MARIANA REGINA ALMAS DO CARMO

Kretzschmaria zonata: FROM THE GENOME TO THE APPLICATION OF THE ENZYME EXTRACT FOR SUGARCANE BAGASSE SACCHARIFICATION

Thesis submitted to the Applied Biochemistry PostGraduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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Co-advisers: Túlio Morgan

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ABSTRACT

CARMO, Mariana Regina Almas, D.Sc., Universidade Federal de Viçosa, May, 2024. ***Kretzschmaria zonata*: FROM THE GENOME TO THE APPLICATION OF THE ENZYME EXTRACT FOR SUGARCANE BAGASSE SACCHARIFICATION.** Adviser: Gabriela Piccolo Maitan-Alfenas. Co-adviser: Túlio Morgan.

Brazil is the largest sugarcane producer in the world and its production is destined for the sugar and ethanol processing industry, which generates tons of lignocellulosic wastes annually. Considering the global need for energy security and environmental concerns, the agro-industrial wastes use as raw materials to generate value-added products is a promising alternative for the sector. Lignocellulosic residues are rich in polysaccharides such as cellulose and hemicellulose, however, they require prior hydrolysis of their chains to be applied in the second-generation ethanol production process. The biomass hydrolysis process consists of the action of several carbohydrate-active enzymes (CAZymes) and it is considered the highest bottleneck in large-scale production, due to the high costs of commercial enzymes. For this reason, the search for new enzymes or enzymes that are more efficient than those currently commercialized has become the subject of research for decades. Overall, filamentous fungi are known to be good producers of plant cell wall degrading enzymes, but phytopathogenic fungi, which secrete several enzymes to infect the host plant and to obtain the nutrients necessary for their survival, have aroused the interest of researchers. Therefore, the present study aimed to sequence the phytopathogen fungus *Kretzschmaria zonata* genome, to predict the genes encoding CAZymes, to obtain its sugar metabolic model, to prospect the lignocellulolytic enzymes of this fungus grown on different carbon sources such as wheat bran, corn cob, barley and sugarcane bagasse, and to apply the crude enzymatic extract in the saccharification of two sugarcane bagasse varieties, a commercial variety - Ridesa RB 867515 - and a variety of energy cane - C-90176. The size of the assembled genome was 30.15 Mbp and a total of 12,135 protein-coding genes were identified, with 575 predicted CAZymes. The repertoire of predicted CAZymes was broad and more similar to the profile presented by the filamentous fungus *Aspergillus niger*, which is a *Eurotiomycetes*, than with the fungus *Trichoderma reesei*, which is a *Sordariomycetes*, as well as *K. zonata*. This suggests that the *K. zonata* is an interesting source of enzymes for biotechnology. *K. zonata* possess a complete metabolic network, except for a gene absence in the galacturonic acid catabolic pathway, which suggests that the fungus can potentially grow on different carbon sources. Maximum cellulolytic activity was obtained for the crude extract obtained after fungal growth on corn cob as carbon source, especially for xylanase, 3.5 U/mL. The extract obtained after growth on wheat bran was the only able to induce all the analyzed enzymes and sugarcane bagasse was the carbon source that induced the lowest diversity of enzymes. For the saccharification experiment, the wheat bran enzymatic extract at a concentration of 2.5 FPU/g of sugarcane bagasse showed the maximum release of glucose and xylose in 96 h, corresponding to 3.1 and 2. 1 g/L, respectively. Thus, these results allowed a better understanding of the enzymatic, metabolic and growth profiles of the fungus *K. zonata*, which can contribute to a more efficiently application of its enzymes in biotechnology processes.

Keywords: Phytopathogenic fungi; Enzimatic profile; Sugar metabolic model; Enzimatic hydrolysis.

RESUMO

CARMO, Mariana Regina Almas, D.Sc., Universidade Federal de Viçosa, maio de 2024. ***Kretzschmaria zonata*: DO GENOMA À APLICAÇÃO DO EXTRATO ENZIMÁTICO NA SACARIFICAÇÃO DE BAGAÇO DE CANA-DE-AÇÚCAR.** Orientadora: Gabriela Piccolo Maitan-Alfenas. Coorientador: Túlio Morgan.

O Brasil é o maior produtor de cana-de-açúcar do mundo e sua produção é destinada à indústria de açúcar e etanol, que gera toneladas de resíduos lignocelulósicos todos os anos. Considerando a segurança energética mundial e a preocupação ambiental, a utilização dos resíduos agroindustriais como matéria-prima para geração de produtos é uma alternativa promissora para o setor. Os resíduos lignocelulósicos são ricos em celulose e hemicelulose, entretanto necessitam de uma hidrólise de suas cadeias para que sejam aplicados na produção de etanol de segunda geração. A hidrólise da biomassa consiste na ação de enzimas ativas em carboidratos (CAZymes) e é considerado o maior gargalo da produção em grande escala. Assim, a busca por enzimas mais eficientes do que as comercializadas atualmente é objeto de pesquisas ao longo de décadas. Os fungos filamentosos são conhecidos por serem bons produtores de enzimas capazes de degradar a parede celular vegetal, porém os fungos fitopatogênicos, têm despertado o interesse dos pesquisadores. Portanto, o presente estudo teve como finalidade sequenciar o genoma do fungo fitopatogênico *Kretzschmaria zonata,* realizar a predição dos genes codificadores de CAZymes, obter o seu modelo metabólico de açúcares, prospectar as enzimas lignocelulolíticas do fungo crescido em diferentes fontes de carbono: farelo de trigo, sabugo de milho, cevada e bagaço de cana-de-açúcar e aplicar o extrato enzimático bruto na sacarificação de duas variedades de bagaço de cana-de-açúcar: a comercial - Ridesa RB867515 - e a cana energia - C-90176. O tamanho do genoma montado foi 30,15 Mbp e um total de 12.135 genes codificadores de proteínas foram identificados, com 575 CAZymes preditas. O repertório de CAZymes foi amplo e mais similar ao perfil apresentado pelo fungo *Aspergillus niger*, que é Eurotiomiceto, do que com o fungo *Trichoderma reesei*, que é Sordariomiceto, assim como o *K. zonata*. Isso sugere que o fungo estudado é uma fonte interessante de enzimas para a biotecnologia, especialmente aquelas aplicadas em hidrólise de biomassas lignocelulósicas. O *K. zonata* possui uma rede metabólica completa, o que sugere potencial crescimento em diferentes fontes de carbono, exceto pela ausência de um gene na via catabólica do ácido galacturônico, confirmada pela ausência de crescimento do fungo usando ácido galacturônico como única fonte de carbono. A máxima atividade celulolítica foi obtida no extrato bruto após crescimento em sabugo de milho como fonte de carbono, especialmente para a xilanase, 3,5 U/mL. O extrato produzido a partir do farelo de trigo foi o único que induziu todas as enzimas estudadas e o bagaço de cana-de-açúcar foi a fonte de carbono que induziu a menor diversidade de enzimas. Na sacarificação do bagaço de cana-de-açúcar, o extrato enzimático obtido a partir do farelo de trigo, na concentração de 2,5 FPU/g de bagaço de cana, mostrou a liberação máxima de glicose e xilose em 96 h de hidrólise, correspondendo a 3,1 e 2,1 g/L, respectivamente. Assim, esses resultados permitiram um melhor entendimento sobre os perfis enzimático, metabólico e de crescimento do fungo, que pode contribuir para a aplicação de suas enzimas nos processos biotencológicos de forma mais eficiente.

Palavras-chave: Fungo fitopatogênico; Perfil enzimático; Modelo metabólico de açúcar; Hidrólise enzimática.

SUMMARY

1. INTRODUCTION

In 2023, at the 28th United Nations Conference on Climate Change (COP 28), Brazil joined the Global Biofuels Alliance program, along with the other two main biofuel producers in the world: USA and India. The initiative brings together 19 countries and 12 international organizations and its main objective is to promote the sustainable production and use of biofuels in the world, as a way of meeting one of the commitments made in the Paris Agreement during COP 21 in 2015. Faced with the challenge of limiting the increase in global temperature to 1.5º C, several countries have come together with the aim of reducing the world's dependence on fossil fuels and consequently the emission of polluting gases that contribute to global warming. According to the International Energy Agency, global production of biofuels needs to triple by 2030 so that the world can achieve net-zero emissions by 2050. Furthermore, the expansion of the use of biofuels in aviation and navigation, which aims to decarbonize these transport sectors, will significantly increase global consumption and result in the expansion of production (IRENA, 2024; Rottammer, 2023; IEA, 2023).

In this context, the growth in global demand for fossil fuels, which are finite resources, the increase in the gases emission responsible for the greenhouse effect, and the interest in diversifying the energy matrix have boosted the study and development of various technologies aimed at sustainable energy sources, such as the production of second generation ethanol. This alternative allows for the ethanol production expansion without the need to expand the planted area, ensuring food security, in addition to reducing environmental impacts with the use of agro-industrial waste as raw materials for the production of this biofuel (Maitan-Alfenas et al., 2015; Martins et al., 2018).

The use of waste to obtain economic and environmental advantages is one of the principles of the circular economy, which is based on cost reduction, sustainability, and increased production capacity in various industrial sectors (Mujtaba et al., 2023). In this sense, lignocellulosic residues, which are rich in polysaccharides such as cellulose and hemicellulose, are considered promising renewable resources, as they are widely available and can be used to generate sustainable energy (Astolfi et al., 2019).

The International Energy Agency's Renewables 2023 report highlighted Brazil as a global protagonist in the energy transition and the data shows that together with other emerging economies, Brazil leads the growth of biofuels. By 2028, the estimate predicted in the document is that the country will contribute alone to 40 % of global expansion. The use of biofuels in road transport remains the main source, corresponding to almost 90 % of the expansion, however positive expectations are reinforced by new policies such as the Fuel of the Future Program, which aims to boost the investment market in the sector, highlighting the fuel sustainable development for aviation (IEA, 2023; Brasil, 2023).

Brazil also stands out as the country with the largest sugarcane production in the world and most of the ethanol produced comes from sugarcane (CONAB, 2024). Therefore, the Brazilian sugar and alcohol industry shows a demand for the implementation of sustainable technologies that can be added to its production chain, aiming to make use of the waste generated. Thus, the second generation ethanol production using lignocellulosic biomass from bagasse or sugar cane straw, appears to be a promising alternative for energy security, for reducing environmental impacts and for partial replacement. the use of fossil fuels.

Lignocellulosic biomass is a complex network consisting mainly of cellulose, hemicellulose, and lignin, as well as other polymers such as pectins and proteins. These compounds are united through covalent and/or non-covalent bonds, giving rise to the plant cell wall. The percentage of cell wall components depends on the nature of the plant; for example, sugarcane bagasse generally has an average composition of 38 to 50 % cellulose, 23 to 32 % hemicellulose, and 15 to 25 % lignin, in addition to small amounts of other extractives (Carvalho et al., 2009; Doran et al., 2000; Ladeira-Ázar et al., 2019).

Lignocellulosic compounds cannot be directly fermented, requiring a prior breakdown, through hydrolysis or redox reactions, of their chains to release fermentable sugars (Banerjee et al., 2010). This depolymerization process consists of the joint and synergistic action of several carbohydrate-active enzymes (CAZymes), such as cellulases and hemicellulases that promote the release of the fermentable sugars hexoses and pentoses (Waghmare et al., 2014). However, in the ethanol production process from lignocellulosic biomass, enzymatic hydrolysis plays an important role in relation to production costs due to the high value of the commercial enzymes that are used. Currently, the enzyme cocktail market is dominated by a few companies such as Novozymes and Dupont (Van Dyk and Pletschke, 2012; Morgan et al., 2022), and studies that look for new enzymes or more efficient enzymes are extremely relevant. In this context, for enzymatic saccharification to be widely used on an industrial scale, it is important to search for fungal enzymes that can act synergistically with existing commercial cocktails, improving their efficiency, or new enzymes that have kinetic and functional properties superior to those present in commercially available mixtures.

Microorganisms play an essential role in the production of enzymes for the saccharification of lignocellulosic biomass. Several microorganisms are involved in the production of cellulases and hemicellulases, and some filamentous fungi stand out in the industrial production of these enzymes, such as fungi *Aspergillus* and *Trichoderma* genus (Florencio et al., 2016). Over the years, phytopathogenic fungi have been studied because they have great potential for secreting these enzymes. The cell wall degradation occurs to allow the fungus to penetrate the host, causing the plant infection and thus obtaining the nutrients necessary for its survival and growth (Maitan-Alfenas et. al., 2019). The interaction between a phytopathogenic fungus and the plant occurs mainly through the action of CAZymes on the plant cell wall, and the monomers released are absorbed by the fungal cells and converted by various sugar catabolic pathways into the compounds necessary for their growth and reproduction (Li et al., 2022; Khosravi et al., 2015). Therefore, the identification and characterization of these enzymes at a molecular level are of great importance (Tavares et al., 2021; Huttner et al., 2017; Park et al., 2018). The set of enzymes secreted by fungi make up the secretome and their analysis allows to obtain a global view of the identity, function and interaction of the extracellular protein arsenal that participates in host infection (Bouws et al., 2008).

The phytopathogenic fungus *Kretzschmaria zonata* belongs to the *Xylariaceae* family and the *Ascomycota* phylum (Rogers and Ju, 1998) and was described for the first time in Brazil in 2021, by Alfenas et al., 2021, on teak trunks. Its virulence capacity proved interesting and studies on the lignocellulolytic enzymes secreted by this organism were carried out. Da Luz Morales et al.,2021 reported for the first time, the fungal enzymatic profile, showing that *K. zonata* is capable of secreting enzymes that can be applied in industrial processes.

Overall, fungi show different plant cell wall decomposition actions, since they present differences in the number and diversity of secreted CAZymes, in addition to differences in the content of genes that encode enzymes involved in metabolic processes (Morgan et al ., 2022; Nogueira et al., 2022). Thus, the fungal genome sequencing and tools combined with bioinformatics allow the broad study of the genome and enable the analysis of its secretome and metabolic network, making it possible to understand in a more comprehensive way the identity, function and interaction of secreted enzymes by these organisms and evaluate their growth under specific cultivation conditions, which can contribute to improving the application of these enzymes in industrial processes (Aguilar-Pontes et al., 2018; Li et al., 2022).

In this sense, the present work reports the complete genome sequence of the phytopathogenic fungus *Kretzschmaria zonata*, its profile of genes encoding CAZymes, its sugar metabolic network, the enzymatic activities of the main plant cell wall degrading enzymes and the application of enzymatic extract of the fungus in the saccharification of sugarcane bagasse. These results made it possible to characterize this important phytopathogenic fungus, understand its growth capacity on different carbon sources and reveal the potential of its enzymatic arsenal for the saccharification of lignocellulosic biomass.

2. GOALS

2.1. General Goal

Develop the genomic characterization of the fungus *Kretzschmaria zonata* to identify the secreted proteins that have the potential to be applied in the enzymatic hydrolysis of lignocellulosic materials and its metabolic profile of sugars to identify the growth potential of the fungus on different carbon sources.

2.2. Specific Goals

CHAPTER I: systematize some computational tools combined with bioinformatics for analyzing the secretome of phytopathogenic fungi and highlight the importance of these analyses for the enzymes market.

CHAPTER II: genome sequence of the phytopathogenic fungus *K. zonata*, prediction of the genes encoding CAZymes, its sugar metabolic profile, and evaluation of its enzymatic profile.

CHAPTER III: evaluate the activities of commercial interest enzymes secreted by the fungus *K. zonata* grown on different carbon sources, such as wheat bran, barley, corncob, and sugarcane bagasse, and apply its crude enzyme extract in the saccharification of two varieties of sugarcane bagasse.

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CHAPTER I – Computational Tools and Phytopathogenic Fungi Secretome: Unraveling the Protein Arsenal

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Highlights

- Phytopathogenic fungi secrete carbohydrases of great appeal to industrial processes
- Secretome analysis of fungal enzymatic profiles are essential for their application
- Advances in fungal enzyme-based bioprocesses are possible using computational tools

ABSTRACT

The industrial enzymes market is mainly segmented into carbohydrases, proteases, and lipases, being the first responsible to catalyze the depolymerization of lignocellulosic biomasses and applied to several industrial process. Microorganisms, plant and animal tissues can be used as sources of these enzymes. However, nowadays the microrganims dominate the market as enzymes sources. The phytopathogenic fungi can secrete several carbohydrases able degrade the host plant cell wall and important for the infection processes, which generates great appeal to produce enzymes from these fungi. Therefore, unraveling all protein arsenal secreted by a phytopathogenic fungus is interesting because these proteins may be useful for different biotechnological processes. Previous researches have showed that several phytopathogenic fungi considered promising for enzymes production, such as *Chrysoporthe cubensis*, *Ceratocystis fimbriata* and *Kretzschmaria zonata*. In addition, the computational tools have significantly contributed to these studies, allowing a better understanding of the fungal enzymatic profile and their biotechnological applications. This work shows a brief overview of the importance of understanding the enzymatic profile secreted by phytopathogenic fungi and their biotechnological applications, as well as a brief overview of computational tools applied for proteomic analysis.

Keywords**:** Phytopatogenic fungi; Secretome; Computational tools; Biotechnological processes.

INTRODUCTION

The study of phytopathogenic fungi secretome is essential to understand the relationship between the fungus and the host plant during the infection [1]. According to [2], the secretome definition is ‘‘the global study of proteins that are secreted by a cell, tissue or organism at any given time or under certain conditions’’. By causing diseases, phytopathogenic fungi secrete enzymes for their penetration and subsequent propagation in the plant tissue. These enzymes can degrade the plant cell wall polymers, which is important for nutriente acquisition by the fungi [3,4].

Secretome analyses are necessary to obtain a global overview of the identity, function, and interaction of the arsenal of extracellular proteins that participate in plant cell wall degradation [5]. Thus, the study of phytopathogenic fungi secretome elucidate the potential role of secreted proteins involved in several metabolic processes from host infection to cell wall degradation and enables the identification and characterization of enzymes that may be applied for biotechnological processes. For instance, the comprehensive identification and quantification of the secretome of phytopathogenic strains may be a useful approach for understanding their enzymatic systems to determine new strategies for energy sources development [6].

Proteomic studies have been carried out to understand plant-fungus interactions, virulence, and fungal pathogenicity, and it is well known that there is a close relationship between virulence and the ability to secrete high levels of hydrolases in phytopathogenic fungi [6]. Furthermore, by using integrated proteomics with computational tools, it is possible to predict the subcellular location, when proteins are expressed, and the different proteins and enzymes produced by fungi. It is also possible to discover the implications for fungi development and changes in environmental responses [7,8,9].

Therefore, the enzymes secreted by phytopathogenic fungi deserve attention due to their potential for industrial application, which encourages research related to genetics and the secretion mechanisms of these enzymes [5].

PHYTOPATHOGENIC FUNGI

Fungi are heterotrophic, eukaryotic, uni or multicellular microorganisms that show a chitinous cell wall as their feature [10]. Fungi may be parasitic, saprophytic or pathogenic, and between these last classification, some can cause diseases in plants, thus, they are denominated phytopathogenic, which can belong to several phylum such as ascomycota, basidiomycota, zygomycota and chytridiomycota [8,11]. Among the large number of identified fungal species, about 10 % can cause diseases in more than 10.000 plants, and their mode of action on the hosts can be varied, depending on the species. For example, some phytopathogenic fungi are able to invade and to colonize all plant tissues, while others act only on specific parts of the host, such as roots, stems, seeds or leaves [12].

Many fungal mechanisms and proteins have been shown to contribute to fungal pathogenicity or virulence, such as extracellular lignocellulolytic enzymes [8]. Thus, a more virulent phytopathogenic fungus shows great appeal for lignocellulolytic enzymes production and these macromolecules can be used in several biotechnological applications, such as the degradation of plant biomasses for the generation of monomeric sugars used for ethanol production [3,4,6].

PHYTOPATHOGENIC FUNGI SECRETOMES

Phytopathogenic fungi can secrete an enzymatic arsenal composed of several enzymes that act in the depolymerization of lignocellulosic biomasses, most of which are classified in the Carbohydrate-Active enZYmes (CAZy) database. The CAZy database classifies the enzymes into families and subfamilies according to their sequence, structure, and biochemical information. Enzyme classes covered by CAZy include glycosyl hydrolase (GH), carbohydrate esterase (CE), polysaccharide lyase (PL), glycosyltransferases (GT), and enzymes with auxiliary activity (AA) [9]. It is known that CAZYmes can be applied in various industrial processes, and many studies are showing the secretion of these enzymes of commercial interest by phytopatogenic fungi [7]. The xylanases, important CAZymes secreted by several pathogen fungus can be used for various biotechnology process such as clarification of juices, pulp bleaching, bioconversion of lignocellulose into fermentable sugars and preparation of animal food and have been reported in many papers [9,13,14,15,16,17].

Over the years, countless research has shown the enzymatic potential secreted by phytopathogenic fungi. CAZymes and accessory enzymes produced by the pathogen fungus *Penicillium funiculosum* are distributed across different subfamilies (GH3, GH5, GH6, GH7, GH10, GH11, GH16, GH30, GH43, GH62, GH71, GH93, CE1, CE5, AA7 and AA9) that are responsible for biomass degradation and can be applied in several biotechnological applications [18]. Recently, our research group published data on the phytopathogenic fungi *Chrysoporthe cubensis* [9], *Ceratocystis fimbriata* [15] and *Kretzschmaria zonata* [16] that aroused interest for being able to secrete enzymes of great commercial appeal. Overall, studied fungi showed wide enzymatic diversity in their exoproteome, thus, the enzymes secreted by these fungi can act in several biotechnological processes, such as lignocellulosic biomass degradation and xylooligosaccharides production contributing with applicable resources in the bioenergy, food and pharmaceutical industries. *Chrysoporthe cubensis* (Figure 1A) is an important pathogen that can strike commercially cultivated eucalyptus species (Figure D) in tropical and subtropical areas of the world, which can generate losses and great damages since it causes stem canker disease of the plant species. According to [9], *Chrysoporthe cubensis* is capable of secreting 313 proteins, including 137 CAZymes classified as Glycosyl Hydrolases (GH), Carbohydrate Esterases (CE), Polysaccharide Lyases (PL) and Auxiliary Activities enzymes (AA). *Ceratocystis fimbriata* (Figure 1B) can cause damage to several agricultural and forestry crops around the world, highlighting root rot, canker, and vascular wilt (Figure E) in economically important species such as eucalyptus [19]. In its exoproteome, there is a β-xylosidase, an accessory enzyme of great importance in the degradation of hemicellulose that may perform an important role in the supplementation of commercial cocktails aiming to improve the yield of xylose and glucose in lignocellulosic biomass saccharification [15]. *Kretzschmaria zonata* (Figure 1C) is a fungus that causes root collar rot disease in teak (Figure 1F) and it was recently reported in Brazil [20]. This fungus produces an arsenal of extracellular enzymes, including xylanase, β-xylosidase and endoglucanases, enzymes with several industrial applications such as second-generation ethanol production [16].

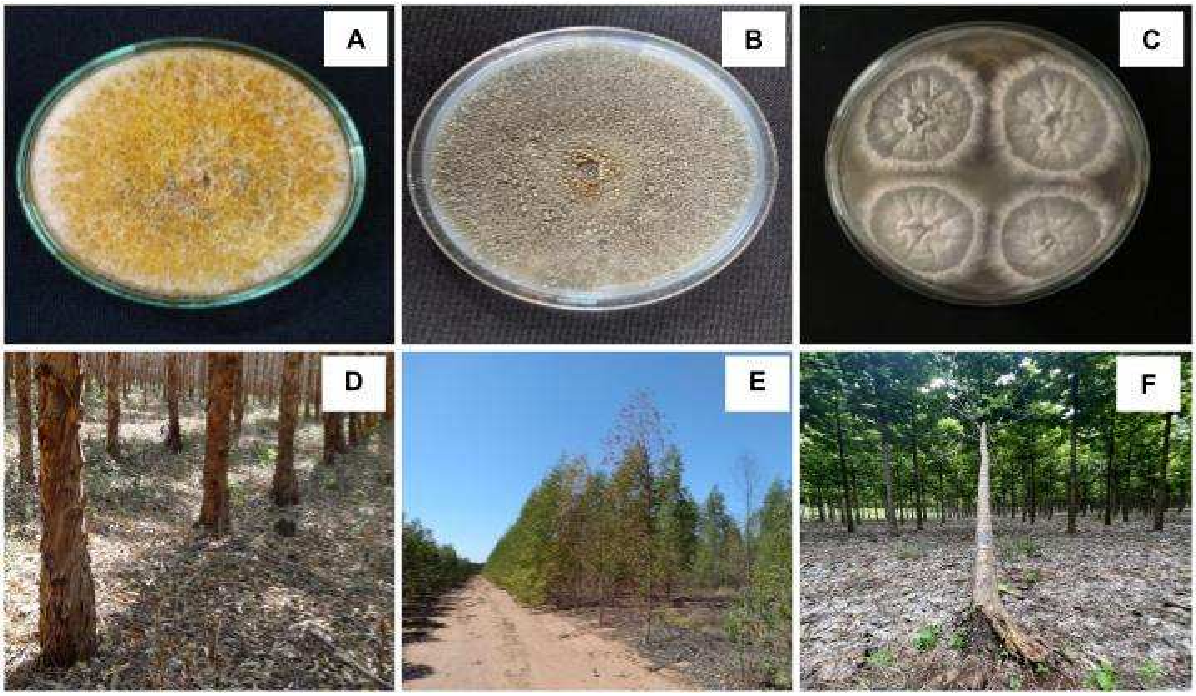

Figure 1: Colony morphology of (A) *Chrysoporthe cubensis* (B) *Ceratocystis fimbriata* and (C) *Kretzschmaria zonata* on malt extract agar culture media. (D) Eucalyptus stem canker caused by *C. cubensis*. (E) Vascular wilt in Eucalyptus caused by *C. fimbriata*. (F) Root collar rot in teak caused by *K. zonata*.

COMPUTATIONAL APPROACHES APPLIED TO THE SECRETOME

Proteomics, in combination with bioinformatics prediction of the secretome and enzymatic activity analyses, constitutes a powerful tool for providing information about pathogenicity, virulence factors and biotechnological potential of the exoproteome of phytopathogenic fungi [7,8,9]. Thus, there are many computational tools that may be employed to predict proteins of interest, their subcellular location, and conditions of expression.

To obtain bioinformatic predictions on secretome, it is necessary to obtain the organism's genome sequence generally using high-throughput sequencing technologies, such as from Illumina and Oxford Nanopore. Thus, from sequenced genome and using appropriate tools, it is possible to unravel all arsenal of proteins secreted by an organism, opening new possibilities for their biotechnological applications. For this, the first step is the genome annotation that may be performed by several programs, such as Geneid [21], one of the first programs to predict full exonic structures of vertebrate genes in anonymous DNA sequences; GeneMark [22], a web software for gene finding in prokaryotes, eukaryotes and viruses; and Augustus [23], a gene prediction program more applied for eukaryotes, that is based on a probabilistic model of a sequence and its gene structure denominated of generalized hidden Markov model. After genome annotation, it is necessary to evaluate the quality and the confidence of the predicted gene sets and, for this, the program Benchmarking Universal Single-Copy Orthologs (BUSCO) [24] can be applied.

To predict the secreted proteins and their subcellular localization, there are several programs available, some perform simpler methods such as SECRETOOL [25] and others more refined, for example, Loctree [26], BUSCO [24] and Deeploc [27]. The programs that perform prediction refined methods can predict the subcellular location that is important in proteomics because proteins can perform a wide diversity of functions inside different cell compartments, the protein function is related to the compartment or organelle where it is located, providing a physiological context for a certain function [28]. The Loctree is a hierarchical tree system combining support vector machines (SVMs) and other method such as gene ortology consortium (GO) for predict the subcellular localization of proteins. It shows good levels of accuracy, but it is not used for predicting localization for membrane protein [26,29]. In contrast the Deeploc program is widely used, their prediction algorithm can differentiate several localizations including cell membrane. Their method is based on deep neural networks that do not depend on annotation of homologues from knowledge databases, relying only on sequence information. It is useful mainly for new proteins prediction that there are no annotated homologues and predict the effects of sequence variants [27].

It can also be used software capable of predicting the presence of putative signal peptides at the N-terminal of protein sequences, and secondary structures and disordered regions necessary for nonclassical secretion methods, as well as capable of postulate amino acid composition and discriminating transmembrane helices containing proteins that will be inserted into the membrane [21,30]. Therefore, the web analysis tool SECRETOOL [25] can be used for this purpose. According to [25], SECRETOOL allows the screening of different properties concerning the proteins of the fungus proteome: location of the predicted N-terminal signal peptide (SP), detection of the presence and location of SP cleavage sites on amino acid sequences, presence of a maximum of one transmembrane domain (TMD) and glycosylphosphatidylinositol (GPI) membrane anchoring. Moreover, the proteins targeted to the nonclassical secretion pathways may be identified using other software such as SecretomeP [30], a program trained on sequence features other than signaling peptides of secreted proteins, although this software is designed for bacteria and mammals and not for fungi. Thus, an alternative is an ortholog-based method to predict secreted proteins via a nonclassical pathway [30].

In phytopathogenic fungi, the CAZymes are the most important secreted enzymes for complex carbohydrate metabolism and, therefore, these are the proteins that generate the most commercial interest and greater appeal for identification [9,13]. CAZymes degrade, synthesize, and modify complex carbohydrates and glycoconjugates in all organisms. In the protein identification step, an approach applied to increase the CAZymes identification is using multiple prediction strategies. For this, the predicted proteome can be subjected to dbCAN2, a meta server for automated CAZymes annotation based on three tools/databases: (i) HMMER search against the dbCAN (hidden markov models) HMMs database; (ii) DIAMOND search against the CAZy database and (iii) Hotpep search against the conserved CAZyme short peptide database [31].

The InterPro is a freely available database used to classify proteins sequences into families and predicts domains and important sites. It is a comprehensive resource that integrating 13 protein signature databases into a single searchable resource.

Sequence research performed into InterPro is powered by the InterProScan, underlying software that perform searches of proteins and nucleic acid against InterPro's signatures [32].

Several software may be used for functional annotation such as GOanna [33], BLASTKoala [34], Blast2GO [35], and eggNOG-mapper v2 [36], which are tools relying on sequence similarity for annotation. The general approach to functional annotation applied by these tools is very similar; initially, the protein sets are scanned for motifs and domains using resources like Pfam [37] and InterPro and mapped to Gene Ontology terms using GO supplied mapping files. In addition, BLAST analysis of full-length sequences may be applied to identify similar sequences which already have GO or pathway annotations linked to them and shorter motifs and domains [38].

The computational tools and approaches mentioned here corroborate to the phytopathogenic fungi secretome research. By properly using the available tools, it is possible to unravel the entire protein arsenal secreted by fungi, from the gene to the functional attribution of the proteins, contributing to a better understanding of the enzymatic profile of these organisms. Table 1 shows some examples of software that were reported in articles for having been used in fungal secretome analysis.

Table 1: Software used in fungal secretome analysis.

Software	**Reference**	**Main functionalities**	**Application in fungi (References)**
Secretool	Cortázar et al., 2014 http://genomics.cicbiogune.es/SECRETOOL/Secretool.php	Predict putative secreted proteins; Infer their domain structure and ortholog relations among fungi.	Tavares et al., 2021; Jing et al., 2022.
Deeploc	Armenteros et al., 2017 https://services.healthtech.dtu.dk/service.php?DeepLoc-1.0	Eukaryotic protein subcellular localization;Can differentiate between 10 different localizations	Tavares et al., 2021.

dbCAN2	Zhang et al., 2018 https://bcb.unl.edu/dbCAN2/	Automated Carbohydrate-active enzyme annotation; Integrates three state-of-the-art tools/databases for automated CAZyme annotation (HMMER, DIAMOND and Hotpep); Combines the results from the three tools and allows visualization of the overlaps as Venn diagram and the detailed results as graphs.	Morgan et al., 2022; Tavares et al., 2021.
Blast2GO	Gotz et al., 2008; Aparicio et al., 2006 https://www.blast2go.com/	Protein sequence annotation; Combination of various annotation strategies and tools controlling type and intensity of annotation; Numerous graphical features such as the interactive GO-graph visualization for gene-set function profiling or descriptive charts.	Tavares et al., 2021.

PERSPECTIVES AND FINAL CONSIDERATIONS

Over the years, research related to the phytopathogenic fungi secretome has aroused much interest because they secrete useful enzymes for biotechnological purposes such as second-generation ethanol production, food and pharmaceutical industries. The integration of computational tools, which have been improved over the years and “omics” data will allow more in-depth study of phytopatogenic fungi, enabling a greater understanding of their enzymatic profile. Therefore, new enzymes and biotechnological applications can be discovered, or applications already carried out in industrial segments can be improved. Thus, with the enzymatic profile of several fungi being unveiled, rapid advances in fungal enzyme-based bioprocesses appear to be imminent in the coming years.

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CHAPTER II – Whole genome sequence, CAZyme repertoire and sugar metabolic model of the phytopathogenic fungus *Kretzschmaria zonata* GFP 132.

PAPER II - **Whole genome sequence, CAZyme repertoire and sugar metabolic model of the phytopathogenic fungus *Kretzschmaria zonata* GFP 132** - Submitted in the Fungal Genetics and Biology - DOI: 10.2139/ssrn.4837909.

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ABSTRACT

Filamentous fungi are of great importance in natural environments because they are producers of several carbohydrate-active enzymes (CAZymes) that are capable of degrading the plant cell wall and thus contributing to the global carbon cycle. Furthermore, the degradation of polysaccharides releases sugar monomers that through a series of enzymatic reactions are metabolized as a source of carbon and energy. The large number of fungi that can be found in the most diverse environments contributes to great genetic variability, reflecting a wide diversity of enzymes being produced. In this study we evaluated the genome, the diversity of CAZymes produced and secreted by the fungus *Kretzschmaria zonata*, its ability to grow on different monosaccharides and polysaccharides, its metabolic network and interconnections and we compared the results with other phylogenetically close fungi, phytopathogens and with fungi known to be good producers of plant cell wall degrading enzymes (CWDE). The size of the assembled genome was 30.15 Mbp and a total of 12,135 protein-coding genes were identified, with putative CAZymes corresponding to approximately 5 % of total protein-coding genes. The repertoire of genes encoding CDWE was broad and showed to be more similar with to *Aspergillus niger* repertoire which is a Eurotiomycetes than to the fungi *Trichoderma reesei* and *Neurospora crassa* that are Sordariomycetes like *Kretzschmaria* zonata. When compared with phylogenetically close fungi, as *Kretzschmaria deusta*, *Hypoxylon submonticulosum* and *Xylaria cf. heliscus,* the CAZymes profile was similar in number and diversity of enzymes, however when compared to other phytopathogenic fungi the difference was more obvious, showing similarity with the fungus *Cryphonectria parasitica* EP155 v2.0 but great discrepancy with the fungi *Bretziella fagacearum* and *Ophiostoma novo-ulmi*. For all the fungi selected for this study, at least one copy of genes-encoding enzymes that catalyze the main reactions of the metabolic pathways were identified. *K. zonata*, possesses a complete metabolic network, except for an absent gene in the galacturonic acid metabolic pathway, and it revealed to have a more extensive set of metabolic genes than *T. reesei*. In addition, the fungus showed good growth on most tested carbon sources, not growing only on galacturonic acid.

Keywords: Plant cell wall; Enzymes; Sugar metabolism; Ascomycete fungi.

1 – INTRODUCTION

The plant cell wall is a rigid barrier composed of a complex network of polysaccharides and other compounds, such as lignin and proteins that provide mechanical strength and defense against microbial infection (Lu, et al., 2020). In nature, enzymes from plant pathogenic fungi have shown the ability to degrade the plant cell wall. This process involves an arsenal of cell wall-degrading enzymes (CWDE) that are usually secreted by these fungi and there is a close relationship between the fungal pathogenicity or virulence and its ability to secrete CWDE (Maitan-Alfenas, et al., 2019). Cell wall degradation occurs to allow the pathogen to penetrate the host, cause the infection, and obtain essential nutrients for its survival, and growth through a variety of sugar catabolic pathways (Maitan-Alfenas, et al., 2019; Li, et al., 2022).

Many enzymes are required for complete plant cell wall degradation, most of which are classified in the Carbohydrate-Active enzymes (CAZy) database (Drula, et al., 2022). The enzymes considered as CAZymes can be related to the breakdown, biosynthesis, or modification of glycoconjugates, oligo- and polysaccharides. The interaction between a phytopathogenic fungus and the host occurs mainly by the action of the CAZymes in the plant cell wall; therefore, the identification and characterization of these enzymes on a molecular level is of great importance (Tavares, et al., 2021; Huttner, et al., 2017; Park, et al., 2018). Previous studies have demonstrated that the production of CWDE by filamentous fungi is controlled at the transcriptional level by a diverse set of transcriptional factors (TFs). Some regulators such as XlnR, CreA, AraR, AmyR, InuR, RhaR, ManR/ClrB, ClrA, GalX, Ace1, and GaaR respond to mono and small oligosaccharides that act as inducers and can also control the metabolic conversion of these compounds (Benocci, et al., 2017). Thus, the efficiency of polysaccharide degradation by the fungus depends on an adequate regulatory system.

Overall, phytopathogenic and non-phytopathogenic fungi show different modes of action in the plant cell wall decomposition, since they show significant differences in the number and diversity of secreted CWDE that are directed by the genetic background. Also, they may present significant differences in the conservation of the genes that encode enzymes involved in sugar metabolism (Morgan, et al., 2022; Nogueira, et al., 2022). Species of the genus *Aspergillus* and *Trichoderma* (non-phytopathogenic fungus) have been the subject of numerous studies and are known as good producers of CWDE, and, in addition, the sugar metabolic network of *Aspergillus niger* is well-established and used in comparison among others fungus using an orthology-based approach (de Vries and Visser 2001; Khosravi, et al., 2015). Previous studies also have reported phytopathogenic fungi producers of CWDE belonging to different phyla such as *Ascomycota, Basidiomycota, Zygomycota*, and *Chytridiomycota* (Zhao, et al., 2013; González-Fernández, et al., 2010).

During the degradation of the plant cell wall process, the complex polysaccharides in this structure that cannot be taken into the cell in this polymeric format are degraded into various monosaccharides used by the fungus metabolism to obtain energy. The monomers are taken up by cells and converted by a variety of sugar catabolic pathways into the compounds needed by the fungal cells for their growth and reproduction (Li, et al., 2022; Khosravi, et al., 2015). Thus, the cell wall degradation is closely connected to the metabolic pathways present in the fungus, and therefore, the investigation and identification of the main enzymes involved in sugar metabolic networks are very important for a better understanding of the enzymatic profile, as well as its growth under specific cultivation conditions and the fungal behavior on its natural habitat, which can contribute to improving their use for industrial applications (Aguilar-Pontes, et al., 2018; Li, et. al., 2022).

Kretzschmaria zonata is an ascomycete fungus that belongs to the *Xylariaceae* family (Rogers and Ju, 1998). It has been characterized as a plant pathogenic fungus, mainly causing root soft rot in crops such as teak and citrus trees (Alfenas, et al., 2021; Cibrián Tovar, et al., 2014). Rogers and Ju (1998) described this species as a significant pathogen distributed in subtropical and tropical regions. Recently, *Kretzschmaria zonata* GFP 132 was isolated and reported in Brazil by Alfenas, et al (2021) and it has previously been reported in Nigeria (West, 1938) and Mexico (Cibrián Tovar, et al., 2014), all in teak trees, causing serious damage to the plantation. da Luz Morales, et al. (2021) described for the first time the enzymatic profile of this fungus, showing that it can secrete an arsenal of CWDE. However, little is known about its genome sequence and annotation of secreted enzymes, as well as its sugar metabolic network.

In this study, we report the whole genome sequence, the profile of CAZyme-coding genes, and the sugar metabolic network of the phytopathogenic fungus *Kretzschmaria zonata* GFP 132. The information shown here provides knowledge of the outstanding ability of *K. zonata* to decompose plant cell wall polysaccharides, aiming to increase the efficiency of existing industrial processes to generate sustainable chemicals from renewable materials. Furthermore, the high-quality genomic data generated here contribute to better characterizing this important fungal plant pathogen, which is poorly covered in biological sequence databases.

2 - MATERIAL AND METHODS

2.1 - Fungal Strain Culture and Genomic DNA and RNA Isolation

Kretzschmaria zonata GFP 132 was obtained from the Forest Pathology Laboratory, of the Federal University of Viçosa, Brazil, and it was cultivated on MEA (Malt Extract Agar) plates for 8 days at 28°C.

For DNA extraction, ten agar plugs (Ø = 5.0 mm) of MEA containing mycelia were added to a sterile 250 mL Erlenmeyer flask, in triplicate, containing 100 mL of medium with the following composition: glucose 10 $g.L^{-1}$, NH_4NO_3 1 $g.L^{-1}$, KH_2PO_4 1 $g.L^{-1}$, $MgSO_4$ 0.5 $g.L^{-1}$ and yeast extract 2 $g.L^{-1}$. The flasks were kept under agitation at 150 rpm for 8 days at 28 °C. The samples were centrifuged for 15 minutes at 15.000 x *g* and DNA was extracted using the Promega® kit (Wizard Genomic DNA Purification Kit), according to the manufacturer's instructions, with maceration in the Tissuelyser equipment using a frequency of 20 Hz per 1 min (2X).

For RNA extraction, ten agar plugs (Ø = 5.0 mm) of MEA containing mycelia were added to 4 Erlenmeyer flasks, in triplicate, containing the same described medium, replacing only the carbon source. The used carbon sources were glucose, sugarcane bagasse, wheat bran, and corn cob. The media were centrifuged at 15,000 × *g* for 15 minutes, and the fungal mycelium from the different culture media was mixed, considering the different growth conditions. RNA was extracted using TRIzol with adaptations. Initially, 25 mL of culture was centrifuged, and the pellet was ground with liquid nitrogen until obtaining a fine dust. A proportion of 1 mL of TRIzol™ Reagent per 50-100 mg of tissue was added to the sample and it was homogenized for 3 minutes using the vortex at maximum speed. The mixture was incubated for 5 minutes to complete the dissociation of the nucleoproteins complex. For the lysis, a proportion of 0.2 mL of chloroform per 1 mL of TRIzol™ Reagent was added and the tube was securely caped. The sample was homogenized in a vortex for 15 seconds and incubated on ice for 3 minutes, then centrifuged for 15 minutes at 12,000 x g at 4 °C. The mixture was separated into a lower red phenol-chloroform, an interphase, and a colorless upper aqueous phase. The aqueous phase, which contained the RNA was transferred to a new tube. Then, the proportion of 0.5 mL of isopropanol per 1 mL of TRIzol™Reagent was added to the aqueous phase, and it was incubated for 2-3 minutes on ice; the sample was centrifuged for 15 minutes at 12,000 × *g* at 4 °C. The aqueous phase containing the RNA was transferred to a new tube and 0.5 mL of isopropanol per 1 mL of TRIzol™ Reagent was added, then it was incubated for 1 hour at -20 °C and centrifuged for 10 minutes at 12,000 × *g* at 4 °C. The supernatant was discarded, and the pellet was resuspended in 1 mL of 75 % ethanol per 1 mL of

TRIzol™ Reagent. Posteriorly, the sample was centrifuged for 5 minutes at 7500 × *g* at 4 °C and the supernatant was discarded. The RNA pellet was air-dried for 5-10 minutes and resuspended in 20-50 µL of RNase-free water.

2.2 - Genome Sequencing and De Novo Assembly

The *Kretzschmaria zonata* GFP 132 genome was sequenced using PacBio 6-10 kb library produced from 2 ug of genomic DNA are treated with DNA Prep to remove single-stranded ends, repair DNA damages and followed by end repair, A-tailed, and ligation with PacBio adapters using SMRTbell Template Prep Kit 1.0 (Pacific Biosciences). The final size selection is with the Sage BluePippin system (Sage Science). PacBio Sequencing primer was then annealed to the SMRTbell template library and sequencing polymerase was bound to them using Sequel II Binding kit 2.0. The prepared SMRTbell template libraries were then sequenced on a Pacific Biosystems' Sequel II sequencer using 8M v1 SMRT cells and Version 2.0 sequencing chemistry with 1x1800min sequencing movie run times. Filtered subread data was processed with the JGI QC pipeline (genome.jgi.doe.gov/lookup?keyName=jgiProjectId&keyValue=1267591) to remove artifacts. Mitochondria were assembled separately with the CCS reads using an in-house tool (assemblemito.py), used to filter the CCS reads, and polished with two rounds of RACON version 1.4.13 racon [-u -t 36] (https://github.com/lbcb-sci/racon). The mitochondria-filtered CCS reads were then assembled with Flye version 2.8.1-b1676 (https://github.com/fenderglass/Flye) [-g 40M --asm-coverage 50 -t 32 --pacbio-hifi] to generate an assembly and polished with two rounds of RACON version 1.4.13 racon [-u -t 36] (https://github.com/lbcb-sci/racon).

The transcriptome was sequenced with Illumina. Plate-based RNA sample prep was performed on the PerkinElmer Sciclone NGS robotic liquid handling system using Illumina's TruSeq Stranded mRNA HT sample prep kit utilizing poly-A selection of mRNA following the protocol outlined by Illumina in their user guide: https://support.illumina.com/sequencing/sequencing_kits/truseq-stranded-mrna.html, and with the following conditions: total RNA starting material was 1000 ng per sample and 8 cycles of PCR was used for library amplification. The prepared libraries were quantified using KAPA Biosystems' next-generation sequencing library qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument. Sequencing of the flowcell was performed on the Illumina NovaSeq sequencer using NovaSeq XP V1.5 reagent kits, S4 flowcell, following a 2x151 indexed run recipe. Filtered RNA-Seq reads were assembled into consensus sequences using Trinity version 2.11.0 (Grabherr, et al., 2011). Raw data is submitted to SRA in accordance with Table 1.

Table 1 – Deposit of genome data in database.

Sequencing Project ID	Library Name	BioProject	BioSample	Project Accession	Run Accession	Load Date
1267595	HSUBW	PRJNA1080816	SAMN40207251	SRP501865	SRR28699446	15/04/2024 13:04
1267596	HNCWH	PRJNA1098161	SAMN40903260	SRP501021	SRR28623120	10/04/2024 23:34

2.3 - Phylogenetic Analysis

Initially, ITS (internal transcribed spacer 1 - 5.8S - internal transcribed spacer 2) sequences from *Kretzschmaria zonata* were searched in the "Nucleotide" Genbank database and the retrieved sequences were used to search the ITS region in *K. zonota* GFP 132 assembly. Furthermore, BLASTn alignments of ITS sequence from *K. zonata* GFP 132 against the NCBI Nucleotide collection (nt/nr) indicated that the fungal species shared more similar sequences to *K. zonata* GFP132. The α-actin (*ACT*) and β-tubulin (*TUB*) sequences from the selected fungal species were downloaded from Genbank and used in the BLASTn searches to identify these genes in the *K. zonata* GFP 132 assembly.

For phylogenetic analysis, reference sequences of *ACT*, *TUB*, and ITS regions were downloaded from GenBank (Supplementary, Table 1). These sequences were aligned using MAFFT v.7.30 (Katoh and Standley, 2013) and jmodeltest v.2.1.10 (Darriba, et al., 2015) was used to infer the best nucleotide substitution model under the Bayesian information criterion. Phylogenetic reconstruction using the maximum likelihood method was carried out in RaxML v.8.2.12 (Stamatakis, 2014) using the TrNef+G4 model with 1000 searches for the best-scoring tree. The tree was visualized in iTOL (Interactive Tree Of Life) (Letunic and Bork, 2021).

2.4 - Gene Prediction and Annotation

The genome was annotated using the JGI Annotation pipeline and made available at JGI MycoCosm genome portal (https://mycocosm.jgi.doe.gov/Krezon1) along with tools for interactive comparative analysis (Grigoriev, et al., 2014). Bother assembly and annotation have been deposited to Genbank under accession (to be provided upon publication).

2.5 - CAZyme Gene Identification and Subcellular Localization Prediction

CAZyme identification was generated by the Carbohydrate-Active enzymes database team (www.cazy.org) according to their annotation pipeline (Lombard, et al., 2014). The subcellular localization prediction was executed by Deeploc-1.0 (https://services.healthtech.dtu.dk/service.php?DeepLoc-1.0).

2.6 - Growth Profiling

For the growth profile, the strain *Kretzschamria zonata* GFP 132 was grown on minimal medium (MM), on 1.5 % (w/v) agar plates with one of the eight monosaccharides or one of the six polysaccharides described in Table 2. Growth was performed at 28 °C. Plates containing only minimal medium, without carbon source, were used as controls. For each specific carbon source, it was considered as no growth in the plates that showed the same growth as those that did not contain any carbon source. Growth was scored based on colony diameter and mycelium density. Growth was performed in duplicate, and no variation was observed between the duplicates for any tested carbon source.

Table 2 **–** Monosaccharides and Polysaccharides used on growth profiling.

Monosaccharides (25 mM)	Polysaccharides (1 %)
D-glucose	Xylan
D-fructose	Galactomannan
D-galactose	Starch
D-mannose	Cellulose
D-xylose	Inulin
L-arabinose	Pectin
D-rhamnose	
D-glucuronic acid	

2.7 - Comparison of CAZymes among various species

Numbers of genes per CAZy family for the selected fungi were obtained from Mycocosm (Grigoriev, et al., 2014) and manually assigned to the substrate they act on as described previously (Vesth, et al., 2018). These were then compared to obtain the polysaccharide degradation potential of each species.

2.8 - Identification of Sugar Metabolism Genes Orthologs and Generation of Sugar Metabolic Models for the Selected Fungal Species

Orthologs are genes in different species that originated from a common ancestor after a speciation event, resulting in the retention of similar functions during evolution. An orthology-based approach can assist us in inferring functions among different organisms. Orthologous SMGs were identified using OrthoFinder (https://github.com/davidemms/OrthoFinder) (Emms and Kelly, 2019). The protein sequences of the fungal species were downloaded from the JGI MycoCosm Portal (https://mycocosm.jgi.doe.gov) (Grigoriev, et al., 2014) as input. The OrthoFinder method provides a fast, accurate and comprehensive platform to infer the complete set of orthologs between selected species based on the phylogenetic information from the ortho-group tree. OrthoFinder was performed using default parameters with DIAMOND (Buchfink, et al., 2015) for sequence similarity searches and Dendro-BLAST for the tree inference of orthogroups.

The identified orthologs were then projected on the previously generated models of *A. niger* and *T. reesei* (Aguilar-Pontes, et al., 2018; Li, et al., 2022), to generate metabolic models for each species.

3 - RESULTS AND DISCUSSION

3.1 - Assembly and annotation of *Kretzschmaria zonata* GFP 132 genome

The *Kretzschmaria zonata* GFP 132 genome sequencing generated 301.508 reads with 2.512.919.225 bases. After quality control (JGI QC pipeline) and raw data filtering, 301.005 reads and 2.508.565.793 bases were kept in the data set, representing 99.8 % of the total data. The total size of the assembled genome was approximately 67 Mbp and the assembled genome contained 12 contigs and 12 scaffolds with the largest scaffolds of approximately 9.4 Mbp. The N50 and L50 parameters were 4 and 7.468.808 bp, indicating a good genome assembly. The total GC count was 24.657.001 bp, corresponding to 36.44 % GC. The main parameters and information about the genome assembly are described in Table 3.

The JGI annotation pipeline predicted the final consensus gene set contained 12.135 protein-coding genes, and a search for Ascomycota and *Sordariomycete* universal single-copy orthologs with BUSCO v5 yielded 97.2 and 97.7 % of completeness, respectively, indicating great genome assembly and gene content.

Table 3 – Summary of the *K. zonata* GFP 132 assembly.

Total sequenced bases	2.508.565.793
Number of scaffolds	12
Number of contigs	12
Largest scaffold (bp)	9371415
Largest contig (bp)	9371415
N50 (scaffolds and contig)	4
L50 (scaffolds and contig – Mbp)	7.47
Sequencing read coverage depth	32.17x
GC content (%)	36.44
Protein-coding genes	12,135
Average exons/gene	3.2
Average introns/gene	16.3
Completeness (*Ascomycota* - %)*	97.2
Completeness (*Sordariomycetes* - %)*	97.7

*The genome completeness was calculated using the *Ascomycota* and *Sodariomycetes* universal single-copy orthologs with BUSCO v3 (Simao et al., 2015).

3.2 - Phylogenetic analysis

Preliminary searches with ITS fungi sequences using BLASTn provided the best hits between *K. zonata* GFP 132 and sequences from *Kretzschmaria deusta* CBS 826.72 with 91.18 % identify and 100 % coverage. The combined multiple sequence alignment of *ACT*, *TUB,* and ITS sequences was used for phylogenetic reconstruction and confirmation of *K. zonata* GFP 132 taxonomic classification.

The maximum likelihood (ML) analysis generated the ML tree (Figure 1). All sections were in accordance with previous studies of *K. zonata* (Alfenas et al. 2021: Pourmoghaddam et al. 2018). *K. zonata* GFP 132 grouped in a clade containing *Kretzschmaria* strains with reliable bootstrap proportions, and thus, we confidently classified this fungus as *Kretzschmaria zonata* GFP 132.

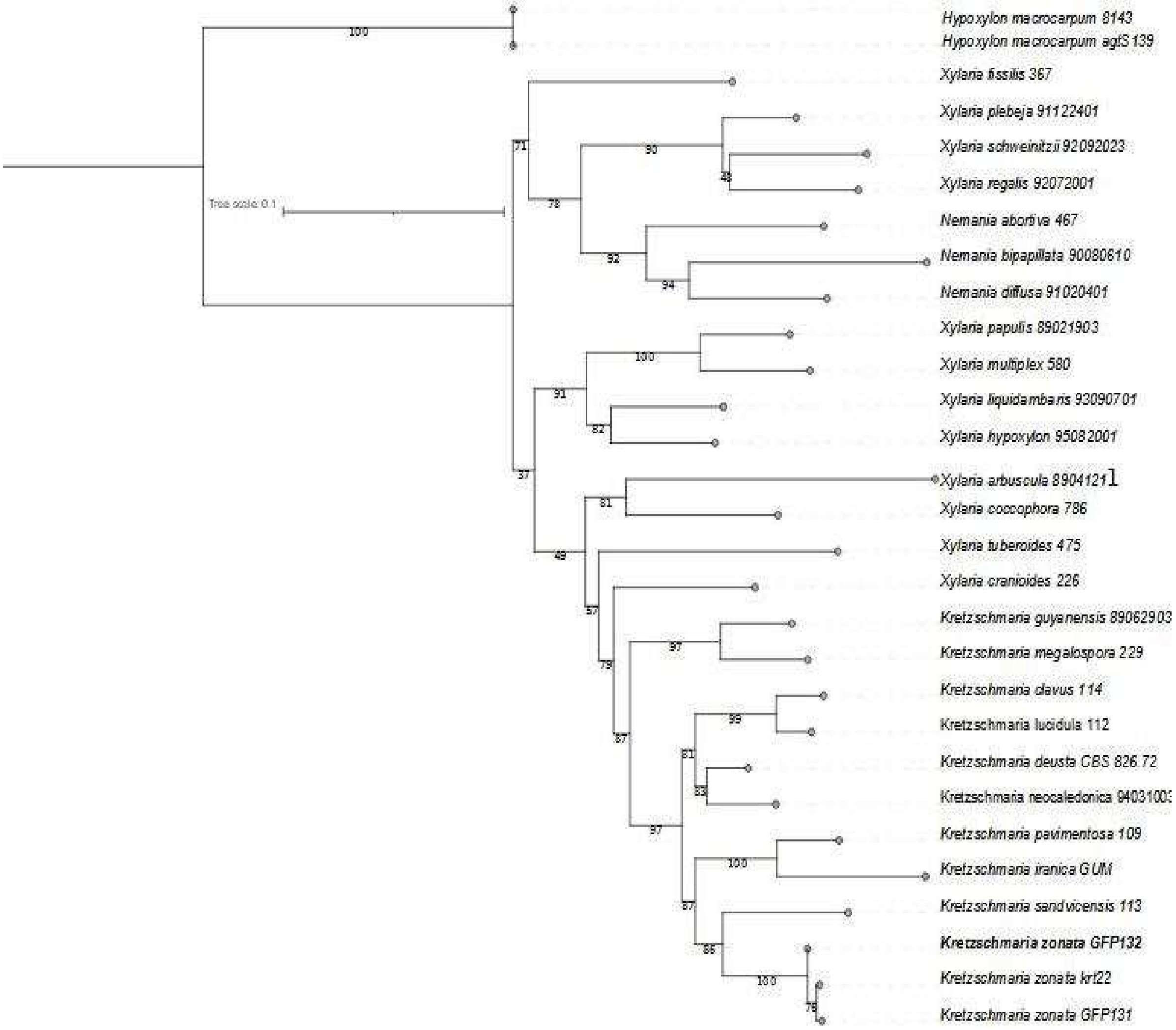

Figure 1 - Best-scoring Maximum Likelihood tree of concatenated sequence alignment of *ACT* (*α – actin), TUB (β – tubulin),* and *ITS* (internal transcribed spacer 1 - 5.8S – internal transcribed spacer 2) from species belonging to *Kretzschmaria*, *Xylaria,* and *Nemania.* The tree is rooted in *Hypoxylon macrocarpum*. The Genbank accession numbers are given in Suplemmentary, Table 1.

3.3 - CAZyme identification

Prediction of CAZymes revealed that the genome of *K. zonata* GFP 132 encoded 575 putative CAZymes, corresponding to approximately 5 % of total protein-coding genes (12.135). These CAZymes consisted of 279 glycosyl hydrolases (GHs), 87 glycosyl transferases (GTs), 18 polysaccharide lyases (PLs), 28 carbohydrate esterases (CEs), 93 auxiliary activity enzymes (AAs), 64 carbohydrate-binding modules (CBMs) and 6 distantly related to plant expansins (Figure 2A), from a total of 139 different families (Supplementary, Table 2). The CAZyme gene sequences were downloaded from the Mycocosm Portal version:17.46 (myco-web-1.jgi.lbl.gov) and the

519 gene sequences available were submitted to Deeploc-1.0 (https://services.healthtech.dtu.dk/service.php?DeepLoc-1.0). Deeploc analysis of CAZymes showed that 293 proteins are secreted by *K. zonata* GFP 132 (Figure 2B), including 180 GHs, 17 PLs, 21 CEs, 63 AAs, 8 CBMs, and 4 EXPNs, most of which may be directly involved in the plant cell wall degradation.

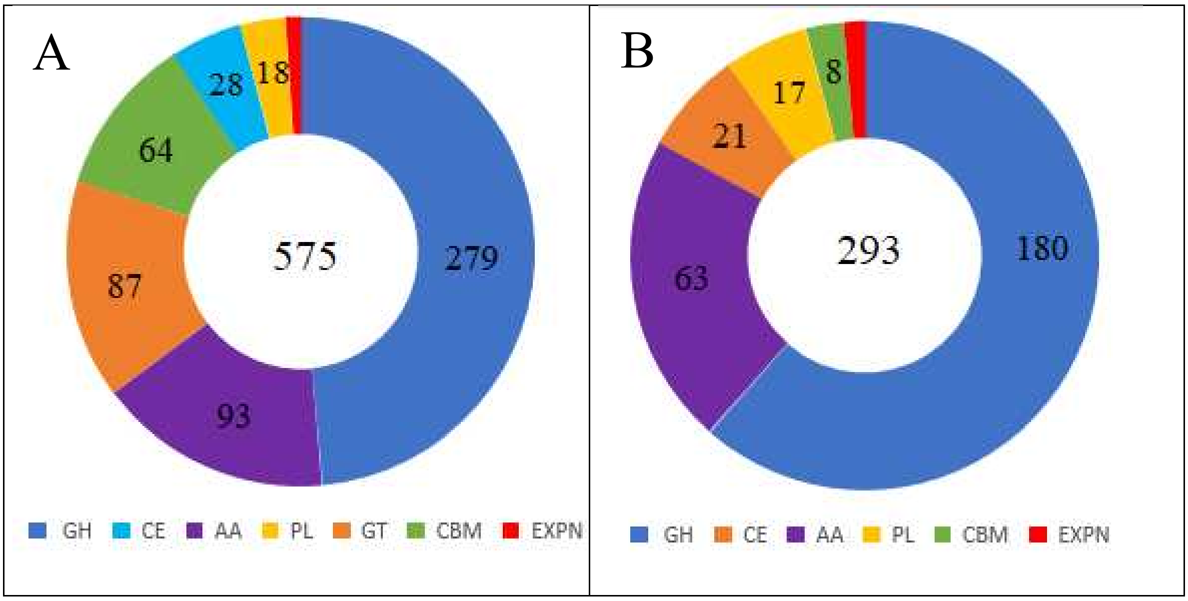

Figure 2 - Carbohydrate-active enzymes of *Kretzschmaria zonata* GFP 132 genome.

With the data obtained from the JGI Mycocosm, a comparison between *Kretzschmaria zonata* and other fungi was performed, and these fungi were divided into three different groups. Group I contained phylogenetically close species: *Kretschmaria deusta* IL1129, *Kretschmaria deusta* CBS 826.72, *Xylaria cf. heliscus* FL0509, and *Hypoxylon submonticulosum* NC0708. Group II contained phylogenetically distant phytopathogenic fungi, such as *Ophiostoma novo-ulmi* subsp. *novo-ulmi* H327, *Cryphonectria parasitica* EP155 v2.0 and *Bretziella fagacearum* C519 v1.0. Finally, group III contained fungi known as good producers of CWDE, *A. niger* and *T. reesei* QM6a. The fungus *N. crassa* was also used for comparison, because it is a well-characterized species among filamentous fungi and also a member of the Sordariomycetes, althought taxonomically distant from *K. zonata*. All of these fungi belong to the Ascomycota phylum.

The total number of putative CAZymes of *K. zonata* GFP 132 was similar to other members of group I (Table 4), suggesting conservation of genome content among the close relatives. However, large differences in CAZy content were observed in comparison to the species of group II (other phytopathogens), suggesting that a similar lifestyle is not accompanied by a similar CAZy content. Interestingly, the number of putative CAZymes of *K. zonata* is more similar to *A. niger* than to *T. reesei* and *N. crassa*, despite the latter two being Sordarimomycetes like *K. zonata*, while *A. niger* is a member of the Eurotiomycetes. While previous studies often showed lower CAZy numbers for Sordariomycetes than for Eurotiomycetes (de Vries, et al., 2017), our study demonstrates that the *Xylariaceae* are as rich in CAZymes as the Eurotiomycetes, which may make them interesting sources of novel enzymes for biotechnology.

Table 4 –Comparasion of CAZymes numbers by selected fungus

	Fungi	CAZymes
	***Kretzschmaria zonata* GFP 132**	**575**
Group I	*Kretzschmaria deusta* IL1129	589
	Kretzschmaria deusta CBS 826.72	593
	Hypoxylon submonticulosum NC0708	566
	Xylaria cf. heliscus FL0509	636
Group II	*Ophiostoma novo-ulmi* H327	342
	Cryphonectria parasitica EP155 v2.0	551
	Bretziella fagacearum C519 v1.0	243
Group III	*Aspergillus niger*	547
	Trichoderma reesei QM6a	401
	Neurospora crassa OR74A	438

3.4 - Enzymatic profile of *Kretzschmaria zonata* GFP 132

The *K. zonata* GFP 132 genome contains 93 AAs, of which 63 are present in the exoproteome and belong to nine families: AA1, AA2, AA3, AA5, AA8, AA9, AA11, AA12, and AA16 (Fig. 3). Of the 64 CBMs, 8 are part of the exoproteome and are associated with GHs, CEs, or AAs. The 28 CE coding genes, belonging to 9 families, include 21 that are in the exoproteome and are distributed as follows: CE1, CE3, CE4, CE5, CE8, CE12, CE15, and CE16. One hundred eighty (180) of the 279 GHs are putatively secreted and belong to 56 different families. The *K. zonata* GFP 132 putative exoproteome showed families with the highest predicted enzyme number as GH3, GH5, GH7, GH10, GH11, GH16, GH18, GH28, GH30_7, GH43, GH51, and GH55. The glycosyl hydrolases constitute families with the highest number of enzymes secreted by *K. zonata* GFP 132, which may contribute to this fungus being a great cellulase producer. Other fungi used in commercial cocktails, such as *Aspergillus* spp., also secrete many cellulases (Falkoski, et al., 2013). Finally, it was predicted 18 PLs, of which 17 are present in the fungus exoproteome, belonging to 7 different families: PL1, PL3, PL4, PL7, PL9, PL20, and PL26.

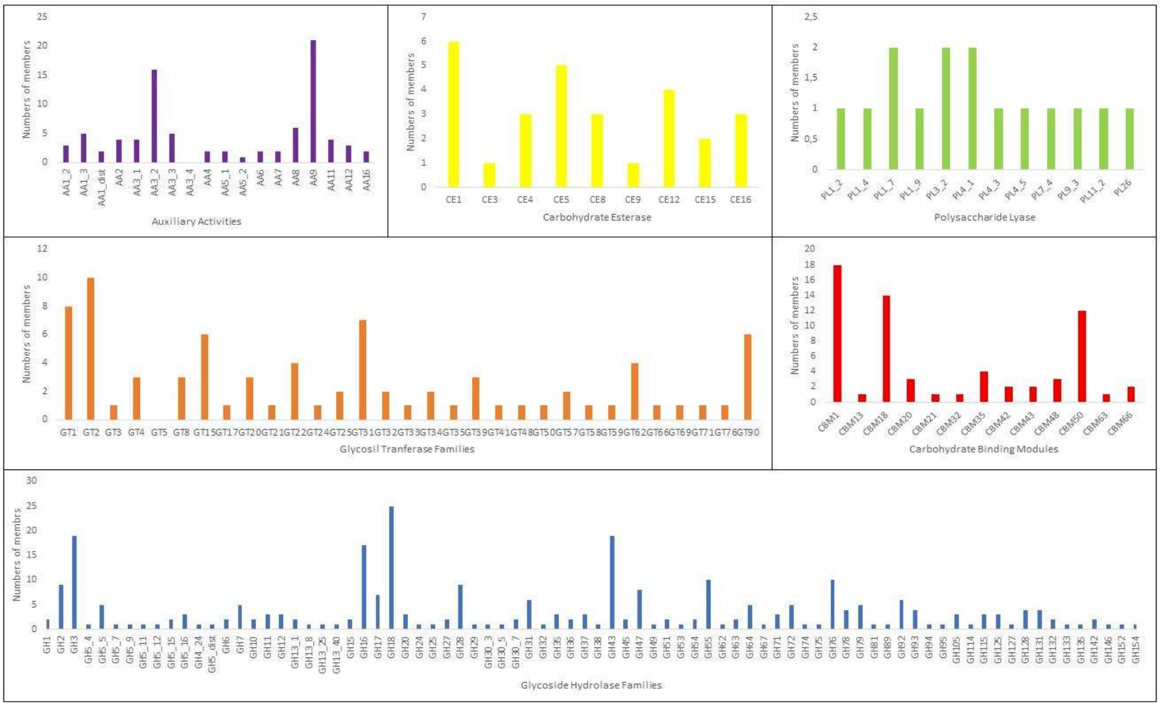

Figure 3 – Diversity of carbohydrate-active enzymes in the *Kretzschmaria zonata* GFP 132 genome.

Some CAZyme families are relevant for plant cell wall degradation, such as enzymes that act on cellulose: GH3, GH5_4; GH5_5, GH6, GH7 and AA9; and enzymes that act on hemicellulose: GH10, GH11, GH30_7, GH43, GH51, CE1, CE5, CE15 and CE16 (Tavares, et al., 2021). In addition, other enzymes may be related to CWDE performance, such as LPMOs from the AA9 family. The fungus *K. zonata* has a great diversity of genes encoding glycosyl hydrolases, auxiliary enzymes and carbohydrate esterases (Figure 3), in this sense the fungus stands out in the number of genes encoding enzymes GH5_5, GH7, GH45, AA9 and CE1 when compared to other fungi. Most of the commercial enzymatic cocktails used for lignocellulosic biomass degradation are produced by fungi belonging to *Trichoderma* and *Aspergillus*. Overall, these fungi show a great ability to secrete a wide range of cellulases and hemicellulases (Florencio, et al., 2016; Vesth, et al., 2018; Rehman, et al., 2014). When we compare *K. zonata* with the fungi *T. reesei* QM6a and *A. niger* NRRL3 significant differences are observed in relation to some enzymes (Table 4). *K. zonata* has a higher number of genes encoding AA9 family enzymes than the selected fungi, which may be interesting for the degradation of the plant cell wall, since Cannela, et al. (2012) showed that enzyme extracts supplemented with LPMO (AA9) resulted in superior saccharification performance.

The *K. zonata* GFP 132 genome showed several CWDE-encoding genes, such as the proteins related to cellulose (43 genes), xylan (32 genes), pectin (50 genes), inulin (1 gene), manan (8 genes), xyloglucan (20 genes), and starch degradation (12 genes) (Supplementary, Table 4). Figure 4 shows a comparison based in substrates of the CAZyme distribuition considering the main families involved in the CWDE of the phytopathogenic fungus *K. zonata* GFP 132 with the previously selected fungi divided into three groups. The lifestyle of fungi was also considered for comparison. It is observed that in group I, very similar profiles were obtained in relation to the main CAZyme families involved in the cellulose, xylan, xyloglucan and pectin degradation, and the number and diversity of enzymes. This similarity suggests that high gene conservation was maintained in taxonomically close fungi. Despite this, some fungi in this group have different lifestyles, some are pathogenic and others are saprobic and yet the profile was similar, suggesting that a different lifestyle among phylogenetically close fungi does not change the CAZyme content. In group II, phytopathogenic fungi that are phylogenetically distant from *K. zonata* showed a large variation in the CAZyme profile, suggesting that a similar lifestyle of taxonomically distant organisms is not accompanied by a similar CAZyme genome content. Still in group II, the *Bretziella fagacearum* C519 v1.0 showed a poor CAZyme profile (Supplementary, Table 3) both in number and in variety of enzymes that act on the polyssacharides, however the fungi *Cryphonectria parasitica* EP155 v2.0 and *Ophiostoma novo-ulmi* subsp. *novo-ulmi* H327 showed a smaller amount of cellulases than *K. zonata,* but the first one showed to have more genes encoding xylanases and pectinases, which may contribute to the plant cell wall degradation. Regarding group III, *Kretzschmaria zonata* showed interesting differences when compared with *A. niger*, *N. crassa* and *T. reesei*. The *K. zonata* obtained a greater number of CAZyme-encoding genes than *T. reesei* and *N. crassa*, which belong to the same class as *K. zonata*, and more closely resembling the profile presented by *A. niger*, which belongs to a different class than other fungi, *K. zonata* also stood out in this group in the number of genes encoding auxiliary enzymes that act in the celulose degradation and in the diversity of putative enzymes that act in the xylan depolymerization. In this context, the study demonstrates that the CAZy profile of the fungus *K. zonata* belonging to the *Xylariaceae* family can be an interesting source of enzymes for biotechnological application.

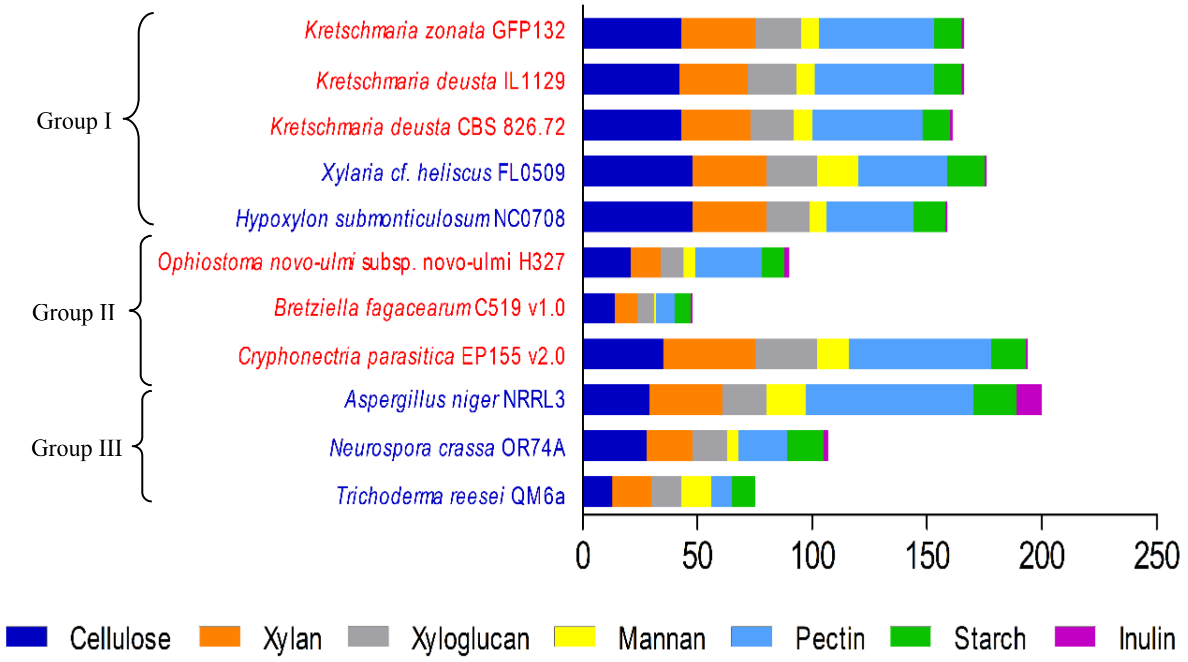

Figure 4 - Distribution of CAZyme genes based on substrate in the genome of the selected fungi based on plant biomass degradation. Phytopathogenic fungi are highlighted in red and saprobes are in blue.

In addition, the relationship between the diversity of predicted CAZymes for *K. zonata* and their growth profile was performed using eigth different monosaccharides and six different polysaccharides as carbon sources (Figure 5). The fungus was able to grow on practically all the tested carbon sources, with very similar growth among the monosaccharides, except for galacturonic acid where no growth was observed. Concerning the polysaccharides, the greatest growth for pectin and celulose corroborates with the number of genes predicted for the fungus, 50 and 43 predicted genes for pectin and cellulose degradation, respectively, being the polysaccharides with the most predicted genes. The low growth in inulin can be explained by the presence of only 1 gene predicted for the degradation of this polysaccharide.

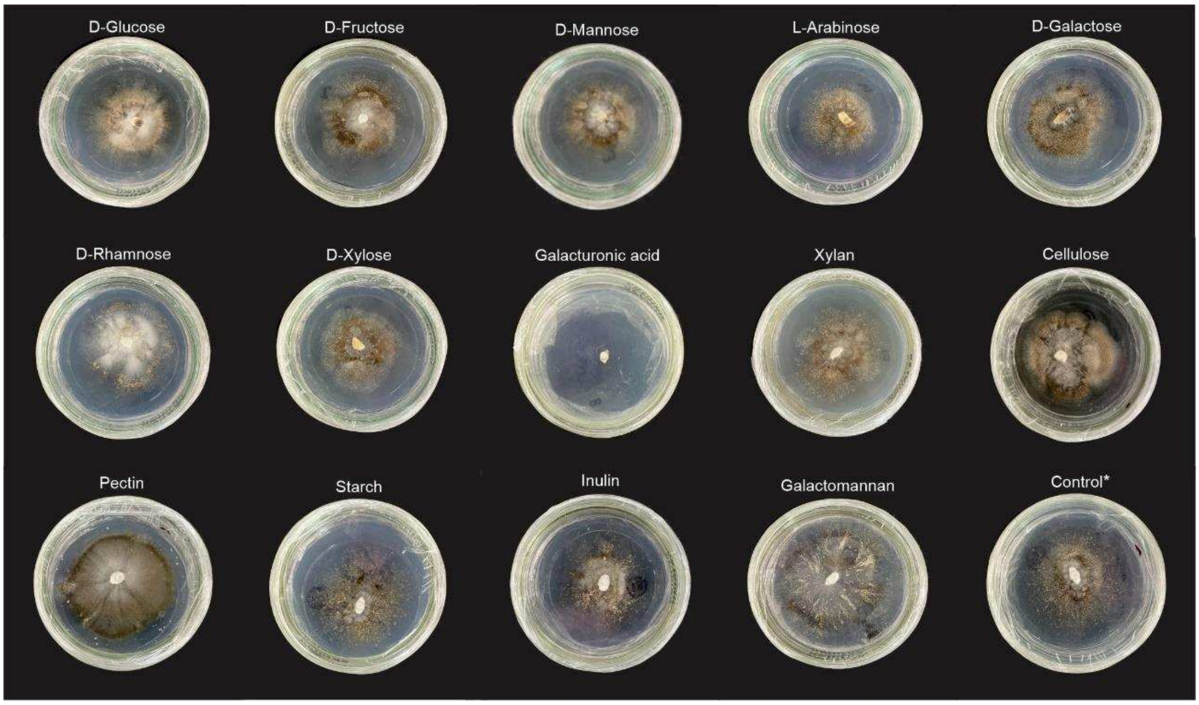

Figure 5 - *Kretzschmaria zonata* growth profile in minimal medium containing different carbon sources (monosaccharides and polysaccharides). *Control, minimal medium without carbon source.

3.5 - Sugar metabolic pathway of different fungal species

The sugar metabolic model revealed that the fungus *K. zonata* possesses genes for most of the main pathways and, when compared to the other selected fungi that were used in the study, it was one of those that showed a more complete metabolic network (Figure 6).

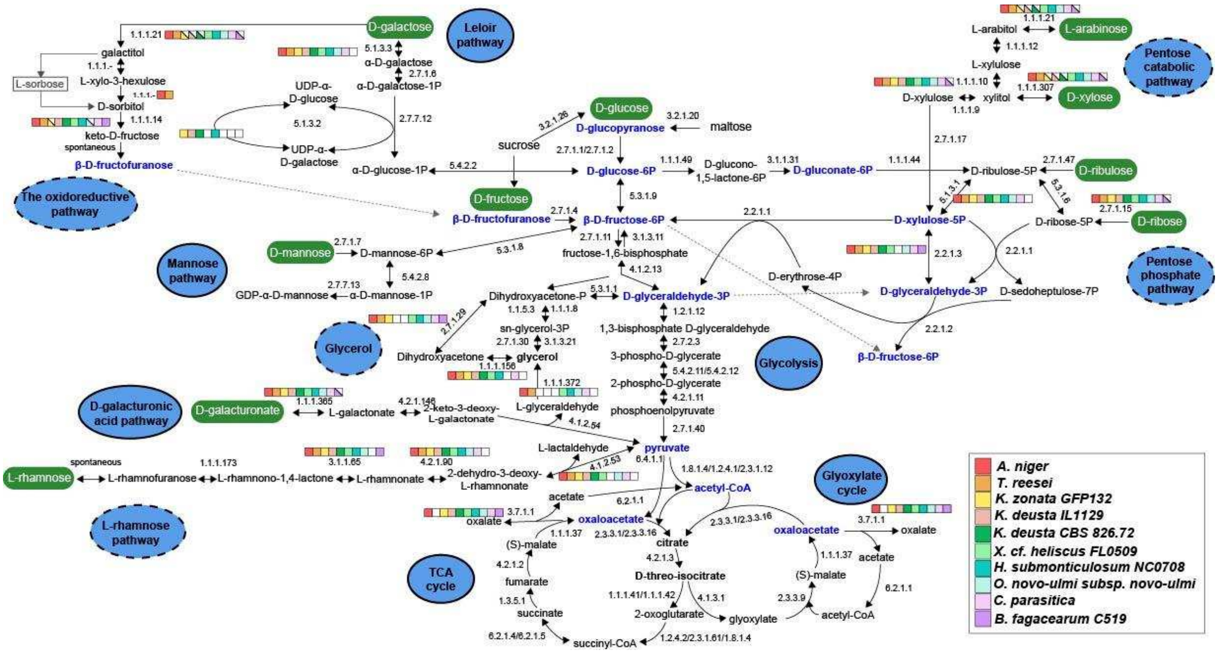

Figure 6 - Sugar metabolic networks of *K. zonata, K. deusta, X. xf. heliscus*, *H. submonticulosum, O. novo-ulmi subsp. novo-ulmi, C. parasítica and B. fagacearum.* The names of the sugar metabolic pathways are shown in blue circles. Reactions are represented with an arrow and reversible reactions with a double arrow. The EC numbers of the enzymes involved in each reaction are indicated. Dashed lines connect metabolites from different pathways. The genes that were present in two orthogroups and had differences are represented as a half-colored square.

For most of the fungi selected for this study, one or more copies of the genes encoding enzymes of the main metabolic pathways were identified. For the fungi of group I, containing the genetically closest species, it was observed that sugar metabolic pathways were highly conserved, and this is well correlated with their taxonomic distance. Among the fungi of group II, the sugar metabolic pathways showed large variations across the fungi and to *K. zonata. Bretziella fagacearum* showed a less complete metabolic network, compared to the other fungi. Although the fungi of this group are all phytopathogenic, the great variation in the metabolic pathways may be explained by the taxonomic distance between them. All are from the same phylum and class as *K. zonata* but belonging to different families and genera. Finally, for group III, *K. zonata* stood out for showing a significant variation in the sugar metabolic pathway compared to *T. reesei*, which does not contain important genes in the glyoxylate cycle and the TCA pathways, for example. However, *K. zonata* showed a profile more similar to *A. niger,* with both fungi presenting a complete metabolic network as shown by (Li, et al., 2022).

The sugar metabolic models revealed that glycolysis, mannose, glyoxylate cycle and the TCA catabolic pathways are well conserved among the selected fungi, but the copy numbers of some genes show a small difference. Nonetheless, Li, et al. (2022) showed that in addition to glycolysis, glyoxylate cicle and TCA, the galacturonic acid catabolic pathway was also well conserved among the tested fungi in their study, and mannose catabolic pathway had moderate conservation. D-glucose is a monosaccharide that is part of cellulose, starch and hemicellulose, and it can be easily catabolized by fungi being generally the preferred source of monomeric carbon (Li, et al., 2022). Therefore, it is common for all fungal species to contain at least one enzyme per reaction for the catabolism of D-glucose and grow in conditions where D-glucose is used as carbon source. *K. zonata* has all the glycolysis genes and was able to grow using D-glucose as a carbon source (Figure 5). *Bretziella fagacearum* was the only fungus that does not have the TPIB gene that encodes the triphosphate isomerase enzyme that catalyzes the conversion of dihydroxyacetone into D-glyceraldehyde-3-phosphate, but it does have TPIA that catalyzes the same reaction. In contrast to the completeness of glycolysis for all studied fungi, the gene copy numbers for different enzymes showed variations across different species. The TPI, PGI, PGM, FBP, PFK, and PGK encoding genes are extremely conserved and presented as a single copy in each fungus, while the copy numbers of other genes showed a slight difference between *K. zonata* and the other species. In the study carried by Li, et al., (2022) only PGI, PGK and PFK were highly conserved and for fungi that are phylogenetically close, there was a great conservation of gene numbers, presenting only a few differences in the number of copies, as also happened with species from the *Kretzshmaria* genus. *K. zonata* and *K. deusta* are very similar, showing almost the same numbers of orthologs identified for each enzymatic reaction step, except for the encoding gene GLK and the putative FBA gene that *K. zonata* shows one more copy, and also for the putative PGM gene, that *K. deusta* has one more copy.

Based on our prediction, almost all the necessary genes for TCA and glyoxylate cycles were identified in the genomes of all the selected fungi, and the CIT, LPD, and ACUA encoding genes are extremely conserved and present as a single copy in each fungus. *K. zonata* showed a predicted gene profile identical to that *K. deusta* and similar to the other fungi for both cycles.

The second most abundant compound in the plant cell wall is hemicellulose, which is formed by several polysaccharides, including mannan and galactomannan.

When these polysaccharides are degraded, they release D-mannose monomers that form their backbone. For mannose metabolization, all the selected fungi possess all the genes that encode the enzymes of the pathway. The MGT, PMM and PMI encoding genes are extremely conserved and presented as a single copy in each fungus, while the HXK encoding gene showed more than one copy only for the fungus *N. crassa.* Corroborating the prediction of all genes of the mannose catabolic pathway for *K. zonata*, the fungus was able to grow using mannose as a carbon source (Figure 5).

Unlike what was reported by Li, et al., (2022), in which all genes of the galacturonic acid catabolic pathway were conserved for all selected fungi, in this study *K. zonata, K. deusta, N. crassa* and *B. fagacearum* do not have the LarA (GaaD) gene, while all enzymes were identified for the fungi *C. parasitica, H. submonticulosum, O. novo-ulmi, T. reesei* and *X. cf. Heliscus*, with *T. reesei* having an extra copy for the GaaC and GaaB genes, *O. novo-ulmi* having an extra copy for the GarB gene and *C. parasitica* having an extra copy for the LarA gene. In agreement with the fact that *K. zonata* does not have all the genes necessary for the galacturonic acid catabolic pathway, this fungus was not able to grow in the medium with galacturonic acid as a carbon source (Figure 5). A previous study that related the growth profiles of several fungi and their predicted metabolic networks (Li, et al., 2022), showed that the absence of enzyme genes that participate in sugar metabolic pathways may imply in abscence or poor growth of the fungus when using the specific substrate as a carbon source, reinforcing what was found in the present study.

Li, et al. (2022) reported that all ascomycetes fungi encode at least one copy of each enzyme involved in the glycerol metabolism and two selected basidiomycetes do not contain the gene gldB (glycerol hydrogenase) that catalyzes the reduction of glycerol into dihydroxyacetone, however our study showed that genomes of the fungi *B. fagacearum* and *O. novo-ulmi* that are ascomycetes fungi also do not contain the gene gldB, suggesting that the absence of this gene is not a characteristic only of basidiomycetes. Still in this pathway, the fungus *K. deusta* is the only one that does not have the gene that encodes the enzyme DakA (glycerone kinase) that phosphorylates dihydroxyacetone. Overall, all other studied fungi showed one or more copies of the genes required for this glycerol catabolism pathway.

L-rhamnose is released from polysaccharides such as pectin. To metabolize rhamnose, four enzymes are necessary and of all the studied fungi, only three did not show at least one copy of all the genes. The fungus *B. fagacearum* did not show copies for the LkaA and LrdA genes encoding 2-keto-3-deoxy-L-rhamnonate aldolase and L-rhamnonate dehydratase enzymes. These enzymes catalyze the conversion of 2-dehydro-3-deoxyl-rhamnonate into L -glyceraldehyde and L-rhamnonate in 2-dehydro-3-deoxyl-rhamnonate, respectively. *C. parasitica* and *Hypoxylon submonticulosum* did not show copies for the LrlA and LkaA genes. In accordance with Li, et al. (2022), the L-rhamnose catabolic pathway was poorly conserved among the studied fungi.

D-galactose is a common monosaccharide that can be metabolized by most filamentous fungi through two different metabolic pathways, Leloir and Oxidoreductive pathway. Overall, for all the studied fungi, the D-galactose metabolism was complete, except for the fungus *B. fagacearum* that lack GalM and GalGc in the Leloir pathway, and the absence of one gene of the oxidoreductive pathway for *X. heliscus, Ophiostoma novo-ulmi* subsp. *novo-ulmi, C. parasítica* and *B. fagacearum*.

For fungi, the pentose phosphate pathway (PPP) and the pentose catabolic pathway (PCP) are very important. The first is the major source of NADPH and this metabolic pathway still provides the intermediate ribose-5-phosphate that is essential for the synthesis of nucleotides and nucleic acids and several intermediates that connect it with glycolysis and PCP pathway is responsible for the L-arabinose and D-xylose metabolization. In this study, all the selected fungi possess all the main genes that encode the enzymes needed for the PCP pathway and at least one copy of the genes that encode the enzymes for all reactions involved in the PPP was identified for the studied fungi, except for *B. fagacearum*, which does not possess the gene encoding RPE, *K. zonata* and *K. deusta* that do not have the gene encoding TktB, and *H. submonticulosum* and *N. crassa*, which do not have the gene encoding TktC. The GsdA, PglA, TAL, GND and TKT encoding genes were extremely conserved with a single copy for all fungi, differently from what was reported by Li, et al. (2022) since only the GsdA and RpeA genes were extremely conserved.

4 – CONCLUSION

This study showed the whole genome sequence, the growth profile and the diversity of the CAZyme-coding genes secreted by the fungus *K. zonata* GFP 132 and its metabolic network. Its enzymatic profile was more similar to that from the fungus *A. niger*, that belongs to a diffent class, than the profiles from *T. reesei* and *N. crassa*, that are Sordariomycetes, such as the fungus of interest, which suggests that *K. zonata* has a more generalistic approach concerning carbon utilization. The fungus also stood out for possessing the genes for all the metabolic pathways of the main sugars, except for galacturonic acid. It also showed more complete pathways than other fungi which are already well studied and used in industrial processes such as *T. reesei*. Furthermore, the fungus also showed good growth on plates using different carbon sources.

These results improve knowledge about the enzymatic profile of *K. zonata* GFP 132, which is rich in CAZymes like the Eurotiomycetes fungi, and its mechanisms for degrading plant biomass, and boost new studies to understand which enzymes generate good performance aiming to increase the efficiency of existing industrial processes. Furthermore, the high quality of genomic data generated allows other genomic analyses to be carried out, which can contribute to a better characterization of this poorly explored fungus.

5 – ACKNOWLEDGMENTS

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CHAPTER III – Hydrolytic enzymes from *Kretzschmaria zonata* induced by different carbon sources for sugarcane bagasse hydrolysis.

PAPER III - **Hydrolytic enzymes from *Kretzschmaria zonata* induced by different carbon sources for sugarcane bagasse hydrolysis.**

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ABSTRACT

The agricultural wastes provide large volumes of lignocellulosic biomass, mainly those originating from sugarcane processing. In this case, the use of lignocellulosic biomass to produce high-added value products generates economic and environmental advantages, and it is closely related to the concept of circular economy. This study evaluated the production of lignocellulolytic enzymes from the fungus *K. zonata* grown on different carbon sources and their potential to hydrolyze different varieties of sugarcane bagasse. The crude enzyme extracts produced using wheat bran, barley, corn cob, and sugarcane bagasse were applied for saccharification of sugarcane bagasse. Two varieties of sugarcane were used in this study, a commercial variety Ridesa RB 867515, and the energy cane. Maximum hemicellulolytic activities were obtained after fungal growth on corn cob, especially concerning xylanase activity, and sugarcane bagasse was the carbon source that induced fewer enzymes. Wheat bran was the only carbon source able to induce all tested enzymes, with the highest laccase activity among the tested extracts. In the saccharification tests, the extract from wheat bran was more efficient with the maximum glucose released after 96 h, 3,1 g/L, while xylose reached 2.1 g/L at the same time, concerning the energy cane hydrolysis.

Keywords: Phytopathogen*;* Lignocellulolytic enzymes; Energy cane; Sugarcane bagasse saccharification;

1 – INTRODUCTION

In 2023, the International Energy Agency report (Renewable, 2023) stood out Brazil among emerging countries as the country with the greatest growth in the biofuels sector. The estimate is that Brazil alone will contribute at least 40 % of the global expansion of biofuels by 2028. This is due to the incentive programs adopted by the country in this sector. Currently, the demand for biofuels in the country is mainly for road transport, which will continue for the next few years, however, at COP28 there were agreements to expand their use for air and sea transport, which will contribute to reducing dependence on fossil fuels and achieving the goal established in the Paris agreement at COP 21 in 2015 in relation to reducing polluting gas emissions. Therefore, with greater demand for biofuels, it will be necessary to further expand the production market (IEA, 2023).

Thus, in recent decades, environmental concerns and efforts to develop more sustainable and efficient products and services have resulted in solutions aimed at reducing waste and also obtaining economic and environmental advantages. The circular economy emerged as an alternative to the linear model of production and resource management and it aims to transform waste into a new resource (Mujtaba et al. 2023; Herrero-Luna et al. 2021). According to the Food and Agriculture Organization of the United Nations (FAO), it estimates that global production of agro-industrial waste reaches 1.3 billion tons per year. In Brazil, the agribusiness sector is large and productive and generates millions of tons of products and waste per year (Conab, 2024). The waste can be used in different segments and their physiochemical properties will suggest what path they may take. The lignocellulosic waste, for example, is rich in polysaccharides such as cellulose and hemicellulose and it is suitable for renewable energy processes whereas wastes with a high concentration of nitrogen and phosphorus are suitable for organic fertilizers. The first estimate of the sugarcane harvest released in April 2024 by the National Supply Company (Conab) indicates that Brazil should produce 685.86 million tons, and in the 2023 harvest, the country had the highest production with 713.2 million tons. Thus, Brazil has enormous potential to develop energy from sugarcane byproducts, mainly as it is the largest global commodity producer (Conab, 2024). In addition, the use of lignocellulosic feedstocks provided by the agribusiness sector has great potential to reduce waste and increase the production of high-value-added products (Mujtaba et al., 2023; Herrero-Luna et al., 2021).

Lignocellulosic residues from sugarcane, such as bagasse and straw, can be used in the bioethanol production, however, due to the recalcitrant nature of the plant cell wall, at least three steps are necessary to access the sugars (Maitan-Alfenas, 2015). The pre-treatment aims to change the biomass structure and facilitate the access of enzymes to polysaccharides; enzymatic hydrolysis converts these polymeric sugars into monomeric sugars that can be used by yeast during the third stage, the fermentation, and being transformed into ethanol (Maitan-Alfenas, 2019). The second-generation ethanol production process, also known as ethanol from waste, still has very high costs, with the main bottleneck being the high costs of the enzyme cocktails used in the saccharification stage and their low yield (Nogueira et al., 2022; Quiao et al., 2022). Fungi are the main producers of enzymes that form an enzymatic blend capable of degrading the polysaccharides from plant cell wall (Maitan-Alfenas, 2019). However, previous studies show that phytopathogenic fungi can play an important role in optimizing this stage, as they secrete in nature the enzymes necessary for the degradation of the plant cell wall seeking to infect the plant (Morales et al., 2021; Tavares et al., 2024; Tiwari et al., 2013).

The phytopathogenic fungus *Kretzschmaria zonata* is described as responsible for causing a soft rooting of the wood. It was recently discovered in Brazil by Alfenas et al., (2021), causing root collar rot disease in teak, and previously it was reported in others countries such as Nigeria (West, 1938) and Mexico (Cibrian Tovar et al., 2014). Alfenas et al. (2021) reported some fungus morphological characteristics such as conidia aseptate, stromata with surface brown to dark-brown and ascospores aseptate dark brown, fusiform to ellipsoid. The infection may cause serious damage to the plantation. Based on the ability of the fungus to degrade the plant cell wall to infect the host, studies were carried out to uncover its enzymatic potential and showed that this fungus secretes lignocellulolytic enzymes as cellulases, hemicellulases, laccases and accessory enzymes and may be able to efficiently catalyze the hydrolysis of plant biomasses such as sugarcane bagasse (Morales et. al, 2021).

The efficient conversion of cellulose and hemicellulose into their respective fermentable sugars occurs through the synergistic action of several enzymes. Most of these enzymes act on carbohydrates and are classified by the Carbohydrate-Active enZYmes (CAZy) database including cellulases, hemicellulases, ligninases, pectinases and auxiliary enzymes (LPMOs, laccases, catalases, peroxidases). Auxiliary enzymes such as laccases (Tavares et al., 2022).

This work aimed to contribute to the production of more efficient enzyme mixtures, in addition to their use in several other biotechnological processes.Therefore, the activities of the main enzymes that act on the plant cell wall degradation secreted by the fungus *Kretzschmaria zonata* were evaluated after cultivation on different carbon sources such as wheat bran, corn cob, barley, and sugarcane bagasse. Moreover, their application in the hydrolysis of two different types of sugarcane bagasse that presented different compositions was performed.

2 - MATERIAL AND METHODS

2.1 – Fungal and culture conditions for enzymatic production

The fungus *Kretzschmaria zonata* was isolated from diseased teak (*Tectona grandis*) roots and trunks and maintained on malt extract agar (MEA) plates. It was provided for this study by the mycological collection of the Forest Pathology Laboratory of the Federal University of Viçosa, Brazil.

The fungus was directly activated in semi-solid state fermentation in 250 mL Erlenmeyer flasks containing 5 g of the carbon source - corn cob, wheat bran, sugarcane bagasse (Ridesa RB 867515), and barley - and 12 ml of the culture medium (60 % moisture final mixture) composed of: NH_4NO_3 1.0 g/L; KH_2PO_4 1.5 g/L; $MgSO_4$ 0.5 g/L; $CuSO_4$ 0.25 g/L; and yeast extract 2.0 g/L. The following trace elements were added to the medium: $MnCl_2$ 0.1 mg/L; H_3BO_3 0.075 mg/L; Na_2MoO_4 0.02 mg/L; $FeCl_3$ 1.0 mg/L; and $ZnSO_4$ 3.5 mg/L. Each flask was autoclaved at 121 °C for 15 min and then inoculated with ten agar disks of 0.5 cm cut from PDA plates and incubated at a controlled temperature of 28 °C. The enzymatic extraction was carried out after 8 days of cultivation. The secreted enzymes were extracted with sodium acetate buffer, 50 mM, pH 5.0, in the proportion of 10:1 (buffer: dry substrate mass), under agitation at 150 rpm for 60 min at room temperature. The solids were separated by filtration in organza fabric and the filtrate was centrifuged at 10,000 *g* for 10 min. Then, the crude extract was frozen and stored for further analysis of enzyme activity.

2.2 – Enzymatic assays

Enzymatic activities were determined on the crude extracts of the fungus *K. zonata* after growth on the different carbon sources. For mannanase, xylanase, endoglucanase, and pectinase activities, the substrates were locust bean gum 0.4 % (w/v), Beechwood xylan 1 % (w/v), carboxymethylcellulose (CMC) 1.25 % (w/v) and polygalacturonic acid 0.25 % (w/v), respectively. The reactions initiated by adding 50 µL of the diluted enzymatic solution and 200 µL of the substrate diluted in sodium acetate buffer, 100 mM, pH 5.0. The reaction occurred at 50 °C for 15 min and it was stopped by adding 250 µL of dinitrosalicylic acid (DNS) for reducing sugars quantification (Miller 1959). Absorbance was read at 540 nm in a spectrophotometer and the standard curves were performed with glucose and xylose. FPase activity was determined using Whatman nb 1 filter paper (1x6 cm or 50 mg) as substrate. The reactions were initiated by adding 100 µL of the diluted enzymatic solution and 1 filter paper and 1,4 mL of sodium acetate buffer 100 mM, pH 5.0. The reaction occurred at 50 °C for 60 min and it was stopped by adding 500 µL of dinitrosalicylic acid (DNS) for reducing sugars quantification (Miller 1959).

Activities of β-glucosidase, β-mannosidase, β-xylosidase, β-cellobiohydrolase α-galactosidase and α-arabinofuranosidase were performed using the synthetic substrates ρ-nitrophenyl-β-d-glucopyranoside (ρ-NPβGlc), ρ-nitrophenyl-β-d-mannopyranoside (ρ-NPβMan), ρ-nitrophenyl-β-d-xylopyranoside (ρ-NPβGXyl), ρ-nitrophenyl-β-d-cellobiopyranoside (ρ-NPβCel), ρ-nitrophenyl-β-d-galactopyranoside (ρ-NPαGal) and ρ-nitrophenyl-β-d-arabinopyranoside (ρ-NPαAra), respectively, according to Visser et al. (2013). The reaction contained 35 µL of sodium acetate buffer 100 mM, pH 5.0, 50 µL of 2 mM synthetic substrate, and 15 µL of diluted crude extract enzyme. The reaction occurred at 50 °C for 15 min and was stopped by adding 100 µL of 500 mM sodium carbonate. The absorbance was read at 410 nm in the spectrophotometer and the values were converted in µmoles of ρ-NP, using a standard curve performed with 0–1.0 µmol of a ρ-nitrophenol solution.

Laccase activity was determined using the substrate ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate)) based on Wolfenden & Willson (1982). The mix reaction was composed of 800 µL of sodium acetate buffer, 100 mM, pH 5.0, 100 µL of ABTS, 10 mM, and 100 µL of diluted enzyme extract. The reaction took place at 50 ºC, in a water bath, for 15 minutes, and the absorbances were read in a spectrophotometer at 420 nm. For quantification of oxidized ABTS, the absorbance values were applied to the equation of Lambert-Beer using $\varepsilon = 3.6 \times 10^4$ cm^{-1} $.mol^{-1}$ .L, referring to the compound.

One unit of enzymatic activity (U) was defined as the amount of enzyme that releases 1 μmol of the corresponding product per minute, under the conditions used for the assay.

The enzymatic assays were carried out in triplicate, and the standard deviation was also calculated for all assays. The values of enzymatic activities on different substrates were analyzed using Minitab 21 software, performing analysis of variance (ANOVA) followed by Tukey's test at a significance level of 5 % ($\alpha = 0.05$). The standard deviation was also calculated for all assays.

2.3 – Protein determination

The protein concentrations of the different crude enzyme extracts were determined by the Bradford method (Bradford 1976), using a standard curve prepared with a 0.2 mg/mL serum bovine albumin solution (BSA).

2.4 – Sugarcane bagasse pretreatment and biochemical composition

Two sugarcane varieties were used in this study: Ridesa RB 867515 and C-90176, both donated by Sugarcane Genetic Improvement Program of the Phytotechnics Department, Federal University of Viçosa, Brazil. RB 867515 is the most planted commercial variety in Brazil, according to the Varietal Census of the Agronomic Institute (IAC), released in December 2023 (Braga Júnior et al., 2023). The second sugarcane variety used is C-90176, which is an energy sugarcane, that is contains a higher concentration of fibers (cellulose, hemicellulose, and lignin) and is ideal for energy production due to the greater generation of bagasse (Lorenzi and Andrade, 2019; Triana et al., 2008).

For the pretreatment, the sugarcane bagasse was washed with cold water and dried using an oven at 50 ºC until reaching a constant mass. Then, it was further milled for particle size less than 20 mesh, and submitted to alkaline pretreatment using NaOH 1% (w/v) at a solid loading of 10 % (w/v). The pretreatment was performed in an autoclave at 121 ◦ C for 60 min. The pre-treated materials were filtered using a Buchner funnel fitted with filter paper. The solid fraction was washed using distilled water and milled for particle size less than 20 mesh. The chemical composition of the alkali-treated sugarcane bagasse was determined as described above.

The composition of *in natura* and pretreated from both sugarcane bagasse were perfomed as described by the National Renewable Energy Laboratory (NREL, USA).

2.4.1 – Extractives

The extractives elimination step was carried out as NREL (2008). Two grams of dry biomass were added in a filter paper (11 x 5 cm) cartridge. The extraction occurred in a Soxhlet system, with 200 mL of distilled water for 6 h, followed by 200 mL of ethanol 95 % for 20 h. The samples were subsequently dried in an oven at 50 ºC for 24 hours. The extractives content was calculated using the cartridge dry mass before and after extraction according to equation 1.

$$Extractives\ (\%) = \frac{(P2) - (P1)\ x\ \%\ total\ solids}{100} \qquad eq.1$$

Where:
P1 ; Cartridge weight + initial sample;
P2 : Cartridge weight + final sample;
% total solids : Obtained by moisture analyzer.

2.4.2 – Carbohydrates and Lignin quantification

The determination of total carbohydrates was performed by acid hydrolysis of biomass, according to NREL (2012). Acid hydrolysis of 0.3 g of extractives-free dry biomass was performed. Initially, the samples were maintained at 30 °C with 3 mL of sulphuric acid 72 %. The material was then transferred to 250 mL Erlenmeyer flasks and diluted to 4 % sulfuric acid concentration and, subsequently, autoclaved at 121 °C for 60 min. The hydrolysate was submitted to vacuum filtration in Gooch crucibles. The glucose values were converted to cellulose contents by the factor 0.9, while xylose was converted to hemicellulose by a factor of 0.88. Total lignin was calculated by adding soluble and insoluble lignin. The insoluble portion of lignin was retained in the crucible and quantified by mass difference. The hydrolysate was analyzed by spectrophotometry in quartz cuvettes in the wavelengths of 215 and 280 nm to calculate soluble lignin (equation 2).

$$Soluble\ lignin\ (\%) = \frac{(4.53\ x\ A215) - A280}{300} \qquad eq. 2$$

Where:
A215: Absorption of the solution at 215 nm;
A280: Absorption of the solution at 280 nm.

2.4.3 – Proteins

Protein content was determined indirectly by nitrogen quantification using Kjeldahl method, through digestion, distillation, and titration, as described (Bradstreet, 1954)). An amount of 0.3 g of dry biomass and 10 mL of digestion solution were added to Kjeldahl tubes. The tubes were gradually heated until 340 °C for 2 h. After cooling, 1 mL of hydrogen peroxide was added and the tubes were kept at 340 °C for more 40 min. The tubes were cooled, and 10 mL of distilled water were added. The digested samples were coupled to a TE-036E (Tecnal) nitrogen distiller, adding sodium hydroxide 50 % until the sample became dark brown. The samples were distilled to a 250 mL Erlenmeyer flask containing 25 mL of boric acid 4 % solution with indicator solution. Distillation stopped when the indicator solution became green, and the total volume was between 100 and 125 mL. The samples were then titrated with HCl 0.05 N until the color changed. The HCl volume was used to calculate the protein content (equation 3).

$$Protein\ (\%) = \frac{V\ x\ f\ X\ N\ x\ 0{,}0014\ x\ Factor}{M}\ x\ 100 \qquad eq. 3$$

Where:
V : HCl 0,05 N volume;
f : HCl 0,05 N correction fator;
N : Normality of HCl solution;
Factor : Nitrogen to protein conversion fator;
M : dried biomass weigth.

2.4.4 – Ashes

To quantify the inorganic matter content of the sample the porcelain crucibles containing 0.1 g of dry biomass were kept in a muffle furnace at 300 ± 25 °C for 1 h, followed by 4 h at 575 ± 25 °C. After cooling in a desiccator, the ash content was calculated by equation 4.

$$Ashes\ (\%) = \frac{Cf - Ci}{M}\ x\ 100 \qquad eq.4$$

Where:
Cf : crucible weight with sample residue after muffle (g);
Ci : empty crucible weight after muffle (g);
M : sample weight.

2.5 – Sugarcane bagasse saccharification

The saccharification of sugarcane biomass was carried out using two sugarcane varieties: Ridesa RB 867515 and C-90176. The crude enzymatic extracts from the fungus *K. zonata* after growth on corn cob, wheat bran, bagasse sugarcane, and barley, and the commercial cocktail Cellic CTec3 were applied for pretreated sugarcane bagasse saccharification. A control without enzymes was performed. Enzymatic saccharification of alkali-treated sugarcane bagasse was performed in 125 mL Erlenmeyer flasks with 25 mL working volume at an initial solid concentration of 5 % dry matter (w/v), corresponding to 1.25 g of biomass, in 100 mM sodium acetate buffer at pH 5.0. The enzyme loading was 2.5 FPase units per gram of biomass. Tetracycline (40 mg/L) and sodium azide (10 mM) were added to the reaction mixture to inhibit microbial contamination. The reactions were performed in an orbital shaker at 200 rpm and 50 ◦ C. Samples of 1.0 mL were taken from the reaction mixture every 24 hours until the 120 hours and they were immediately heated to 100 °C to denature the enzymes, cooled, centrifuged for 5 min at 15,000 x g, and stored at – 20 °C. The assays were executed in triplicate for each treatment.

2.6 – Hydrolysis products quantification

Glucose and xylose were quantified using high-performance liquid chromatography (HPLC) with a Shimadzu series 10 A chromatograph (Shimadzu, Kyoto, Japan). The HPLC had an Aminex HPX-87 P column (300 ×7.8 mm) and a refractive index detector. The column was eluted using water at a flow rate of 0.6 mL min^{-1} and operated at 80° C. The concentration of monosaccharides was determined through the equation of the straight line obtained by analytical curves with D-glucose and D-xylose standards in concentrations from 0.1 to 10 g/L, eluted under the same conditions. The standard deviation was calculated for all assays.

The glucose values were converted to cellulose contents by the factor 0.9, while xylose was converted to hemicellulose by a factor of 0.88. The biomass conversion efficiency was calculated considering the biochemical composition of the pretreated sugarcane bagasse, by the cellulose and xylan hydrolysis rate, according to equations 5 and 6 (Falkoski et al., 2013).

$$Cellulose\ hydrolysis\ rate\ (\%) = \frac{Mass\ of\ glucose\ (g)\ x\ 0.9}{Mass\ of\ cellulose\ (g)}\quad x\ 100 \qquad eq.5$$

$$Xylan\ hydrolysis\ rate\ (\%) = \frac{Mass\ of\ xylose\ \ (g)\ x\ 0.88}{Mass\ of\ xylan\ (g)}\quad x\ 100 \qquad eq.6$$

3 - RESULTS AND DISCUSSION

3.1 – Enzymatic activities secreted by the fungus *K. zonata* after growing on different carbon sources

Overall, the fugus *Kretzschmaria zonata* was able to secrete several enzymes involved in the plant cell wall degradation, for the four tested carbon sources, highlighting xylanase, pectinase, and laccase activities after growth in wheat bran (Table 1).

Table 1 - Crude extract activities (U/mL) from *K. zonata* after growth on different carbon sources.

	Carborn source			
	Barley	**Corn cob**	**Sugarcane bagasse**	**Wheat bran**
ctivity (U/mL)				
Endoglucanase	0.747^{a} ±0.244	0.429^{b}±0.053	0.259^{c}±0.027	0.669^{a} ±0.036
FPase	0.544^{b} ±0.046	0.241^{c}±0.050	0.511^{a}±0.206	0.469^{a}±0.046
Laccase	0.068^{b} ±0.001	0.024^{c}±0.001	ND	0.507^{a}±0.003
Mannanase	0.793^{a} ±0.082	0.206^{c}±0.066	0.255^{c}±0.023	0.644^{b}±0.143
Pectinase	1.179^{a} ±0.148	0.687^{c}±0.112	0.823^{b}±0.053	1.255^{a}±0.055
Xylanase	1.460^{b}±0.351	3.541^{a}±0.263	0.542^{c}±0.044	3.020^{a}±0.374
α-Galactosidase	0.016^{c}± 0.001	0.011^{d}±0.001	0.029^{b}±0.005	0.136^{a}±0.002
α-Arabinofuranosidase	0.018^{a}±0.005	0.009^{b}±0.001	0.003^{c}±0.001	0.027^{d}±0.001
β-Cellobiohydrolase	0.020^{a}±0.001	0.010^{b}±0.001	ND	0.004^{c}±0.001
β-Glucosidase	0.020^{c}±0.001	0.106^{b}±0.004	0.021^{c}±0.001	0.279^{a}±0.001
β-Manosidase	ND	ND	ND	0.001^{a}±0.000
β-Xylosidase	ND	0.001^{b}±0.000	ND	0.006^{a}±0.000

After growth on wheat bran, the crude extract of *K. zonata* showed 0.396 mg/mL of protein (Table 2) and this was the carbon source that induced all tested enzymes. The highest specific activities were found for xylanase, pectinase and mannanase, respectively (Table 3). Morales et al., 2021 also tested the cultivation of this fungus in wheat bran and showed a similar result to that obtained for the protein content: 0.36 mg/mL. The activities for xylanase, endoglucanase and FPase were also close. The difference in activity values can be explained by the different origins and compositions of the biomass that can molecularly alter the expression of enzymes. Previous studies showed that wheat bran is a good inducer of lignocellulolytic enzymes and its structure is composed of a large amount of hemicellulose and proteins (Bernardi et al., 2023; Tavares et al., 2024) that can contribute to fungal growth and enzyme induction and explain the best results for xylanase and mannanase. The crude extract of the fungus grown on wheat bran also showed a great result for laccase, an important enzyme classified as a ligninnase that acts in the oxidation of several complex compounds such as aromatics, diphenolics, polyphenols, diamines, and phenols (Tavares et al., 2024). Laccases act by catalyzing the oxidation of these compounds, removing electrons and concomitantly reducing oxygen molecules to water, producing only water as a by-product of the reaction. Thus, the production of this enzyme has a great appel as it can be used in several areas, mainly for lignin degradation during plant cell wall depolymerization (Tavares et al., 2024).

Table 2 - Concentrations of the total proteins (mg/mL) from the crude extracts produced by *Kretzschmaria zonata*.

	Carborn source			
	Barley	**Corn cob**	**Sugarcane bagasse**	**Wheat bran**
Proteins (mg/mL)	0.019^{b}± 0.01	0.391^{a}± 0.013	0.082^{c} ± 0.008	0.396^{a} ± 0.039

The crude extract obtained when barley was used as carbon source was capable of inducing all tested enzymes (Table 1), except β-mannosidase and β-xylosidase. However, as this extract showed a low amount of protein, 0.019 mg/mL (Table 2) the specific activity was higher for all enzymes, compared to the other tested carbon sources, which makes this inductor an interesting carbon source. The specific activities of xylanase, pectinase, and endoglucanase stood out as the highest (Table 3). Xylanases can be applied in various biotechnological processes to obtain value-added products (Maitan-Alfenas, 2016) and endoglucanases are important cellulases in the degradation of the plant cell wall.

It is important to highlight that the concentrations of total proteins from the crude extracts are not only inherent to the fungus, but the difference is also due to the protein content found in each biomass used as a carbon source. For example, wheat bran and corn cob are reported in previous studies as carbon sources that have a high protein content in their composition (Wu, 2019; Falkoski, 2013), whereas sugarcane bagasse contains approximately 3 % of protein and barley is also poor in proteins. Thus, the protein content is not only inherent in fungi but also in the biomass that is used as a carbon source.

Table 3 - Specific activities (U/mg) from the crude extracts produced by *Kretzschmaria zonata*.

	Carborn source			
	Barley	**Corn cob**	**Sugarcane bagasse**	**Wheat bran**
Specific activity (U/mg)				
Endoglucanase	39.316^{a}± 2.47	1.097^{d} ± 0.07	3.165^{b} ± 0.29	1.689^{c} ± 0.15
FPase	28.667^{a}± 1.04	0.616^{c} ± 0.09	6.233^{b} ± 1.77	1.186^{c} ± 0.07
Laccase	3.579^{a} ± 0.06	0.061^{c} ± 0.01	ND	1.280^{b} ± 0.01
Mannanase	41.754^{a}± 2.84	0.506^{d} ± 0.05	3.115^{b} ± 0.20	1.626^{c} ± 0.18
Pectinase	62.008^{a}± 2.58	1.770^{d} ± 0.09	10.046^{b}± 0.46	3.169^{c} ± 0.14
Xylanase	71.079^{a}± 5.76	9.056^{b} ± 0.67	6.555^{c} ± 0.44	7.640^{c} ± 0.68
α-Galactosidase	0.842^{a} ± 0.03	0.028^{d} ± 0.00	0.354^{b} ± 0.04	0.343^{c} ± 0.04

α-Arabinofuranosidase	1.035^{a} ± 0.05	0.023^{b} ± 0.00	0.040^{d} ± 0.01	0.068^{c} ± 0.01
β-Cellobiohydrolase	1.053^{a} ± 0.06	0.026^{b} ± 0.00	ND	0.010^{c} ± 0.00
β-Glucosidase	1.053^{a} ± 0.05	0.271^{c} ± 0.01	0.260^{d} ± 0.01	0.705^{b} ± 0.02
β-Manosidase	ND	ND	ND	0.003^{a} ± 0.00
β-Xylosidase	ND	0.003^{b} ± 0.01	ND	0.015^{a} ± 0.00

After growing on corn cob, the fungus *K. zonata* was able to produce most of the enzymes, except β-mannosidase. For xylanase activity, this inducer showed the greatest activity, corroborating the results found by Morales et al., 2021. However, this carbon source was not the best inducer. According to Wu et al., 2019, corn cob contains a large amount of xylan in its composition, which may be responsible for the high induction of xylanase.

Sugarcane bagasse induced the lowest number of enzymes, showing lower activities, and the low induction of enzymes may be related to the low protein content of this biomass. However, for FPase this extract showed the best activity among all tested sources tested (Table 1). Sugarcane bagasse generally has a large amount of cellulose in its composition (Bernardi et al., 2023), which may explain the greater activity for FPase. However, the extract had low endoglucanase activity, and it did not show activity for cellobiohydrolase, both cellulases. This extract also showed a low concentration of proteins, 0.082 mg/mL (Table 2), which makes this carbon source interesting for the induction of enzymes, since the specific activities were the second highest for most of the tested enzymes.

Filamentous fungi of the genus *Trichoderma*, *Aspergillus* and *Penicillium* are known to be good producers enzymes that degrade the plant cell wall (Morgan et al., 2022). These fungi secrete several enzymes that are commercially used in biomass saccharification for the production of ethanol or other value-added products, mainly from sugarcane bagasse, however the use of enzymes for the degradation of biomass represents a significant proportion of the costs for generate products from lignocellulosic materials (Humbird et al., 2011). Furthermore, the market for enzyme mixtures is dominated by companies such as Novozymes and Dupont and producing these enzymes more cheaply is still one of the sector biggest challenges. Therefore, studies that look for new or more efficient enzymes are extremely important (Lorenzi and Andrade, 2019). The present study demonstrates the diversity of enzymes secreted by the fungus *K. zonata*, the high production of laccases after growth in wheat bran, and the high specific activities using barley as an inducer, which suggest that *K. zonata* enzymes have potential to be applied in biotechnological technologies.

3.2 – Biomass composition

The *in natura* biomass composition for the two sugarcane bagasse varieties is described in Table 4. The two varieties show differences in cellulose, hemicellulose, and lignin content, which can result in different concentrations of sugars released during the hydrolysis process.

In relation to pre-treated sugarcane, the cellulose was approximately 43 % for Ridesa and 45 % for C-90176, the hemicellulose content was 26 and 28 %, and the lignin was 15 and 16 %, with a mass recovery of 66 and 68 % for Ridesa and C-90-176, respectively. The result of the composition after alkaline pre-treatment indicates that there was a reduction in the lignin content of around 40 %. This reduction is expected in this type of pre-treatment, which is capable of degrading the lignin fraction, facilitating enzymatic action in the saccharification stage (Falkoski et al., 2012). The values found were close to those presented by Maitan-Alfenas et al., 2015.

Table 4 - *In natura* and pretreated biomass composition of sugarcane varieties Ridesa RB 867515 and C-90176.

	C-90176 (*In natura* 100 g)	C-90176 (Pretreated 68 g)	Ridesa (*In natura* 100 g)	Ridesa (Pretreated 66 g)
Lignin (%)	24.37^{a} ± 2.34	16.48^{a} ± 0.24	22.72^{a} ± 1.57	15.22^{b} ± 0.68
Cellulose (%)	31.55^{a} ± 1.12	45.43^{a} ± 1.13	30.69^{a} ± 1.64	43.18^{b} ± 0.92
Hemicellulose (%)	22.54^{a} ± 0.53	28.86^{a} ± 2.31	19.81^{a} ± 3,56	26.52^{a} ± 0.88
Protein (%)	$2{,}23^{b}$ ± 0.11	-	2.96^{a} ± 0.62	-
Ashes (%)	1.40^{a} ± 0.18	-	1.82^{a} ± 0.49	-
Total Extratives (%)	15.42^{b} ± 1.36	-	19.77^{a} ± 1.35	-
Oil (%)	1.34^{b} ± 0,01	-	1.66^{a} ± 0.03	-

3.3 – Enzymatic hydrolysis of alkali-pretreated sugarcane bagasse

Saccharification was performed for the two sugarcane varieties using 5 % of pre-treated dry bagasse. The hydrolysis was carried out with the same enzyme loading of 2.5 FPase units/g of dried alkaline pretreated bagasse for two sugarcane varieties. Crude extracts of the fungus *K. zonata* after cultivation in wheat bran, barley, corn cob, and sugarcane bagasse, and the commercial cocktail Cellic CTec3 by Novozymes were used for saccharification.

For the pretreated RB 867515 sugarcane bagasse, the commercial cocktail Cellic CTec3 released the higher amounts of sugars at 96 h, producing 22.7 and 12.3 g/L of glucose and xylose, respectively, with a conversion rate of 95 % for cellulose and 75 % for hemicellulose. The saccharification performed with the commercial cocktail Cellic CTec3 showed a conversion rate of 76 % for cellulose and 72 % for hemicellulose, considering the C-90176 hydrolysis. The sugars released had a peak at 96 h, with 19 g/L of glucose and 10 g/L of xylose. The results showed that the conversion rate for cellulose using the commercial cocktail was lower in the energy sugarcane saccharification. The C-90176 variety possess a higher lignin content than commercial sugarcane, which may have reduced the access of enzymes to the cellulose fraction.

The sugars released after saccharification of RB 867515 and C-90176 sugarcane varieties up to a time of 120 h by the fungal crude extracts are shown in Figure 1. The results for RB 867515 hydrolysis (Figure 1A and 1B) showed that for the extract obtained after growth in wheat bran, the highest concentration of glucose and xylose were released at 96 h with 2.12 g/L for glucose and 1.97 g/L for xylose. This was the extract that released the greatest amount of xylose among the tested extracts. Additionally, this extract showed one of the highest activities for xylanase, which corroborates the results obtained. The highest released glucose (2.26 g/L and 2.37 g/L) was showed for corn cob and barley extract, respectively, and there is no statistical difference between them (Supplementary Table 1). These extracts showed high specific activities for endoglucanase, cellobiohydrolase, and β-glucosidase enzymes. For the barley extract, there was no release of xylose, despite it showed the highest specific activity for xylanase. Sugarcane bagasse extract surprisingly reached the lowest concentration of glucose and xylose released, with their peaks at 120 h, corresponding to 1.56 and 0.87 g/L. respectively It was expected that the release with this inducer would be higher since the same biomass was used for induction and saccharification, but this extract showed the least diversity and activities for the main enzymes, which may have contributed to the low efficiency of this extract for saccharification

In relation to biomass conversion efficiency and considering 43 % of cellulose and 26 % of hemicellulose from pretreated sugarcane bagasse, the highest cellulose conversion was 9.92 % for barley extract while the highest hemicellulose conversion was 13,33 % for wheat bran extract. The small number of enzymes, in terms of FPU/g of biomass, was applied in this study, which can explain the low conversion rates. The test aimed to evaluate the enzymatic potential of the extracts using the smallest volume. For instance, da Luz Morales et al., 2021 tested the sugarcane bagasse saccharification with corncob extract using 10 % of biomass and 10 U/g of xylanase enzyme. The conditions tested in this study are unprecedented for this fungus in our research group.

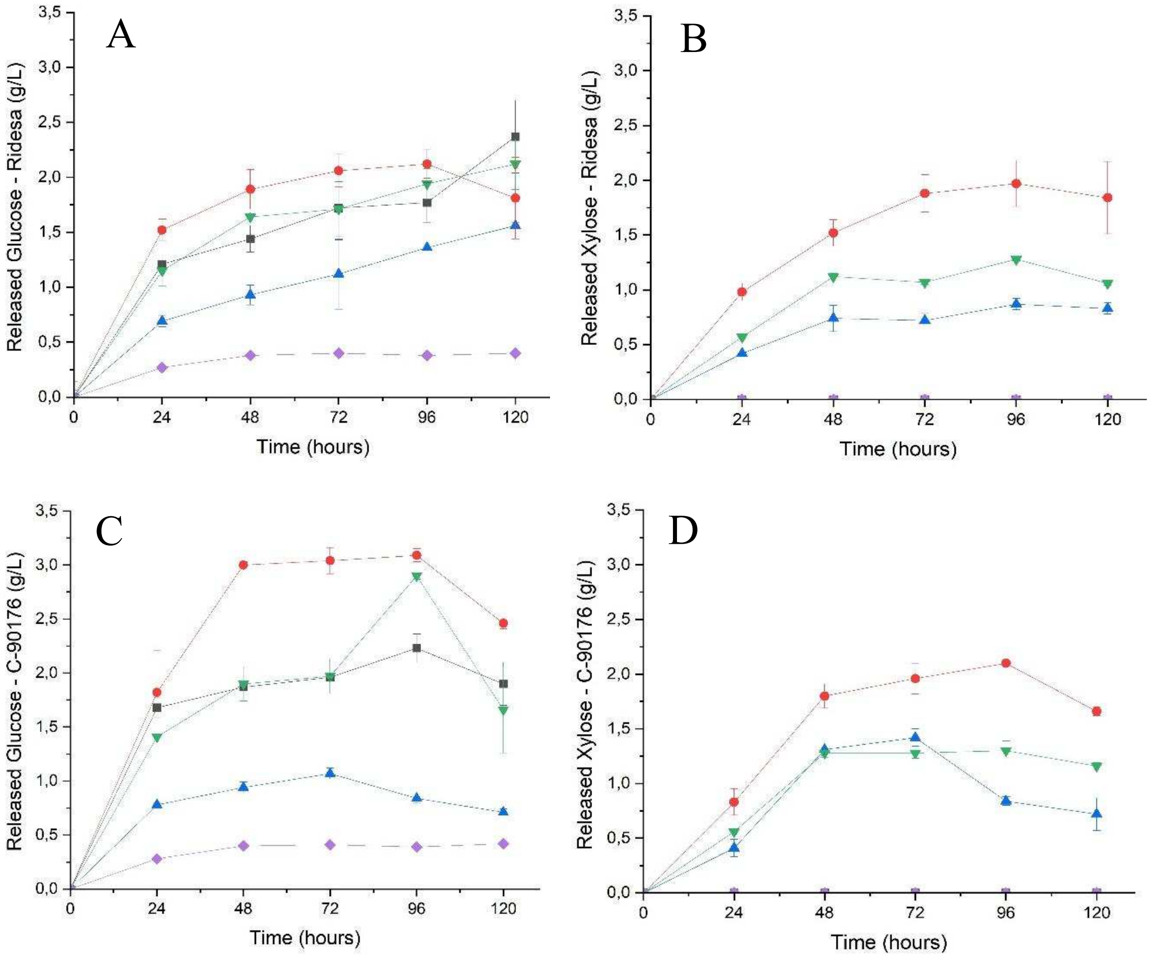

Figure 1 - Released glucose (A) and xylose (B) after pretreated Ridesa RB 867515 sugarcane bagasse saccharification and released glucose (C) and xylose (D) after pretreated C-90176 energy sugarcane bagasse saccharification. Enzyme-free extract (purple diamond), crude extract from *Kretzschmaria zonata* using wheat bran (red dots), barley (black square), corn cob (green inverted triangle), and sugarcane bagasse (blue triangle).

The results from C-90176 saccharification (Figure 1C and 1D) showed that the crude extract obtained after wheat bran induction released the highest glucose and xylose concentration 3,16 and 2.16 g/L, respectively. The biomass conversion efficiency for C-90176 considering 45 % cellulose and 28 % hemicellulose from pretreated sugarcane bagasse obtained the highest cellulose and hemicellulose conversion for wheat extract with 7.11 % and 13.57 %, respectively.

The energy cane variety showed the highest release of sugars than the Ridesa. However, the rate of cellulose conversion was lower for this variety. Energy sugarcane has several advantages over commercial sugarcane, such as a higher fraction of cellulose and hemicellulose, less susceptibility to diseases, and better adaptation to adverse environmental conditions (Meena et al., 2024). Therefore, further studies that optimize the use of this variety in enzymatic hydrolysis to produce second-generation ethanol are interesting for the energy sector.

4 – CONCLUSION

The production of lignocellulolytic enzymes by *K. zonata* was different for each tested carbon source and the enzymatic extract of the fungus grown on corn cobs stood out for presenting the highest activity for the xylanase enzyme, 3.5 U/mL. In the sugarcane bagasse saccharification, the enzymatic extract grown on wheat bran showed the best results for the release of glucose and xylose at 96 h of hydrolysis, with 3.1 and 2.1 g/L, respectively. The percentage of degraded cellulose and hemicellulose was lower compared to that obtained by the commercial cocktail, which can be explained by the low tested enzyme load.

This study showed the ability of *K. zonata* to secrete important enzymes for lignocellulosic biomass degradation, among them laccase. The difference in the diversity and activity of these enzymes using different carbon sources and their potential in the saccharification of sugarcane bagasse, which is an abundant waste industrial in Brazil, was shown. These results may contribute to the application of these enzymes in biotechnological processes such as biomass saccharification.

5 – ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

Table Supplementary 1 - Released glucose by Ridesa RB 867515 saccharification.

Time (hours)	**Control**	**Wheat bran**	**barley**	**corn cob**	**sugarcane bagasse**
0	0	0	0	0	0
24	0,27^{E}	1,52^{A}	1,21^{B}	1,15^{c}	0.69^{D}
48	0.38^{E}	1.89^{A}	1.44^{C}	1.64^{B}	0.93^{D}
72	0.4^{D}	2.06^{A}	1.72^{B}	1.71^{B}	1.12^{C}
96	0.38^{E}	2.12^{A}	1.77^{C}	1.94^{B}	1.36^{D}
120	0.4^{D}	1.81^{B}	2.37^{A}	2.26^{A}	1.56^{C}

Table Supplementary 2 - Released xylose by Ridesa RB 867515 saccharification.

Time (hours)	Control	Wheat bran	Barley	Corn cob	Sugarcane bagasse
0	0	0	0	0	0
24	0^{D}	0,98^{A}	0^{D}	0.57^{B}	0.42^{B}
48	0^{D}	1.52^{A}	0^{D}	1.12^{B}	0,74^{C}
72	0^{D}	1.88^{A}	0^{D}	1.07^{B}	0,72^{C}
96	0^{D}	1.97^{A}	0^{D}	1.28^{B}	0,87^{C}
120	0^{D}	1.84^{A}	0^{D}	1,06^{B}	0,83^{C}

Table Supplementary 2 – Released glucose by C-90176 saccharification.

Time (hours)	**Control**	**wheat bran**	**barley**	**corn cob**	**sugarcane bagasse**
0	0	0	0	0	0
24	0,28^{E}	1,82^{A}	1,68^{B}	1,41^{c}	0.78^{D}
48	0.40^{D}	3.00^{A}	1.87^{B}	1.90^{B}	0.93^{C}
72	0.41^{D}	3.04^{A}	1.96^{B}	1.97^{B}	1.08^{C}
96	0.39^{E}	3.16^{A}	2.22^{C}	2.90^{B}	0.84^{D}
120	0.42^{E}	2.46^{A}	1.90^{B}	1.67^{C}	0.71^{D}

Table Supplementary 2 – Released xylose by C-90176 saccharification.

Time (hours)	Control	Wheat bran	Barley	Corn cob	Sugarcane bagasse
0	0	0	0	0	0
24	0^{D}	0,83^{A}	0^{D}	0.57^{B}	0.42^{C}
48	0^{C}	1.86^{A}	0^{C}	1.28^{B}	1,31^{B}
72	0^{D}	1.96^{A}	0^{D}	1.28^{C}	1,42^{B}
96	0^{D}	2.16^{A}	0^{D}	1.30^{B}	0,84^{C}
120	0^{D}	1.66^{A}	0^{D}	1,16^{B}	0,72^{C}

4. FINAL CONSIDERATIONS

The present work summarizes and showed the genomic analysis importance of phytopathogenic fungi and showed some computational tools that can be used so that, based on genomic data, the enzymatic profile of these organisms can be evaluated. The commercial interest enzymes identification and characterization can guide the application of these biomolecules in biotechnological processes, achieving more efficient results.

The phytopathogenic fungus *K. zonata* genome sequencing and assembly allowed the genes encoding carbohydrate-active enzymes (CAZymes) identification, providing a better understanding of its enzymatic profile. This fungus belongs to the *Xilariacea* family and the *Sordariomycete* class, and its profile was more similar to that showed by the fungus *Aspergillus niger*, which is a *Eurothiomycete*, than to that of other fungi belonging to the same class such as *Trichoderma reesei*. This demonstrates that organisms from the *Xilariacea* family can express several commercial interest enzymes. The sugar metabolic model was also evaluated, along with its growth profile on different carbon sources, identifying that *K. zonata* has almost all genes involved in the main sugar metabolic pathways and was capable of growing on all sources carbon tested, except for galacturonic acid. This result highlights the growth versatility of the fungus and may contribute to the study of new enzyme-inducing sources.

The lignocellulolytic enzymes production by *K. zonata* was different for each carbon source tested and the enzymatic extract grown on corn cobs stood out for presenting the highest activity for the xylanase enzyme, 3.5 U/mL. The composition of corn cob is rich in xylan and proteins, which may contribute to the greater induction of this enzyme. Wheat bran was the only carbon source that induced all tested enzymes. Its composition rich in proteins and minerals and its low lignin content may have contributed to fungal growth and better enzyme induction. Sugarcane bagasse was the compound that induced the lowest diversity of enzymes. Its composition is rich in polysaccharides, however, it has a significant percentage of lignin, which can make it difficult for the fungus to access them.

In the sugarcane bagasse saccharification, the enzymatic extract of the fungus grown on wheat bran showed the best results for the release of glucose and xylose in

96 h of hydrolysis, with 3.1 and 2.1 g/L, respectively. The percentage of degraded cellulose and hemicellulose was lower compared to that obtained by the commercial cocktail, which can be explained by the low enzyme load tested.

The results obtained in the present work improve the understanding of the fungus *K. zonata*, which until then was little explored and little described in the literature. The high-quality genomic data obtained enables new lines of study, such as the identification of genes related to plant-pathogen interaction, the development of heterologous proteins, the understanding of the regulatory process of metabolic pathways, among others.