0	0	0	1	1	1	0	1	0	0	0	0	0	0	0
M43B	Cytophagalysin	1	1	0	1	1	0	1	1	1	1	1	0	1	1	1	2	0	0	0
M48A	Ste24 peptidase	1	1	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

M48C	Oma1 peptidase	1	4	1	2	1	6	1	1	1	1	1	2	1	1	1	1	1	1	1
M48	not assigned to subfamily	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M49	dipeptidyl-peptidase III	2	1	0	2	2	2	1	1	1	1	1	1	1	1	1	1	2	2	0
M50A	site 2 peptidase	0	0	1	0	0	2	0	0	0	0	0	1	0	0	0	0	0	0	1
M54	Archaelysin	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0
M67A	RPN11 peptidase	4	6	11	6	4	11	4	5	4	4	4	7	4	5	4	4	4	5	7
M67C	STAMBP isopeptidase	1	1	2	0	1	4	1	1	1	1	1	2	1	1	1	1	1	1	2
M67	not assigned to subfamily	2	2	3	3	2	3	2	2	2	2	2	2	2	2	2	2	2	2	3
M76	Atp23 peptidase	1	1	2	2	1	2	1	1	1	1	1	2	1	1	1	1	1	1	2
M77	tryptophanyl aminopeptidase 7-DMATS-type peptidase	2	1	0	4	7	0	0	0	0	0	1	0	1	2	2	0	0	0	0
M79	RCE1 peptidase (Saccharomyces cerevisiae)	1	1	0	2	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1
M80	Wss1 peptidase	3	3	1	2	2	4	2	3	3	3	2	2	2	2	2	3	3	3	2
M81	microcystinase MlrC	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
P2B	polycystin-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
P1	DmpA aminopeptidase	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N9	intein-containing V-type proton ATPase catalytic subunit A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
S1A	chymotrypsin A	0	0	1	1	0	2	0	0	0	0	0	1	0	0	0	0	0	0	0
S1C	DegP peptidase	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
S1D	lysyl endopeptidase	1	1	4	3	1	6	1	1	1	1	2	3	2	1	1	1	1	1	3
S1E	streptogrisin A	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
S3	Togavirin	0	0	0	2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
S8A	subtilisin Carlsberg	5	20	31	20	4	36	7	8	9	9	2	17	2	1	1	9	8	8	18
S8B	Kexin	1	2	3	2	1	3	1	1	1	1	2	2	1	1	1	1	1	1	2
S9A	prolyl oligopeptidase	0	0	2	0	0	2	0	0	0	0	0	1	0	0	0	0	0	0	1
S9B	dipeptidyl-peptidase IV	1	5	4	6	4	7	4	4	4	3	0	2	1	2	1	3	2	1	2
S9C	acylaminoacyl-peptidase	22	15	10	12	11	10	6	8	7	8	7	12	8	7	7	8	11	9	8
S9		69	52	21	45	39	33	21	20	24	27	30	23	37	17	16	36	19	29	10

S10	carboxypeptidase Y	14	7	8	7	12	11	3	4	6	6	5	4	9	4	4	3	3	5	4
S12	D-Ala-D-Ala carboxypeptidase B	10	11	0	10	6	2	1	1	5	5	6	1	7	1	1	5	2	7	2
S13	D-Ala-D-Ala peptidase C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
S14	peptidase Clp	1	2	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1
S16	Lon-A peptidase	2	4	6	1	2	6	2	2	2	2	1	6	2	2	2	2	2	2	5
S26A	signal peptidase I	2	3	0	2	2	3	1	2	2	1	2	2	2	2	2	2	1	2	2
S26B	signalase 21 kDa componente	1	2	1	1	1	0	1	1	1	1	2	1	2	1	1	2	1	1	1
S28	lysosomal Pro-Xaa carboxypeptidase	3	5	1	9	3	4	2	2	3	3	3	2	0	2	2	0	4	2	0
S33	prolyl aminopeptidase	60	54	22	39	41	43	23	25	32	31	38	22	29	17	17	21	27	30	13
S41A	C-terminal processing peptidase-1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
S49B	protein C	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S53	Sedolisin	4	7	0	6	5	0	5	2	6	3	8	0	3	2	0	5	2	7	0
S54	rhomboid-1	3	3	6	6	3	8	3	2	3	3	4	5	3	3	3	3	3	3	5
S59	nucleoporin 145	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1
S66	murein tetrapeptidase LD-carboxypeptidase	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T1A	archaean proteasome, beta componente	11	13	23	15	12	20	11	11	11	12	12	11	12	11	11	11	11	11	14
T1	not assigned to subfamily	3	6	5	5	3	4	5	6	4	6	3	3	4	4	4	5	5	5	3
T2	glycosylasparaginase precursor	2	8	1	2	3	3	6	3	7	6	3	1	3	3	3	6	6	6	1
T3	gamma-glutamyltransferase 1	5	3	4	1	3	10	3	2	3	3	5	2	3	3	3	5	3	3	3
T5	ornithine acetyltransferase precursor	1	1	1	2	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1

Table 6. Comparison of amino acid composition in peptidases from mesophilic and thermophilic fungi.

Amino Acid	Mesophilic (%)	Thermophilic (%)
Alanine (Ala)	**8.2±0.013**	**9.14±0.01**
Cysteine (Cys)	**1.49±0.01**	**1.4±0.004**
Aspartic acid (Asp)	**8.2±0.01**	**9.14±0.01**
Glutamine (Glu)	**6.08±0.01**	**6.17±0.01**
Phenylalanine (Phe)	**3.73±0.01**	**3.52±0.01**
Glycine (Gly)	**6.63±0.12**	**7.15±0.01**
Histidine (His)	**2.49±0.01**	**2.4±0.004**
Isoleucine (Ile)	**5.02±0.01**	**4.45±0.01**
Lysine (Lys)	**5.31±0.01**	**4.72±0.01**
Leucine (Leu)	**8.87±0.01**	**8.74±0.01**
Methionine (Met)	**2.33±0.01**	**2.16±0.00**
Asparagine (Asn)	**3.95±0.01**	**3.37±0.00**
Proline (Pro)	**5.82±0.01**	**6.42±0.01**
Glutamine (Gln)	**4.05±0.01**	**3.95±0.01**
Arginine (Arg)	**6.09±0.01**	**6.95±0.01**
Serine (Ser)	7.96±0.01	7.97±0.01
Threonine (Thr)	**6.01±0.01**	**5.76±0.01**
Valine (Val)	6.18±0.01	6.17±0.01
Tryptophan (Trp)	1.45±0.00	1.44±0.00
Tyrosine (Tyr)	**2.94±0.01**	**2.7±0.004**
Charged	**27.39±0.03**	**29.38±0.02**
Polar	**23.31±0.02**	**21.05±0.01**
Hydrophobic	**44.44±0.02**	**51.90±0.02**

Bold numbers represent significant difference (p<0.05).

Table 7. Validation parameters computed for built 3D protein of the Aspartic peptidase sequence.

Fungi	QMEAN	Z-score	Lgscore	Ramachandran (%)*			
				MFR	AAR	GAR	DR
Aspergillus fumigatus	0.54	-9.09	5.179	86.4	10.2	2.7	0.7
Aspergillus niger	0.6	-7.73	4.454	84.7	12.3	2.7	0.3
Chaetomium globosum	0.32	-7.16	5.878	86.6	10.7	2.4	0.3
Chaetomium thermophilum	0.53	-7.92	5.539	84.6	11.9	3.1	0.3
Myceliophthora fergusii	0.5	-7.18	5.853	87.5	9.5	2.7	0.3
Myceliophthora sepedonium	0.5	-6.87	5.887	85.6	11.3	2.7	0.3
Myceliophthora thermophila	0.5	-7.18	5.853	87.5	9.5	2.7	0.3
Myriococcum thermophilum	0.49	-7.66	5.791	85.5	11.4	2.4	0.7
Rasamsonia byssochlamydoides	0.59	-8.47	5.154	85.4	11.8	1.7	1.0
Thermoascus crustaceus	0.58	-8.1	5.052	88.4	9.9	1.4	0.3
Thielavia australiensis	0.55	-6.8	5.893	87.8	8.7	2.8	0.7
Thielavia terrestris	0.34	-7.5	5.566	85.5	11.8	2.8	0

* MFR: Most Favored Region; AAR: Additionally Allowed Region; GAR: Generally Allowed Region; DR: Disallowed Region

Table 8. Characterization of the Aspartic Peptidase protein and the three-dimensional structure.

Fungi	Mol. Mass (Kda)	pI	α-helix	β-strands	β -sheet	Superficial area (Å^2)	Volume (Å^3)	Nº of cavities
Aspergillus fumigatus	49.99	5.88	6	25	3	12.76	44.29	8
Aspergillus niger	46.72	4.56	6	27	4	12.67	43.50	5
Chaetomium globosum	80.29	5.52	7	25	3	13.44	43.52	5
Chaetomium thermophilum	49.38	7.82	3	25	3	13.49	43.72	5
Myceliophthora fergusii	52.44	4.57	7	24	3	13.57	44.01	3
Myceliophthora sepedonium	52.07	4.67	6	24	3	13.09	44.33	6
Myceliophthora thermophila	52.45	4.39	7	24	3	13.57	44.01	3
Myriococcum thermophilum	52.08	4.84	5	25	3	13.45	44.10	4
Rasamsonia bysoclamydoides	45.41	4.73	8	26	3	12.45	42.15	4
Thermoascus crustaceus	47.43	4.89	8	26	3	13.26	43.40	5
Thermoascus aurantiacus	45.57	7.22	6	25	4	12.89	43.62	2
Thielavia terrestris	74.85	5.13	7	25	3	13.21	42.21	3

FIGURE CAPTIONS

Figure 1. Cluster analysis of profiles of genes encodinf for peptidases from thermophilic (red), thermotolerant (blue) and mesophilic (green) fungal species.

Figure 2. Predicted three-dimmensional structures of selected peptidases of fungi. (A) *Aspergillus fumigatus*; (B) *A. niger*; (C) *Chaetomim globosum*; (D) *C. thermophilum*; (E) *Myceliophthora fergusii*; (F) *M. sepedonium*; (G) *M. thermophila*; (H) *Myriococcum thermophilum*; (I) *Rasamsonia bycochlamydoides*; (J) *Thermoascus crustaceus*; (K) *Thielavia australiensis*; and (L) *T. terrestris*.

FIGURES

Figure 1

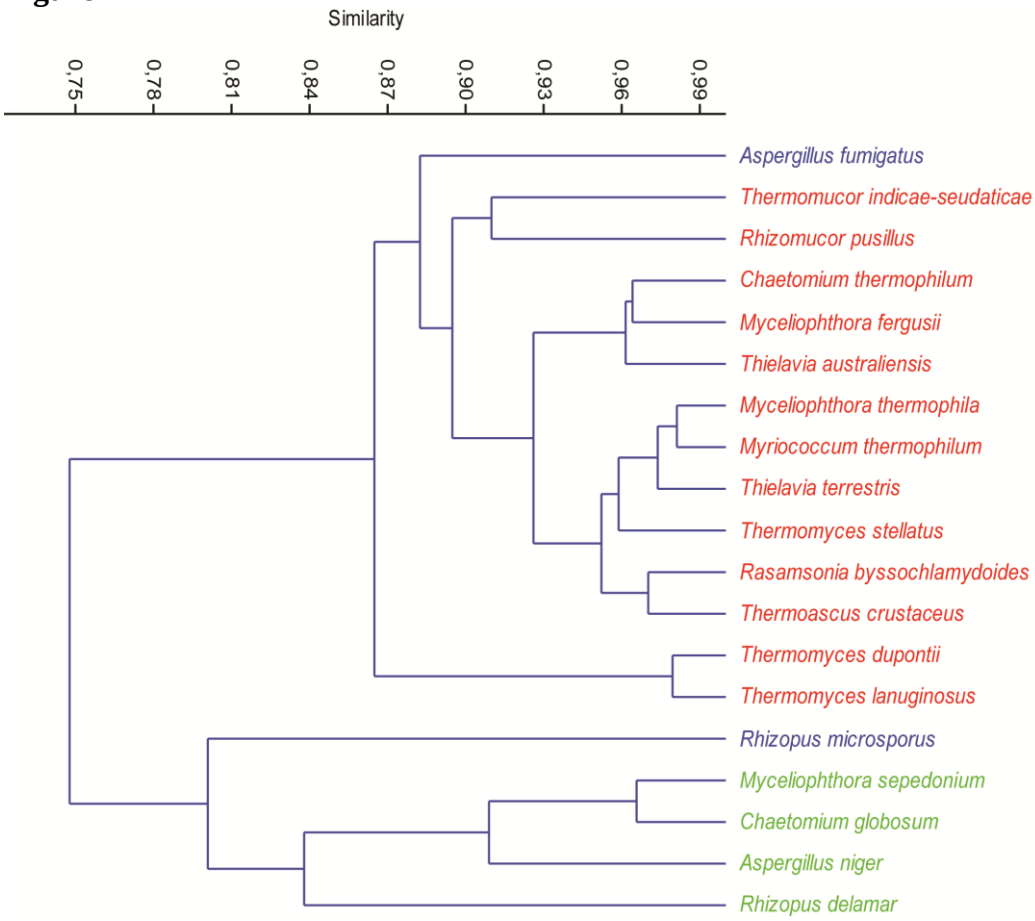
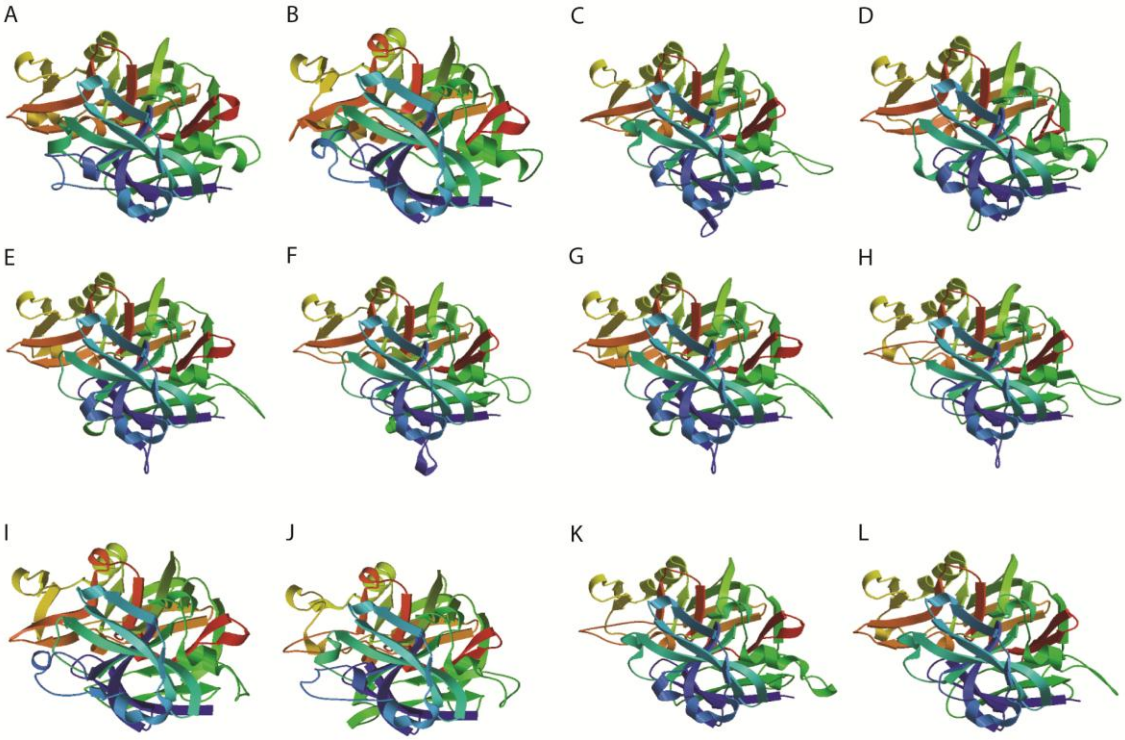


Figure 2



5 CONSIDERAÇÕES

A torta de filtro é um resíduo que pode apresentar riscos à saúde humana, por conter inúmeras espécies de fungos patógenos oportunistas, e que, portanto, requer cuidado em seu manuseio, como o uso de equipamentos de proteção pessoal (luvas, máscara etc). Além disso, o estudo mostrou que o tramento deste resíduo, como a compostagem, reduz a carga desses fungos, evitando a constante reinoculação dos mesmos no campo e a exposição dos manipuladores a esses patógenos.

A compostagem, não apenas é efetiva na redução desses fungos como também cria um ambiente propício para a prospecção de espécies com potencial biotecnológico, como demonstrado para os fungos termofílicos e termotolerantes isolados durante o processo (seis e três espécies, respectivamente), os quais são capazes de produzir enzimas termoestáveis para a degradação de biomassa. Além disso, estes fungos são interessantes modelos para estudos de termofília, pois possuem no genoma informações chaves sobre as adaptações destas enzimas às condições de altas temperaturas.

Apesar do potencial biotecnológico dos fungos termofílicos, a taxonomia desse grupo carecia de uma revisão atualizada, o que constitui em uma barreira para aqueles que trabalham com micologia aplicada. Assim, espera-se que a organização taxonômica realizada no presente trabalho, auxilie os pesquisadores envolvidos nos estudos aplicacados a encontrarem o material necessário e de fácil acesso para seus trabalhos com os fungos termofílicos.

ANEXOS

Figura 1. Macro e micromorfologia de *Aspergillus fumigatus* isolado de pilhas de compostagem de torta de filtro. A. Morfologia da colônia cultivada em MA2% a 45 °C, durante cinco dias. B-D. Microscopia detalhando os conidióforos com vesículas unisseriadas (ou seja, com apenas uma camada de células condiogênicas – fiálides).

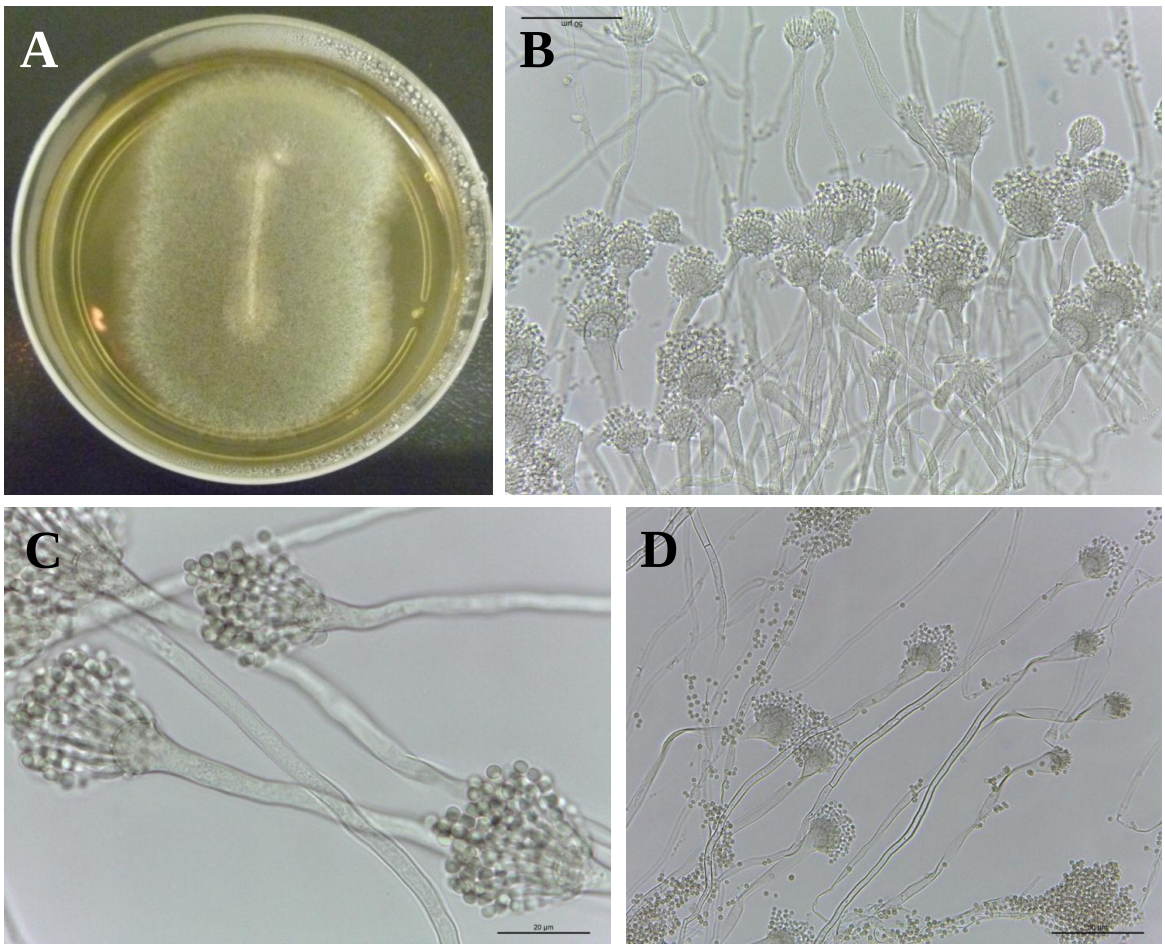


Figura 2. Macro e micromorfologia do fungo *Thermomyces lanuginosus* isolado de pilhas de compostagem de torta de filtro. A-C. Diferentes morfologias da colônia cultivada em MA2% a 45 °C, durante dois dias. D-E. Observam-se os aleuroconídios e a presença de esporo único por célula conidiogênica. F-G. Conídio de cor marrom, globoso, não septado e com parede rugosa.

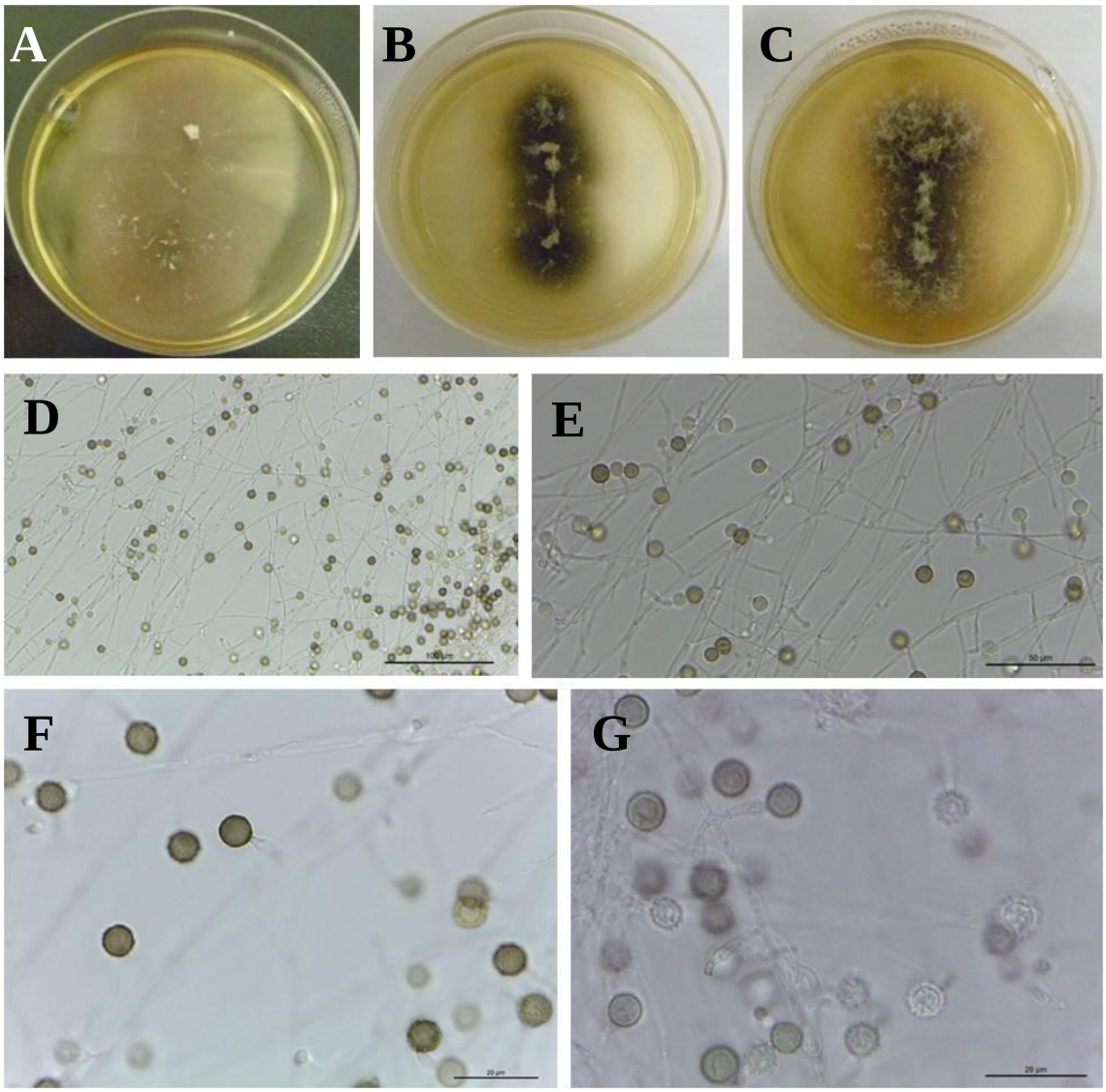


Figura 3. Macro e micromorfologia do fungo *Myriococcum thermophilum* isolado de pilhas de compostagem de torta de filtro. A. Morfologia da colônia cultivada em MA2% a 45 °C, durante dois dias. B-D. Conidiogênese intercalar (meristemática), representada nas fotos pelas cadeias de conídios (seta vermelha). E. Conídio marrom, globoso, liso e com dupla membrana.

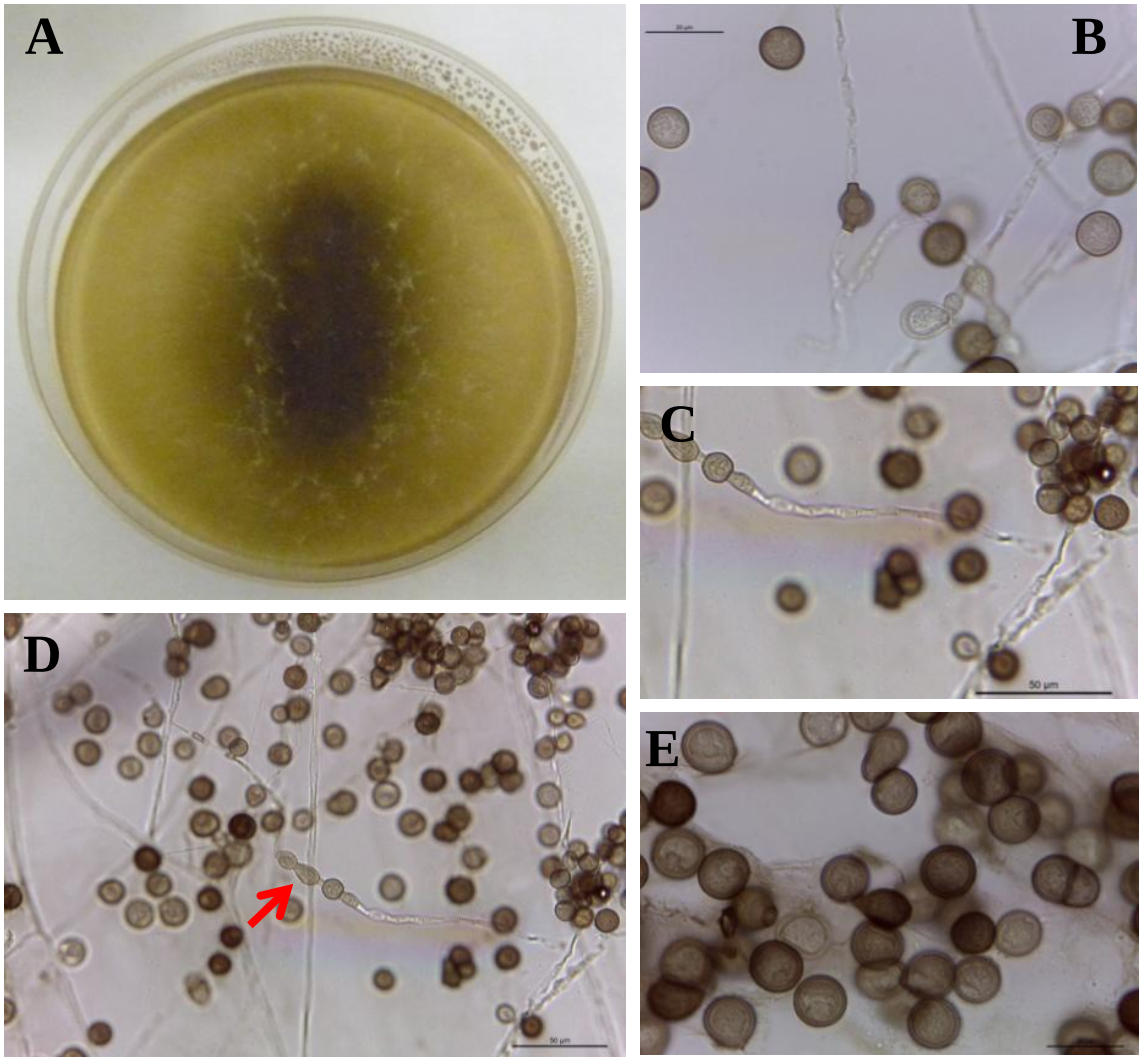


Figura 4. Macro e micromorfologia do fungo *Myceliophthora fergusii* isolado de pilhas de compostagem de torta de filtro. A. Morfologia da colônia cultivada em MA2% a 45 °C, durante dois dias. B-C. Conídios obovóides e lisos surgindo diretamente de hifas não diferenciadas. D-E. Aspecto geral dos conidióforos.

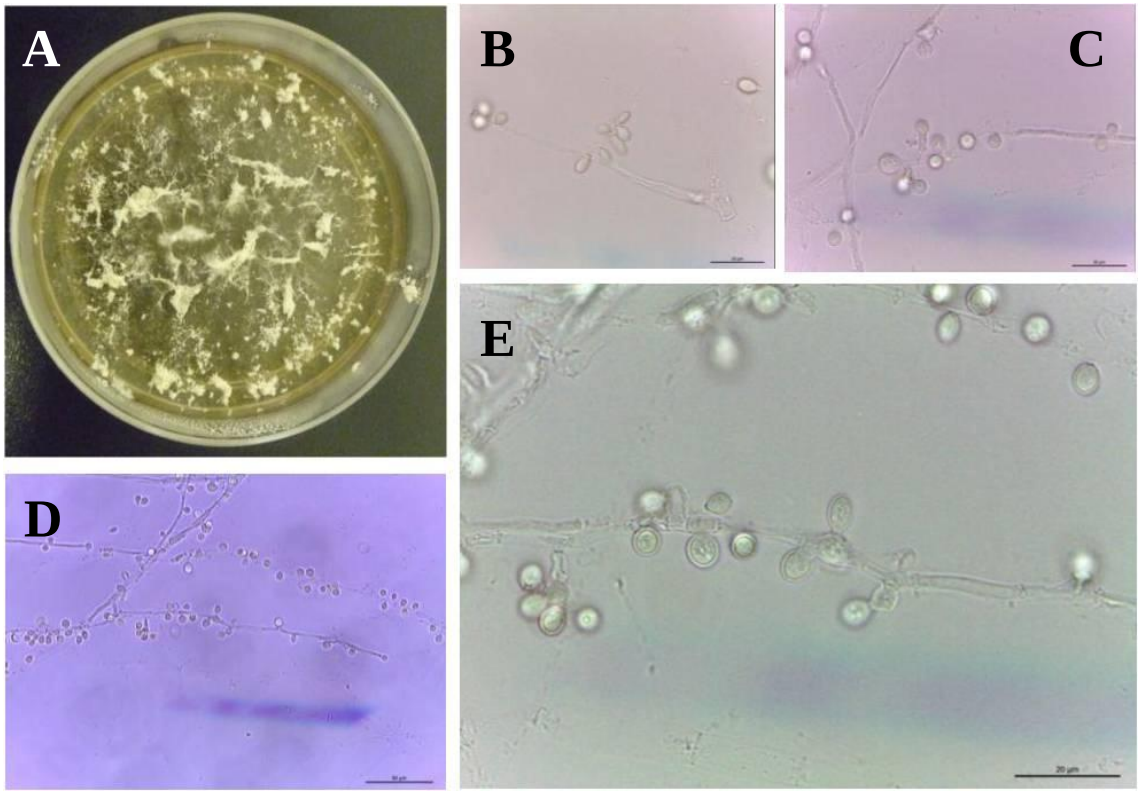


Figura 5. Macro e micromorfologia do fungo *Myceliophthora thermophila* isolado de pilhas de compostagem de torta de filtro. A. Morfologia da colônia cultivada em MA2% a 45 °C, durante dois dias. B-D. Observam-se as células ampuliformes e os conídios não pigmentados, lisos, obovoides a clavados, com base truncada.

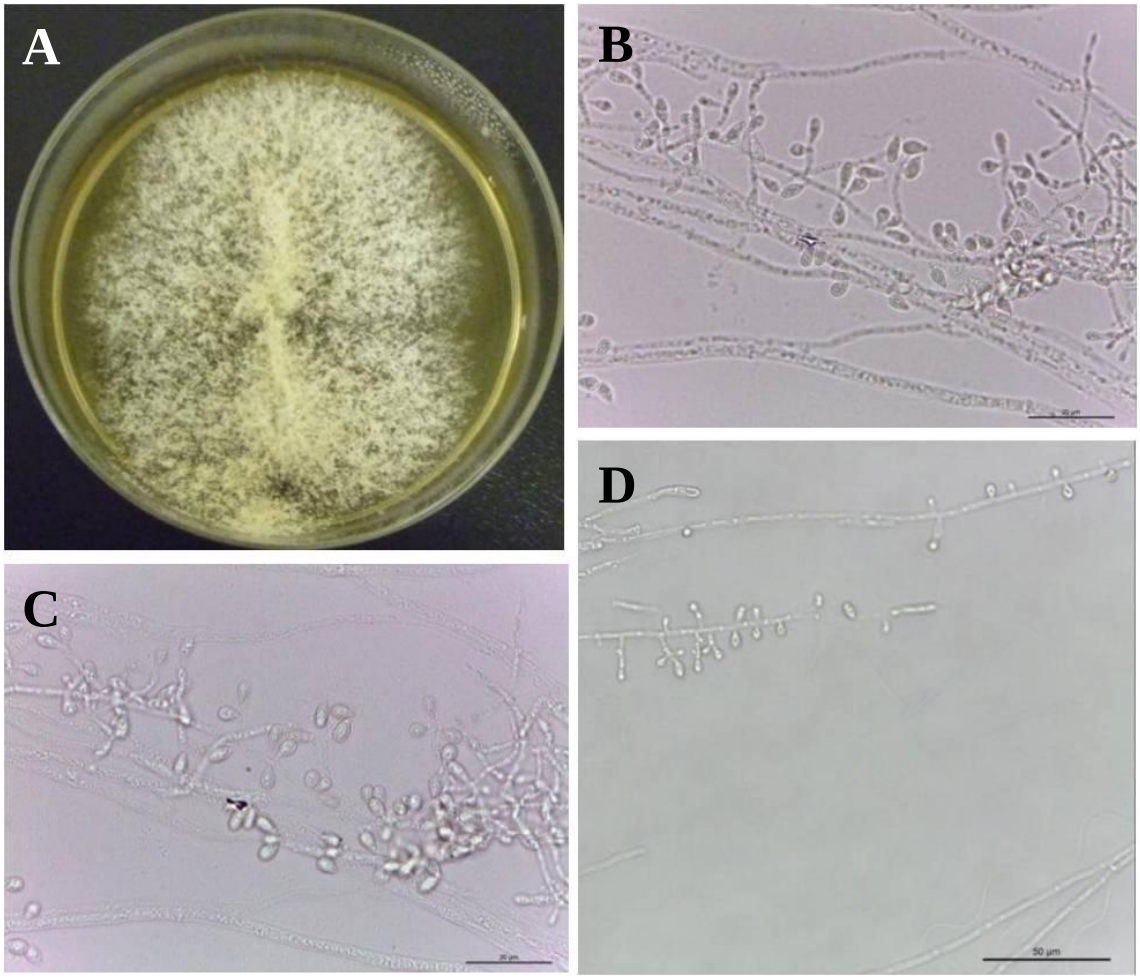


Figura 6. Macro e micromorfologia do fungo *Lichtheimia ramosa* isolado de pilhas de compostagem de torta de filtro. A. Morfologia da colônia cultivada em MA2% a 45 °C, durante dois dias. B-C. Observa-se a presença de columela piriforme e esporangiósporos.

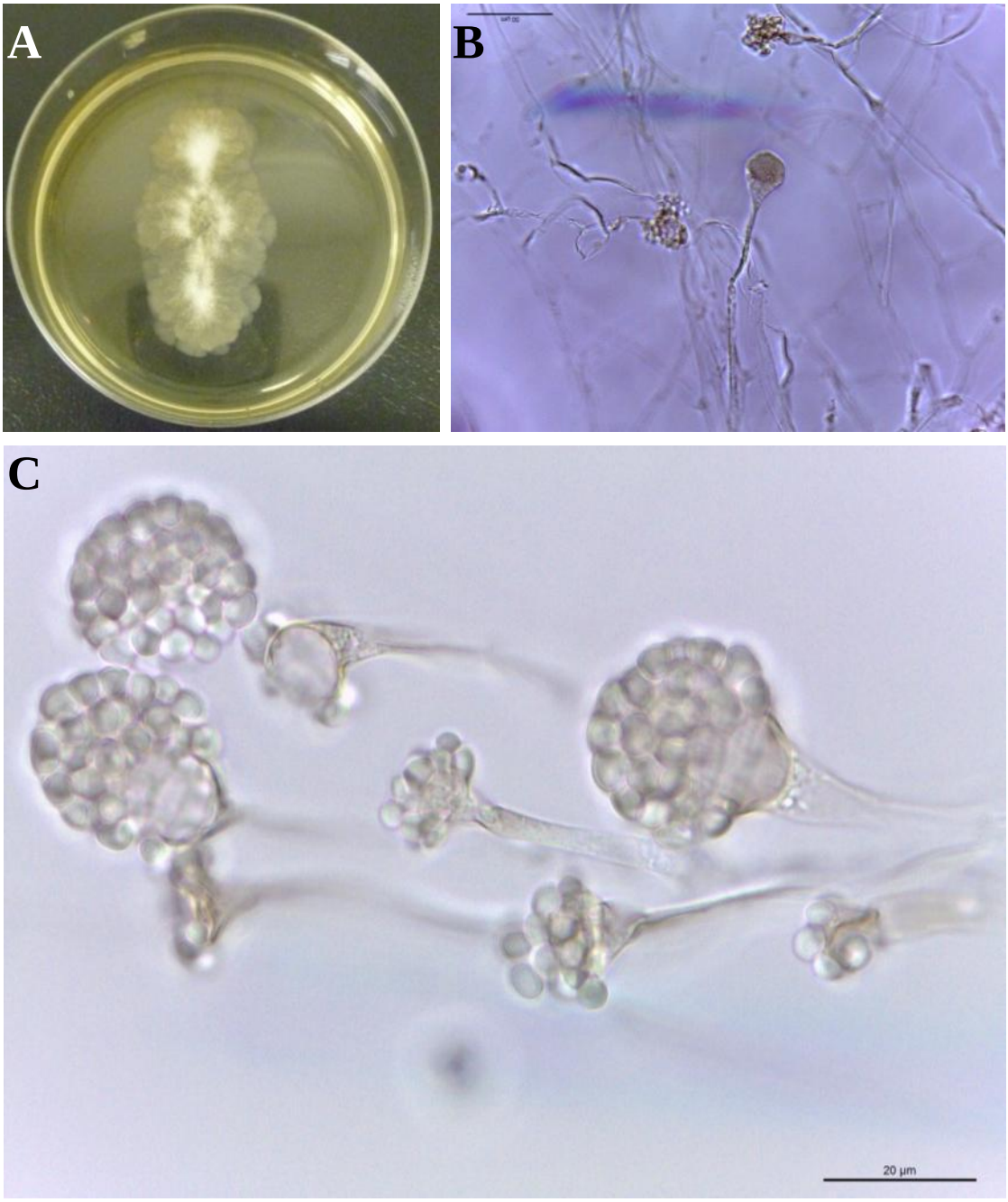


Figura 7. Macro e micromorfologia do fungo *Rhizopus microsporus* isolado de pilhas de compostagem de torta de filtro. A. Morfologia da colônia cultivada em MA2% a 45 °C, durante dois dias. B. Observa-se a presença de rizóide. C. Esporângio. D. Esporangiósporos escuros ao redor da columela.

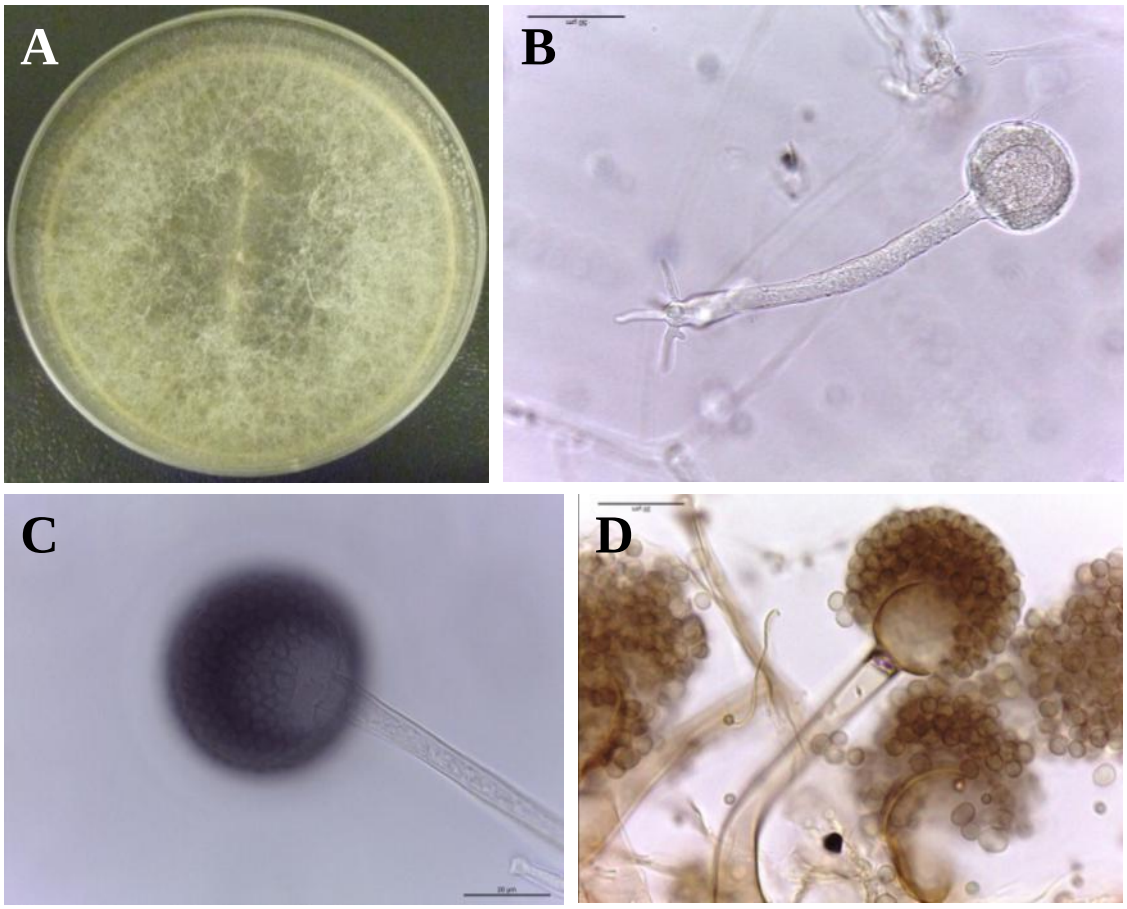


Figura 8. Macro e micromorfologia do fungo *Thermomucor indicae-seudaticae* isolado de pilhas de compostagem de torta de filtro. A. Morfologia da colônia cultivada em MA2% a 45 °C, durante dois dias. B. Esporangióforo ramificado. C-D. Esporângio apresentando esporangiósporos. E. Presença de rizóide.

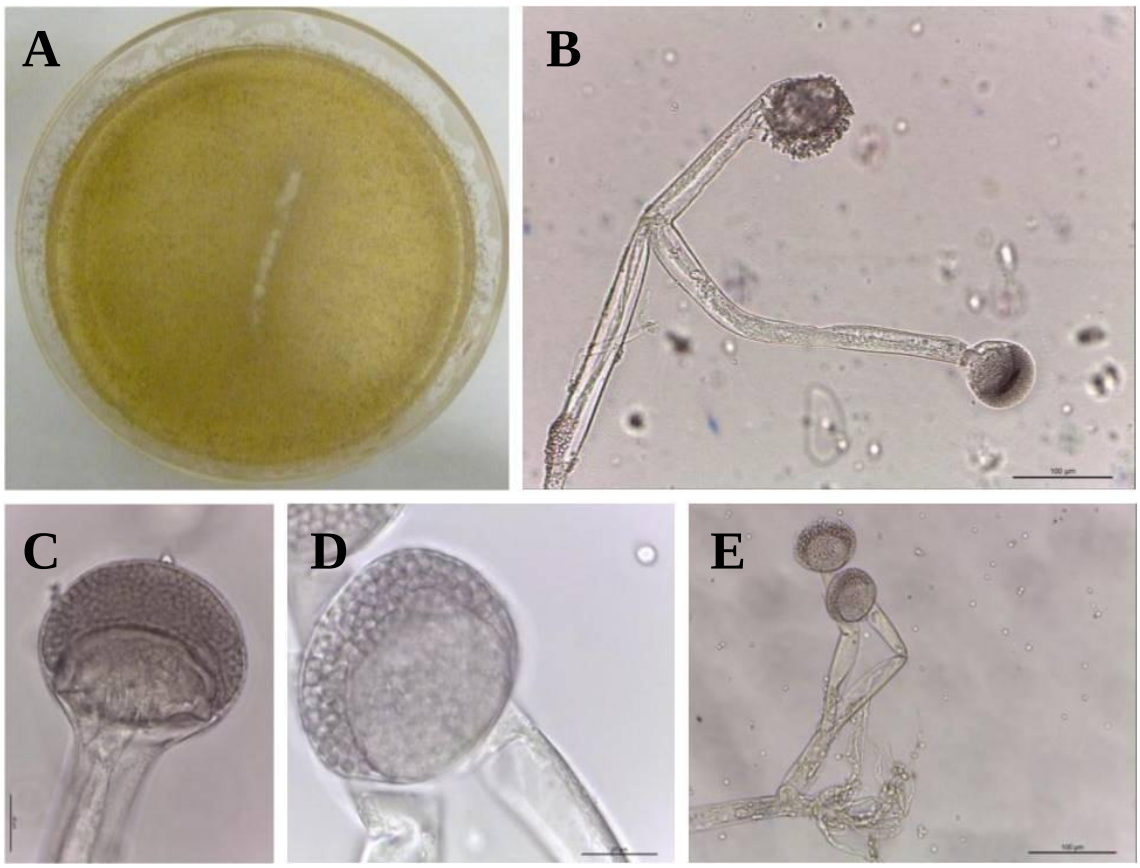


Figura 9. Macro e micromorfologia do fungo *Rhizomucor pusillus* isolado de pilhas de compostagem de torta de filtro. A. Morfologia da colônia cultivada em MA2% a 45 °C, durante dois dias. B. Esporângio com esporangiósporos. C-D. Presença de columela.