ISABELLE LIMA LOPES

BIOPROSPECTING OF ENZYMES FROM FUNGI ISOLATED FROM CANASTRA CHEESES FOR INDUSTRIAL APPLICATION

Dissertation submitted to the Agricultural Microbiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

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Co-advisers: Marisa Vieira de Queiroz
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"Vai. E se der medo, vai com medo, mesmo."

(Thiago Melo e Amorim)

“A ciência nunca resolve um problema sem criar pelo menos outros dez”.

(George Bernard Shaw)

ABSTRACT

LOPES, Isabelle Lima, M.Sc., Universidade Federal de Viçosa, May 2022. **Bioprospecting of enzymes from fungi isolated from Canastra cheeses for industrial application.** Adviser: José Guilherme Prado Martin. Co-advisers: Marisa Vieira de Queiroz and José Humberto de Queiroz.

The microbial diversity of Brazilian artisanal cheeses is far from being fully explored. Among frequently isolated microorganisms, bacteria and fungi stand out. Filamentous fungi can be highlighted for their significant impact in the quality of surface mould-ripened cheeses, besides that several metabolites produced, including enzymes. Fungal enzymes are widely used in industry, due to the easier extraction and purification compared to other enzymes sources. In this context, the aim of this study was to evaluate the proteolytic and lipolytic activity of 58 filamentous fungi isolated from artisanal cheeses produced in Canastra region, Minas Gerais State, Brazil. Submerged fermentation assays were performed considering casein as a protease inducer and olive oil as a lipase-inducing substrate. The proteolytic activity was evaluated by the caseinolytic method using a spectrophotometric assay; lipolytic activity, by potentiometric titration of fatty acids released by fungi lipases. *Penicillium steckii* and *Aspergillus versicolor* showed the highest proteolytic activity, with with 31.14 U/mL and 29.35 U/mL, respectively. Regarding lipolytic activity, *Geotrichum candidum* and *Penicillium steckii* presented the highest activities, with 62.91 U/mL and 55.44 U/mL, respectively. Considering the results of the proteolytic activity and their potential use in the dairy industry, a *Geotrichum candidum* isolate (GC-31) was selected and submitted to ultraviolet light (UV) mutation induction assay; then, the mutants obtained were evaluated for proteolytic activity. In total, 18 mutant strains were obtained; GC-M10 showed an increase in proteolytic activity up to 274% (29.96 U/mL) in comparison to the wild type (10.94 U/mL); therefore, the UV induction method was efficient in increasing the enzymatic activity of the isolated evaluated. This study showed that fungi isolated from artisanal cheeses produced in Canastra region have potential for biotechnological exploration and the production of industrial enzymes.

Keywords: Protease. Lipase. Canastra cheese. *Geotrichum candidum*. Mutante. Epifluorescence microscopy.

RESUMO

LOPES, Isabelle Lima, M.Sc., Universidade Federal de Viçosa, maio de 2022. **Bioprospecção de enzimas de fungos isolados de queijos da Canastra para aplicação industrial**. Orientador: José Guilherme Prado Martin. Coorientadores: Marisa Vieira de Queiroz e José Humberto de Queiroz.

Queijos artesanais brasileiros apresentam uma complexa microbiota, ainda longe de ser totalmente explorada. Dentre os microrganismos frequentementes isolados, destacam-se bactérias, leveduras e fungos filamentosos; estes últimos merecem destaque por impactarem significativamente na qualidade do produto, bem como por se constituírem de fonte importante de diversos metabólitos, principalmente enzimas. Enzimas fúngicas são amplamente utilizadas na indústria, pela facilidade de extração e purificação em relação a outras fontes. Nesse contexto, o objetivo deste estudo foi avaliar as atividades proteolítica e lipolítica de 58 isolados fúngicos coletados de queijos artesanais produzidos na região da Canastra, estado de Minas Gerais, Brasil. Ensaios de fermentação submersa foram realizados considerando-se a caseína como indutora de protease e o azeite de oliva como substrato indutor de lipase. A atividade proteolítica foi avaliada pelo método caseinolítico, por meio de ensaio espectofotométrico; a atividade lipolítica, por titulação potentiométrica de ácidos graxos liberados pela enzima. *Penicillium steckii* e *Aspergillus versicolor* demonstraram maior atividade proteolítica, com 31.14 U/mL e 29.35 U/mL, respectivamente. Em relação à atividade lipolítica, *Geotrichum candidum* e *Penicillium steckii* demonstraram maior atividade, com 62.91 U/mL e 55.44 U/mL, respectivamente. A partir dos resultados de atividade proteolítica, o isolado de *G. candidum* (GC-31) foi selecionado para ensaios de indução de mutação por luz ultravioleta (UV); os mutantes foram, então, avaliados quanto à atividade proteolítica. No total, 18 estirpes mutantes foram obtidas; GC-M10 demonstrou aumento na produção de proteases de 274% (29.96 U/mL) em comparação com a estirpe selvagem (10.94 U/ml). Portanto, o método de indução por UV mostrou-se eficiente no aumento da atividade enzimática de *G. candidum*. Este estudo demonstrou que fungos isolados de queijos artesanais produzidos na região da Canastra, bem como estirpes mutantes, apresentam potencial de exploração biotecnológica para produção de enzimas de interesse industrial.

Palavras-chave: Protease. Lipase. Queijo Canastra. *Geotrichum candidum*. Mutante. Microscopia de epifluorescência.

SUMMARY

1. INTRODUCTION

Artisanal cheeses produced in Minas Gerais have been used in several studies about fungi isolation and characterization (BORELLI, 2006; LIMA, 2009; DORES, 2013; LUIZ, 2017; KAMIMURA, 2019). In general, its production involves family labor and the use of fresh raw milk, salt, rennet and "pingo", which is an endogenous ferment collected from the last batch of the previous day. Instead of the "pingo", some producers use a grated portion of the cheese as starter (a practice known as "rala") (OLIVEIRA, 2018). Serra da Canastra is a microregion in the southwest of Minas Gerais, which is one of the most important producer regions of artisanal cheeses. The microbiota of artisanal cheeses is far from being fully explored, as the uniqueness of each region, dairy farm and the seasons significantly contribute to its diversity (MONTEIRO, 2018). Cheese aroma and flavor are also influenced by climate, topography, raw material and manufacturing practices (FIALHO *et al*, 2018; MONTEIRO, 2018; CAMPOS *et al*, 2021).

Microorganisms isolated from artisanal cheeses often include yeasts, lactic acid bacteria (LAB) and filamentous fungi, responsible by sensorial characteristics throughout ripening (FOX et al., 2004; KAMIMURA, 2019). Regarding Canastra cheeses mycobiota, Aragão (2018) isolated filamentous fungi during ripening and the more abundant genera found were *Paecilomyces*, *Aspergillus*, *Fusarium*, *Geotrichum*, *Microascus* and *Acremonium*. On the other hand, a previous study performed by our research team highlighted the presence of *Fusarium solani*, *Geotrichum candidum*, *Aspergillus versicolor*, *Penicillium westerdijkiae* and *Penicillium steckii* (CESAR, 2019).

Filamentous fungi are recognized by their metabolites production with industrial application in different areas, such as food, pharmaceutical, medical, among others (ALBERTI, FOSTER and BAILEY, 2017). They are the most frequently involved in enzyme production, mainly extracellular enzymes, promoting and facilitating their extraction from the fermentation media. Proteases, lipases, bioactive peptides and antimicrobial substances are obtained on an industrial scale using filamentous fungi (BORTOLAZZO, 2011). The use of microbial enzymes is an advantage, as well as other microbial metabolites, because it avoids the use of toxic and polluting extraction agents, reducing their impacts on the environment. Besides that, it also requires lower temperature and pressure conditions than chemical processes (JISHA et al, 2013).

Fungal enzymes play an important role in the dairy, leather, textile and pharmaceutical industries (JISHA et al, 2013). *Aspergillus nidulans*, *Aspergillus niger*, *Rhizomucor miehei*,

Penicillium camemberti and *Penicillium roqueforti* are recognized as good producers of enzymes (MAMO & ASSEFA, 2018; KUMAR, 2020). Some filamentous fungi species, for example *Geotrichum candidum*, are frequently isolated from ripened cheeses around the world (BIOLCATI *et al*, 2020). It is a worldwide-distributed specie and its enzymes are related to dairy and milk products (ELISKASES-LECHNER, F.; GUÉGUEN, M.; PANOFF, 2011). Besides that, it is frequently isolated from raw milk and raw milk cheeses, such as artisanal cheeses, soft cheeses, Camembert cheese, semi-fresh goat's cheese and ewe's milk cheese (MARCELLINO *et al*., 2001; GABORIT, MENARD & MORGAN, 2001; BOUTROU & GUÉGUEN, 2005; PRACHAROVA *et al*., 2019). *G. candidum* is used as secondary culture in cheese ripening, where its proteolytic and lipolytic enzymes generate volatile compounds related to flavor and aroma (BOUTROU & GUÉGUEN, 2005; PRACHAROVA *et al*., 2019).

Proteases stand out in the enzyme market, corresponding to the most used enzyme in the pharmaceutical, leather, detergent, textile and food industries (Table 1) (JISHA et al, 2013). In food production, proteases are mainly used by dairy industries, where cheese coagulation processes require aspartic proteases (JACOB et al., 2011). On the other hand, lipases are versatile enzymes, with a wide variety of applications in industrial processes, for example in detergent, pharmaceutical, energy, cosmetics, fine chemicals, paper and food industries (Table 2). As well as proteases, it is important for dairy industries, since is related to the flavor due to the short-chain fatty acids released, resulting from lipase action in cheese ripening (PENG et al. 2014). Therefore, ripened artisanal cheeses are a significant source for prospecting fungi enzymes with potential industrial applications.

2. LITERATURE REVIEW

2.1. Artisanal Minas Cheese

Artisanal Minas Cheeses (AMC) are nationally and internationally recognized for their quality and sensory characteristics (DORES, 2013). The product has won awards in several competitions, such as *Mondial du Fromage et des Produits Laitiers* in France (FIEMG, 2019). Studies about nutritional benefits, culture and sensory quality of them have been developed last few years, mainly focused on microbiota composition and biochemical and physical changes during the ripening (KAMIMURA *et al*., 2019; SILVA, 2020; HOSKEN, 2021).

Table 1. Aspartic protease-producing fungi involved in cheese and food production.

Species	**Application of proteases**	**References**
Pleurotus sajor-caju	Substitute for animal rennet in cheese production	Moharib (2007)
Mucor bacilliformis	Substitute for animal rennet in cheese production	Machalinski *et al*. (2006)
Rhizomucor miehei	Substitute for animal rennet in cheese production	Mamo & Assefa (2018)
Aspergillus niger	Substitute for animal rennet in cheese production (Heterologous Production)	Mamo & Assefa (2018)
Aspergillus oryzae MTCC 5341	Food industry and milk coagulation for cheese production	Vishwanatha & Singh (2009)
Penicillium camemberti	Substitute for animal rennet in cheese production	Chrzanowska *et al*. (1995).
Penicillium roqueforti	Blue Cheeses manufacturing	Larsen, Kristiansen, & Hansen (1998)
Rhizopus SMC	Protein hydrolysates preparation	Ramamurthy *et al*. (1991)
Rhizopus oryzae	Substitute for animal rennet in cheese production; protease with interesting physicochemical characteristics for cheese maturation.	Kumar *et al*. (2005)
Thermoascus aurantiacus	Industrial processes that require high temperature proteolytic action	Merheb *et al*. (2007)
Thermomucor indicae-seudaticae N31	Substitute for animal rennet in cheese production	Merheb-Dini *et al*. (2010)

Source: Adapted from Mamo & Assefa (2018).

Cheeses's characteristics depend on the particularities of each producing region, such as climate conditions, topography, temperature and humidity, raw material, and cheesemaking process, which can impact microbiota and the physical-chemical properties (MONTEIRO, 2018). Artisanal cheeses are produced with fresh raw milk obtained from the original farm and must present characteristic color and flavor, firm consistency and uniform mass, with no addition of coloring and preservatives, with or without mechanical holes (MINAS GERAIS, 2008).

Table 2. Examples of industrial lipase uses.

Species	Application of lipases	References
Rhizopus chinensis	Biodiesel production	He *et al.* (2008)
Rhizopus oryzae	Biodiesel production	Arumugam & Ponnusami (2014)
Candida rugosa	Food industry (synthesis of methyl acetate and butyl butyrate)	Kuo, Shaw & Lee (2015) Trbojevic *et al.* (2016) Ng & Yang (2016)
Aspergillus awamori BTMFW032	Bioremediation (treatment of oil-laden effluents)	Basheer *et al.* (2011)
Candida antarctica	Chemical industry (synthesis of lipophilic antioxidants); Food and Pharmaceutical Industry (Glycerolysis of corn oil and vitamin A biosynthesis)	Cai *et al.* (2016) Liu *et al.* (2012)
Hypocrea pseudokoningii	Detergent industry (improvement of derivatives in organic solvents)	Pereira *et al.* (2015)
Thermomyces lanuginosus	Food and Pharmaceutical Industry (Synthesis of butyl butyrate)	Matte *et al.* (2016)
Penicillium aurantiogriseum	Production of monoacylglycerides	Lima *et al*. (2004)
Geotrichum sp.	Selective application in the hydrolysis of conjugated linoleic acid (CLA)	Burkert, Maugeri & Rodrigues (2004)
Yarrowia lipolytica	Biocatalyst in pharmaceutical, food and environmental industries	Brígida (2014)

Source: Adapted from MELANI, TAMBOURGI & SILVEIRA (2020).

Canastra, a microregion in the southwest of Minas Gerais state (Figure 1), produces one of the most famous Brazilian artisanal cheeses and includes more than two thousand farms. The manufacturing of AMC produced in Canastra region is based on knowledge transmitted from generation to generation for centuries. For its production, after the mechanical or manual milking, the milk is filtered and transferred to a tank, where rennet and an endogenous ferment known as "pingo" are added (DORES, 2007). After, the curd is cut and partially drained using a synthetic cloth popularly known as "*volta ao mundo*" (DORES, 2007). The whey collected from this stage consists of "pingo" and is used in the next batch of production (BORELLI, 2006). After, the cheese is salted on both sides. The final stage consists of the ripening for at least 14 days (MINAS GERAIS, 2021). This stage plays an important role in cheese flavor,

texture and aroma, especially when filamentous fungi are present. During ripening, microbial proteolytic and lipolytic enzymes act on proteins and fats in the curd, resulting in compounds such as aldehydes, peptides, free amino acids and ketones, important for the cheese quality (GUTIERREZ et al., 2004; DORES, 2007; DE ASSIS SALES, 2015). Also, cheese microbiota can contribute to the inhibition of undesirable microorganisms.

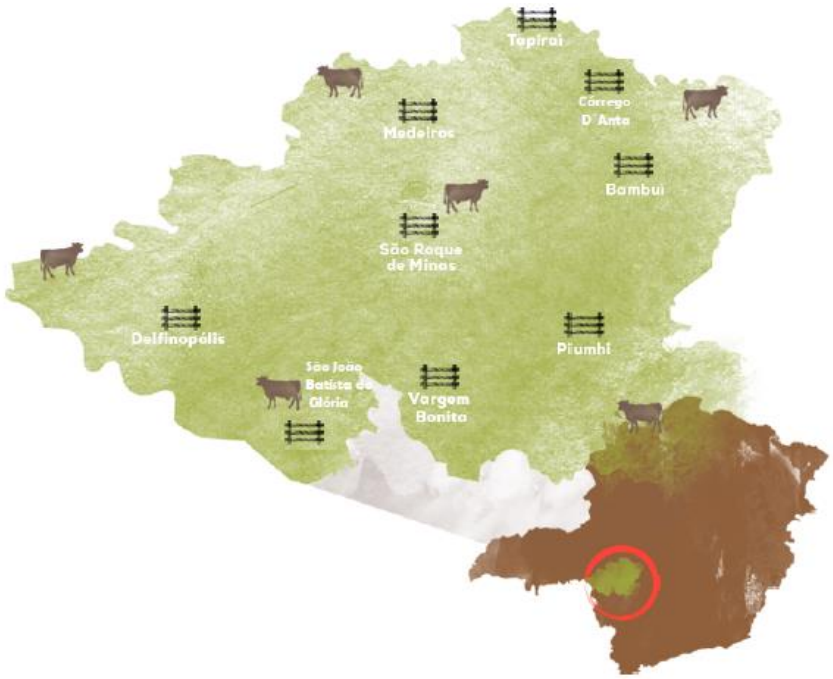

Figure 1. Producing cities in Canastra region, Minas Gerais state. Source: QUEIJO DA CANASTRA (2022). https://queijodacanastra.com.br.

2.2. Filamentous fungi and bioprospecting

Fungi comprise a diverse group, widely found in different natural environments. They range from unicellular (yeasts) to multicellular (filamentous fungi) forms; these latter are characterized by the presence of hyphae – structural units that constitute the mycelia – involved in different functions, such as nutrient assimilation, fixation, growth and reproduction. The set of mycelia results in the formation of colonies, which can be easily recognized (ORLANDELLI, 2012). Fungi are eukaryotes with more complex morphological features then bacteria, such as the presence of a chitin cell wall, genetic material organized by a nuclear membrane and specialized organelles. Reproduction can occur by asexually (by mitosis) or

sexually (by plasmogamy, karyogamy and meiosis) ways (SILVEIRA, SOUZA & KOLLER, 2002). Some fungi present dimorphism, displaying filamentous fungi or yeasts form, depending on environmental conditions, such as different temperature – somes species appear as filamentous fungi at 25°C or as yeasts, unicellular and without mycelia at 35-37°C (NOGUEIRA & CARDOSO, 2000).

The fungi group is recognized for its biotechnological importance, mainly for the production of extracellular enzymes, since they release enzymes in several types of substrates, with absoption nutrition; this feature favors the extraction process, with lower costs (BORTOLAZZO, 2011). Considering the importance of metabolites with biotechnological potential for several industrial sectors, the fungi bioprospecting is useful to provide novel substances with high value-added (CARVALHO et al, 2005). In this context, raw milk used in cheesemaking process provide an ideal substrate for microbial growth, including filamentous fungi. *Penicillium*, *Geotrichum*, *Aspergillus*, *Mucor* and *Fusarium* can be highlighted as the most common genera found in artisanal cheeses (LAVOIE, et al. 2012; OZTURKOGLU-BUDAK, 2016; CESAR, 2019).

2.3. Industrial uses for proteases and lipases

Enzyme's market can reach $11.92 billion in 2030 (RESEARCH AND MARKETS, 2020). In general, the exploitation of microbial enzymes results in lower costs as no high temperature and pressure are used, besides it doesn't require heavy chemical components, contributing for a cleaner process (MUGHAL *et al*., 2009). Proteases are the market leader enzymes, and are used in the detergent sector, bakery, breweries, dairy, food, waste treatment, textile industry, leather industry, animal feed, chemical, pharmaceutical, medical and cosmetic industries (THERON, DIVOL, 2014; JISHA et al, 2013). On the other hand, lipases appear in the third position and are used in dairy, cosmetics, detergent, pharmaceutical, energy, fine chemicals and paper industries (PENG et al. 2014).

Proteases, hydrolytic enzymes responsible for the cleavage of peptide bonds in proteins, are the main group of enzymes in the current market. They can be classified as exopeptidases (when the target of the reaction is amino acids at the ends of the chain), and as endopeptidases (when they cleave peptide bonds within the protein chain) (ZAHA, 2014). The optimal conditions of pH, temperature and stability profile of proteases are specific and complex, demanding more knowledge about each enzyme (KUMAR et al., 2010). Filamentous fungi are

the most important industrial source of proteases, due to their extracellular production, fast growth, separation from media by simple filtration, the possibility of solid-state production and relatively easy genetic manipulation (MAZOTTO, 2010). Aspartic proteases, also called acid proteases, are part of the group of endopeptidases that are widely used in the food and beverage industry, and they are also used to replace animal rennet in cheese production (MAMO & ASSEFA, 2018; SHARMA et al, 2019).

On the other hand, lipases are classified as glycerol ester hydrolases, which catalyze mainly the hydrolysis of carboxylic ester bonds in triacylglycerols, releasing diacylglycerols, monoacylglycerols, glycerol and fatty acids. They are considered quite versatile enzymes, as they also perform other reactions, such as esterification, interesterification, transesterification, acidolysis and aminolysis (ZAHA, 2014). The efficiency of lipases performance strongly depends on the medium features, with optimal pH ranging from 4 to 9 and optimal temperature ranging from 30 to 40 °C (CORTEZ et al., 2017). Lipases are obtained from several sources, as plants, animal tissue and microorganisms, mainly filamentous fungi (TREICHEL et al, 2010).

In Brazil, cheese consumption increased 29.2% in 2020 in comparison to 2019 (MILKPOINT, 2020). In this context, the research for novel enzymes may supply alternatives to the dairy industry. Coagulation and ripening stages play an important role in cheese production, as proteolytic and lipolytic enzymes contribute to the quality of the final product. In coagulation, milk caseins are hydrolyzed by the coagulant or rennet. Previously, animal origin rennet generally extracted from calves' stomach was used. Its use presented some limitations, such as little availability, difficulty in extraction and issues with animal welfare. Then, the extraction of microbial enzymes represents an important alternative for the sector. Besides that, fungal enzymes can be improved by genetic manipulation, present greater stability, faster growth and lower production costs (JACOB et al., 2011; YEGIN; DEKKER, 2013). Although there are already microbial enzymes substitute for bovine pepsin on the market, such as *Aspergillus niger* chymosin marketed by Chr-Hansen®, there are still improvements to be provided and other possible sources of proteases with desirable characteristics to be explored (SILVA, 2017).

Briefly, proteolytic reactions during the ripening process occur in a primary proteolysis stage from the hydrolyze of caseins by residual coagulant in the curd or indigenous proteinases from raw material, releasing peptides; and a second stage, from the hydrolysis of the peptides generated in the first stage by proteases and peptidases produced by LAB, non-starter LAB (NSLAB) or secondary starter (as filamentous fungi, for example) (FEIJOO-SIOTA et al.,

2014). Currently, for industrial cheese production, microbial proteases are used to speed up the ripening process, enhance flavor and aroma and produce functional cheeses with reduced food allergy content through the hydrolysis of proteins (PANESAR, 2010). However, the characteristics of the ripening process depend on the type of cheese and may range from a few weeks to months or years. Decreasing ripening time may be interesting to reduce costs whereas doesn't affect cheese quality (FEIJOO-SIOTA et al., 2014; RANI & JAGTAP, 2019).

Regarding lipolysis, fatty acids are formed during the cheese ripening by the activity of lipolytic enzymes produced by microbiota or added in the industrial process (FOX, 2004). As well as proteases, lipases were originally extracted from calf stomachs, mainly for the production of Italian cheeses, such as Romano and Provolone; currently, microbial and pregastric lipases may be used for cheese production with enhanced characteristics (BATTISTOTTI & CORRADINI, 1993; ZHAO et al., 2022; DEETH, 2022). Releasing of fatty acids, such as butyric, caprylic, capric and lauric provides a characteristic flavor to cured cheeses (FOX, 1993). However, lipases require temperature and pH stability to provide satisfactory effects on cheese quality (YEGIN; DEKKER, 2013).

3. MATERIAL AND METHODS

3.1. Fungal Isolates from Canastra's cheeses

The filamentous fungi isolates (n = 58) used in this study were previously isolated from cheeses produced in 16 different properties from Serra da Canastra (CESAR, 2019) (Table 3). The isolates, identified by sequencing the ITS region, have been kept by Castellani method (CASTELLANI, 1939) and are part of the Microorganisms Collection of the Microbiology of Fermented Products Laboratory (FERMICRO/UFV). The isolates were reactivated in Petri dishes with malt extract agar (MEA) and 10 ml of chloramphenicol alcoholic solution (10 mg/ml) which was added for each liter of medium.

Table 3: Fungi isolates used in enzymatic activity assays.

(To be continued)

Isolate Identification	Species	Origin
GC-02	*Geotrichum candidum*	São Roque de Minas
GC-03	*Geotrichum candidum*	São Roque de Minas

GC-04	*Geotrichum candidum*	São Roque de Minas
DH-05	*Debaryomyces hansenii*	São Roque de Minas
GC-06	*Geotrichum candidum*	São Roque de Minas
FS-07	*Fusarium solani*	São Roque de Minas
AV-08	*Aspergillus versicolor*	Vargem Bonita
PS-09	*Penicillium steckii*	Vargem Bonita
SB-10	*Scopulariopsis brevicaulis*	Vargem Bonita
AW-11	*Aspergillus westerdijkiae*	Vargem Bonita
GC-13	*Geotrichum candidum*	Vargem Bonita
FO-14	*Fusarium oxysporum*	Vargem Bonita
DI-15	*Diaporthe infecunda*	Vargem Bonita
PS-16	*Penicillium steckii*	Vargem Bonita
FS-17	*Fusarium solani*	Vargem Bonita
AV-18	*Aspergillus versicolor*	São Roque de Minas
AV-19	*Aspergillus versicolor*	São Roque de Minas
FS-20	*Fusarium solani*	São Roque de Minas
TR-21	*Trichothecium roseum*	São Roque de Minas
GC-22	*Geotrichum candidum*	São Roque de Minas
FS-23	*Fusarium solani*	São Roque de Minas
FS-24	*Fusarium solani*	São Roque de Minas
GC-25	*Geotrichum candidum*	São Roque de Minas
AV-26	*Aspergillus versicolor*	Piumhí
FS-28	*Fusarium solani*	Piumhí
FL-29	*Fusarium lichenicola*	Piumhí
FS-30	*Fusarium solani*	Piumhí
GC-31	*Geotrichum candidum*	Piumhí
MU-32	*Mucor sp.*	Medeiros
MU-33	*Mucor sp.*	Medeiros
MC-34	*Mucor circinelloides*	Medeiros
GC-35	*Geotrichum candidum*	Medeiros
MC-36	*Mucor circinelloides*	Medeiros
FS-37	*Fusarium solani*	Medeiros
FO-40	*Fusarium oxysporum*	Córrego d'Anta
AV-41	*Aspergillus versicolor*	Tapiraí
AW-44	*Aspergillus westerdijkiae*	Tapiraí
AV-45	*Aspergillus versicolor*	Tapiraí

(Conclusion)

AV-46	*Aspergillus versicolor*	Tapiraí
PS-47	*Penicillium steckii*	Medeiros
FS-48	*Fusarium solani*	Tapiraí
AV-49	*Aspergillus versicolor*	Bambuí
AW-50	*Aspergillus westerdijkiae*	Bambuí
FS-51	*Fusarium solani*	Bambuí
ML-52	*Moniliella sp*	Bambuí
GC-53	*Geotrichum candidum*	Bambuí
PS-54	*Penicillium steckii*	Bambuí
KO-55	*Kodamaea ohmeri*	São João Batista do Glória
FS-56	*Fusarium solani*	São João Batista do Glória
AN-57	*Aspergillus nomius*	São João Batista do Glória
AW-58	*Aspergillus westerdijkiae*	São João Batista do Glória
AV-59	*Aspergillus versicolor*	São João Batista do Glória
PS-60	*Penicillium steckii*	São João Batista do Glória
AW-61	*Aspergillus westerdijkiae*	Delfinópolis
PC-62	*Penicillium citrinum*	Delfinópolis
FS-63	*Fusarium solani*	Delfinópolis
AW-64	*Aspergillus westerdijkiae*	Delfinópolis

3.2. Fungi inoculation

Submerged fermentation (FS) was performed for the enzymatic assays, i.e., proteolytic and lipolytic activities. Filamentous fungi were grown in MEA at 25°C during 7 days. After, the mycelia were scraped using a Drigalski's loop and tween 80 (0.85%) solution, in order to obtain a conidia suspension. The conidia number was verified using a Neubauer chamber in an optic microscope. The amount of around $1x10^7$ conidia/mL was inoculated in each Erlenmeyer flask containing the respective culture media (as described in the next section). The fungi inoculation was performed in triplicate and the fungi that did not reach the required conidia number after two attempts were excluded.

3.3. Screening of proteolytic activity

3.3.1. Tyrosine standard curve

In order to calculate the amount of tyrosin released by fungal extracts and to reach U/mL values, a tyrosine standard curve was created from increasing concentrations of tyrosine (200 μg/mL), which was diluted in 0.2 M HCl. The tyrosin solution concentrations used were 0 μg/mL, 25 μg/mL, 50 μg/mL, 75 μg/mL, 100 μg/mL, 125 μg/mL, 150 μg/mL, 175 μg/mL and 200 μg/mL. The absorbance was read using a microplate reader at 280 nm (Thermo Fisher Scientific, Multiscan Go). This assay was performed in triplicate.

3.3.2. Proteolytic activity

Proteolytic activity screening was performed by using 125 mL Erlenmeyer flasks containing 50 mL of Manachini medium (MANACHINI; FORTINA; PARINI, 1987) composed by 2 g/L of potassium dihydrogen phosphate (KH_2PO_4), 1 g/L of ammonium sulfate ($(NH_4)_2SO_4$), 0.1 g/L of magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), 0.9 g/L of sodium phosphate dibasic ($Na_2HPO_4.2H_2O$), 1 g/L of yeast extract, 0.5% casein (CRQ, Diadema, São Paulo) with pH value adjusted for 8.0. Fungi inoculation was carried out as described in item 3.2. The flasks were incubated in a refrigerated shaker (Tecnal, Brasil) at 25℃ for 7 days at 100 rpm. After, the cultures were removed using a sterile gauze and the filtrate was centrifuged at 3,000 g for 10 minutes to obtain the enzymatic extract. Subsequently, the proteolytic assay was carried out by the caseinolytic method, according to Soares et. al. (2015), with modifications. To measure the proteolytic activity, 500 μL of casein (1% w/v, pH 8) and 400 μL of citrate-phosphate buffer pH 7.0 were distributed into 2 Eppendorffs tubes, kept at 50 °C in a water bath. Then, 100 μL of the enzymatic extract was transferred to each tube. To stop the enzymatic reaction, 1 mL of trichloroacetic acid (TCA 10% w/v, Êxodo científica, Hortolândia, São Paulo) was added to each tube after 30 minutes of incubation. The tubes were centrifuged at 11,200 g for 5 minutes and the supernatant was collected to determine the absorbance at 280 nm in a plate reader (Thermo Fisher Scientific, Multiscan Go). The time 0 was calculated placing the 1 mL of TCA 10% w/v, before the enzyme extract. These tests were carried out in triplicate. In this study, the enzyme activity unit (U) was defined as the amount of enzyme capable of releasing 1 μmol of tyrosine per minute.

3.4. Screening of the lipolytic activity

Lipase enzymatic extract was obtained using 125 mL Erlenmeyer flasks containing 50 mL of a nutrient medium composed of 10 g/L of olive oil; 5 g/L of yeast extract; 20 g/L of peptone; 0.5 g/L of magnesium sulfate heptahydrate ($MgSO_4.7H_2O$); 0.4 g/L of zinc sulfate heptahydrate (ZnSO4 .7H2O); 1 g/L of ferrous sulfate heptahydrate (FeSO4 .7H2O); 0.2 g/L of manganese sulfate (MnSO4) with pH value adjusted for 7.0 (TOSCANO, 2011). Inoculation was carried out as described in item 3.2. The flasks were incubated in a refrigerated shaker (Tecnal, Brasil) at 30℃ for 4 days at 130 rpm. Thus, the cultures obtained were removed using sterile gauze and the filtrate was centrifuged at 4 °C for 20 minutes at 12,000 g to obtain the enzymatic extract. It was used to evaluate the lipolytic activity according to Rodrigues (2015) and De Morais Junior (2016). A volume of 19 mL of emulsion (1% Triton X-100 and 5% olive oil) in McIlvaine buffer (pH = 7.0) and 1 mL of the enzymatic extract were transferred to 125 mL Erlenmeyer flasks and incubated under agitation at 37 °C for 30 minutes at 130 rpm. Then, 20 mL of acetone:ethanol solution (1:1 v/v) was used in order to stop the enzymatic reaction. The released fatty acids were titrated with 0.3 M NaOH solution until pH 11 by potentiometric titration (ION, model pHB 500). The lipolytic activity was calculated using the follow equation (Maldonado, 2006):

$$Lipolytic\ activity\ (U/mL) = \frac{(Vtf - Vti)\ x\ 1000\ x\ Fd\ x\ N}{t}$$

Where:

U = μmol of fatty acid released by the action of the enzyme.min^{-1}

Vtf = Volume of NaOH after 30 min of reaction;

Vti = Volume of NaOH used to titrate time 0, with all reagents at the start including the enzyme extract;

N = Molarity of NaOH;

t = total reaction time (min);

Fd = Dilution factor of the enzymatic crude extract.

3.5. Assay for obtaining mutant strains using ultraviolet light (UV)

As frequently isolated species from cheeses and closely related to the dairy industry, *G. candidum* isolate with the highest proteolytic activity (GC-31) was used in a mutation induction assay to reach strains with improved protease activity. Protease activity of the mutant strains was performed considering the relevance of *G. candidum* for the ripening cheese process. The current analysis comprised the conidium core analysis, spore viability, UV light survival curve and mutation induction and proteolytic activity.

3.5.1. Conidium core analysis by epifluorescence microscopy

G. candidum was grown in MEA supplemented with chloramphenicol at 25 °C for 2 days. After, the colonies were scraped off with 1 mL of tween 80 solution (0.85%) using a Drigalski strap and filtrated to obtain a conidia solution free of mycelia. Next, the conidia suspension was transferred to a 2 mL Eppendorf and diluted. Thus, 10 microliters of each dilution were placed on microscope slides and spread out (in duplicate). After its drying and fixation, 200 µL of staining coloring solution (1 µL/mL 10 000 × SYBR Green in 10 mM KH2PO4 and 18% glycerol) was applied at 25 °C for 5 min. Subsequently, the staining solution was rinsed with 2 mL of sterile water and a coverslip was placed over the slide. An epifluorescent microscope (Thermo Fischer Scientific® EVOS M5000) was used to observe the conidia core (excitation filters GFP and DAPI).

3.5.2. Spore viability

G. candidum was grown in MEA supplemented with chloramphenicol at 25 °C for 2 days. After, the colonies were scraped off with 1 mL of tween 80 solution (0.85%) using a Drigalski strap and filtrated to obtain a conidia solution free of mycelia. The number of conidia in the solution was measured using a Neubauer Chamber, from which 150 conidia were taken and inoculated in 3 Petri dishes containing 20 mL MEA with chloramphenicol. The number of colonies in the Petri dishes was counted after 48 hours of incubation at 25 °C; the conidia viability was calculated and expressed as a percentage.

3.5.3. UV light survival curve and mutation induction

After the growth of *G. candidum* in MEA and chloramphenicol at 25 °C for 48 hours, a conidia suspension (10^7 conidia/mL) was prepared using 0.85% saline solution, with a final volume of 4.0 mL distributed in each tube. This content was transferred into a Petri dish and exposed to ultraviolet light (Mineralight, San Gabriel, California) in increasing exposure times (0, 1, 2, 3, 4, 5, 6, 7, 8 and 9 minutes) at a distance of 15 cm from the irradiation source. The, conidia suspensions were diluted in Tween 80 solution (0.1%) and 0.1 mL of each suspension were seeded in Petri dishes containing MEA and chloramphenicol medium. Colonies were maintained in a dark chamber for 24 hours for thymine-thymine (T-T) dimmers stabilization. Non-irradiated conidia were also seeded (time point = zero). After 48 hours of incubation at 25 °C, the colonies were counted and the number of surviving colonies per mL and the percentage of survival were calculated for each treatment time; results were expressed as CFU per mL and as percentage. The number of colonies grown on time point zero was considered as 100% growth. Then, 0.1 mL of each sample was taken from the spore suspension and submitted to the mutagenesis step, which was incubated in Petri dishes with MEA and chloramphenicol. The largest colonies were selected for proteolytic activity tests.

3.5.4. Proteolytic activity for the mutant strains

The enzymatic activity for the mutant strains was performed according described in 3.3 section.

3.6. Statistical analysis

Significant differences between the proteolytic and lipolytic isolates activity were evaluated by One-way ANOVA with Dunnett's multiple comparisons test. Both tests were performed using GraphPad Prisma version 9.3.1 (San Diego, California, USA). The level of statistical significance of the tests was $P < 0.05$. In order to access which were the best enzymatic results, a Kruskal Wallis test was applied using Rstudio (Northern Ave, Boston, MA, USA).

4. RESULTS AND DISCUSSION

4.1 Protease Activity

From 58 isolates evaluated in FS screening step, 33 were able to produce conidia enough to obtain 1x10^7 conidia/mL, in triplicate. The tyrosine standard curve and its equation are represented in Figure 2.

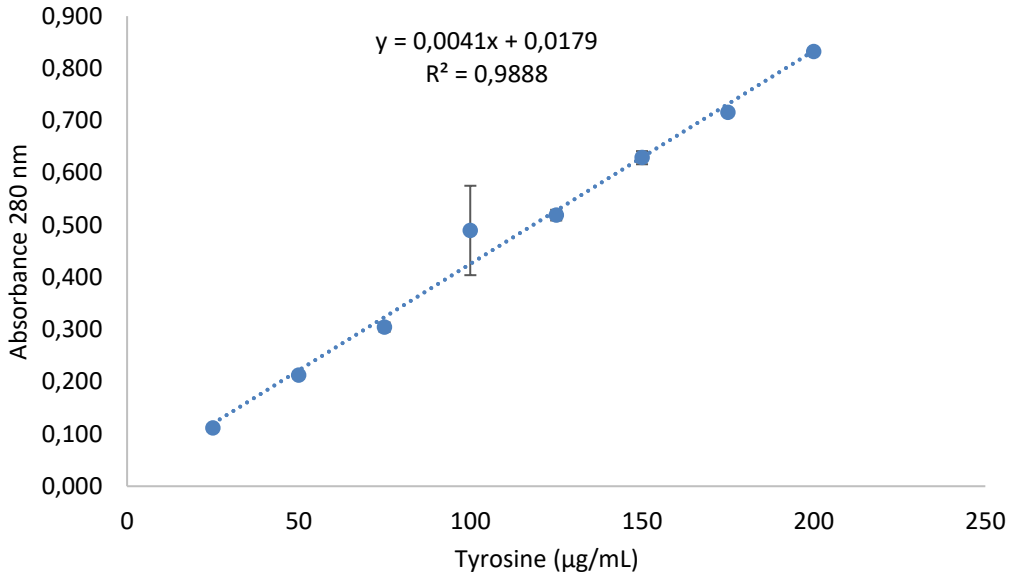

Figure 2. Tyrosine standard curve and equation.

Protease activity results are presented in Figure 3. The highest protease activity was observed for PS-09 (31.14 U/mL), followed by AV-41 (29.35 U/mL) and PS-60 (29.03 U/mL) isolates. Ozturkoglu-Budak (2016) evaluated the protease activity of bacteria, yeasts and filamentous fungi isolated from artisanal raw ewe's milk cheese in the Netherlands and showed highest values for *Penicillium* genus isolates: *Penicillium brevicompactum* (14.19 U/mg), *Penicillium corylophilum* (13.44 U/mg) and *Penicillium chrysogenum* (11.85 U/mg), which values are lower than the observed in this study.

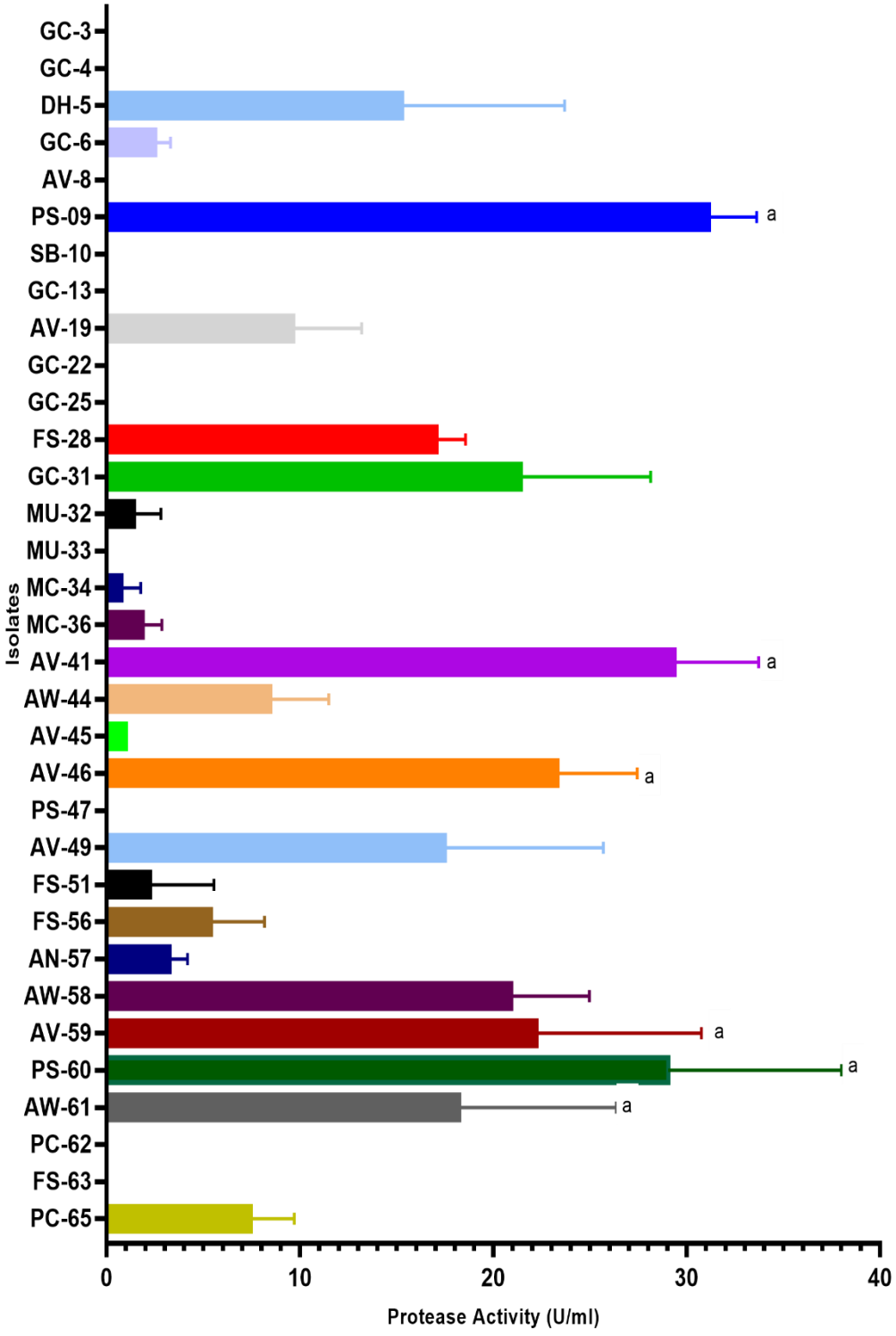

Figure 3. Protease activity of filamentous fungi isolated from Canastra cheeses. The letter *a* indicates the best results with statistical difference.

Aspergillus and *Penicilium* genera are excelente protease producers, with several applications in food and dairy industries (OZTURKOGLU-BUDAK, 2016; BEN MEFTEH *et al*., 2019; COTON *et al*., 2020; GAO *et al*., 2020; MUSTEFA BEYAN *et al*., 2021). For example, a chymosin for cheese production is obtained from *Aspergillus niger var. awamori* (Ha-la®). Besides that, *A. niger* is a versatile filamentous fungus and its enzymes have an important role in fermented food production, such as fermented meat, sauce and vinegar (TE BIESEBEKE & RECORD, 2008). Finally, mixed cultures (*A. niger* and *Aspergillus oryzae*) have improved the sensory characteristics and decreased the fermentation time in fish sauce production (ZHAO et al., 2017); the same culture (*A. oryzae*: *A. niger* in a ratio of 3:1) was evaluated as an alternative to be used as beef potentiator, a popular savory flavor important used in food industry. It presented superior contents of flavor substances, such as alcohols, pyrroles, sulfurous compounds and pyrazines, reinforcing the potential of *Aspergillus* strains for industrial uses (GAO et al., 2014). The use of agroindustrial wastes as substrate for *Aspergillus ochraceus* BT21 produce proteases resulted in thermostable proteases with alkaline pH stability, which are important characteristics for the detergent industry. Furthermore, *A. ochraceus* BT21 increased two-fold protease production using wheat bran as substrate (EL-KHONEZY, 2021).

A. versicolor's alkaline protease was described as a substitute for conventional silver recovering from X-rays films and as compatible detergent industry protease, since it is stable at alkaline pH and high temperatures (CHOUDHARY, 2012; CHOUDHARY, 2013; SHARMA, 2019). *A. versicolor* is an important glucose oxidase producer, an enzyme that catalyzes the oxidation of β-D-glucose resulting in D-gluconolactone (KUDDUS, 2018). The importance of *A. versicolor* for protease production for milk coagulation was demonstrated in previous studies several decades ago (ABDEL-FATTAH & SALEH, 1979).

P. steckii is a saprophytic fungus living in the soil and is also described as an entomopathogenic specie (BONNET et al., 2021). It is related to food spoilage in temperate regions and resistance to hypersaline conditions (ZAJC et al., 2012). *P. steckii* isolated from cheese samples produced in Yemen showed no protease activity in skim milk agar. The authors demonstrated that *A. niger* and *Aspergillus sydowii* were the best protease producers, with moderate enzymatical activity (HUMAID *et al*., 2020). Although in the present work the tests were carried out using a different culture medium, *P. steckii* isolates produced significant amounts of protease (Figure 2). In another study, extracellular caseinase and gelatinase activity from 1108 filamentous fungi isolated from sailfin sandfish egg masses was evaluated (PARK

et al., 2018). In total, 54 strains of *P. steckii* showed no halo for gelatinase and moderate activity for caseinase. There are no studies regarding the enzymatic activity of *P. steckii* isolated from artisanal cheeses; so, the results showed in this study can contribute to a better understanding of its role on surface mold-ripened cheeses, besides suggesting its potential for biotechnological exploitation for industrial uses.

However, is noteworthy that both *P. steckii* and *A. versicolor* does not have a Generally recognized as safe (GRAS) status by the Food and Drug Administration (FDA), since they are recognized as potential mycotoxins producers in food (MALMSTRØM, J.; CHRISTOPHERSEN, C.; FRISVAD, 2000; MEYER *et al*., 2016; EZEKIEL *et* al, 2020; NAVALE, 2021). *A. versicolor* is well known to produce sterigmatocystin (STC) and cyclopiazonic acid (NAVALE, 2021). STC is one of the most powerful carcinogenic mycotoxins, being usually detected in dairy products, green coffee beans, food grains and spices (NAVALE, 2021). On the other hand, *P. steckii* was described as a citrinum producer by later studies (COX *et al*., 1979; MALMSTRØM, J.; CHRISTOPHERSEN, C.; FRISVAD, 2000). For this reason, the use of transcriptomics, proteomics and metabolomics could provide further information about the presence of mycotoxins or toxigenic compounds in food (AFSHARI *et al*., 2020). Considering the impossibility to apply these species to produce enzymes to be used in food matrices, other applications could be considered. *A. versicolor,* for example, was used for protease and lipase production during the treatment of Jatropha seed cake, a byproduct of the oil extraction during biodiesel production; since it has many anti-nutrient compounds (phytic acids, tannins and lectins) which could be reduced by the fungal enzymes (VEERABHADRAPPA, SHIVAKUMAR & DEVAPPA, 2014).

4.2 Lipase Activity

All 33 isolates showed some lipase activity in FS screening (Figure 4). The best results were obtained for *G. candidum* GC-06 (62.91 U/mL), *P. steckii* PS-09 (55.44 U/mL) and *A. versicolor* AV-08 (55.33 U/mL).

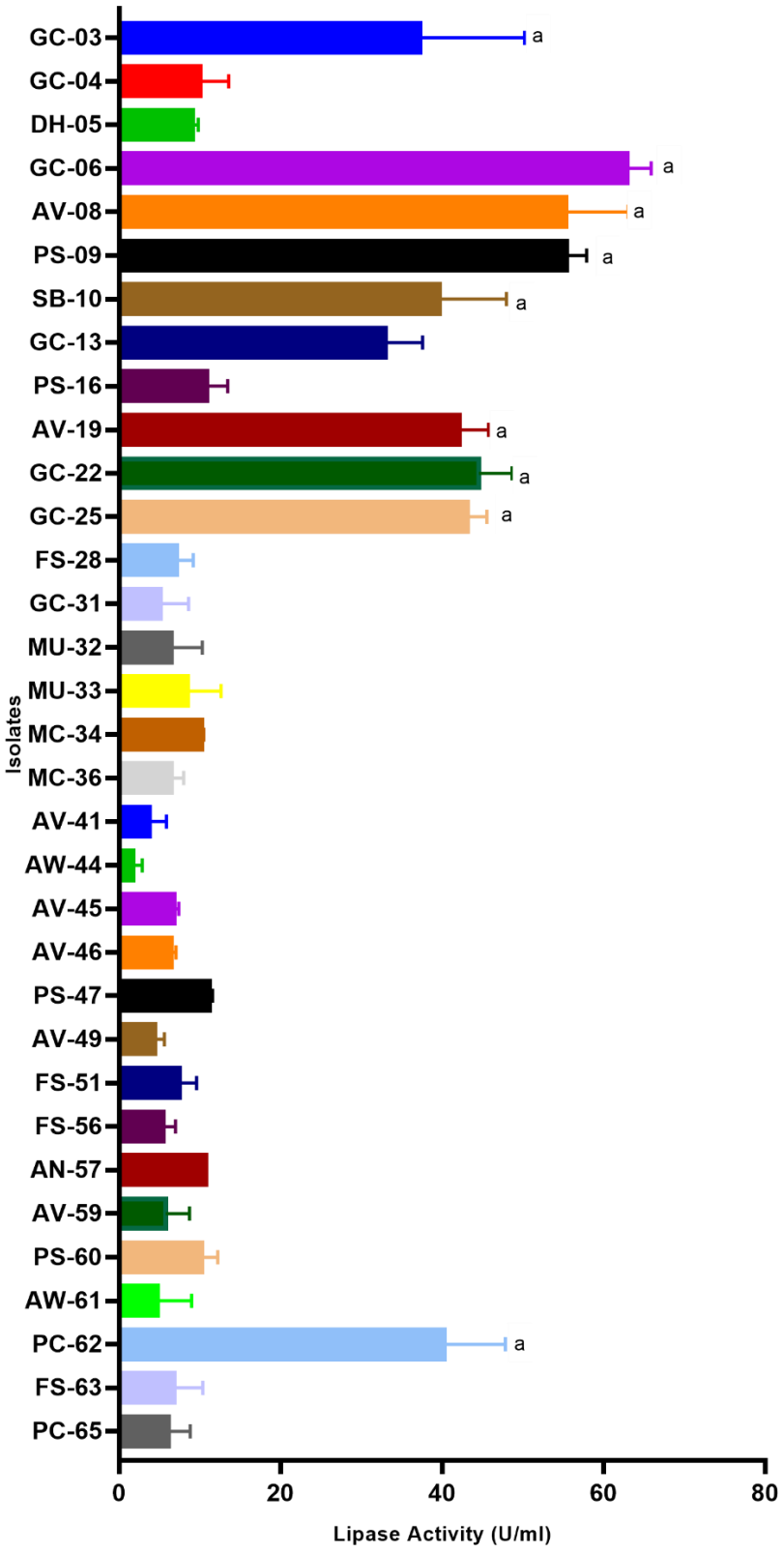

Figure 4. Lipase activity of filamentous fungi isolated from Canastra cheeses. The letter *a* indicates the best results with statistical difference.

Filamentous fungi are frequently reported as lipase producers with several applications. In addition to *Aspergillus*, *Fusarium*, *Mucor*, *Rhizopus* and *Penicillium, Geotrichum* is a genus able to produce lipases with industrial importance, for example as wastewater treatment lipases, for the synthesis of lipophilic antioxidants in sunflower oil and cheese ripening (KUDDUS, 2018; NIMKANDE & BAFANA, 2022). *G. candidum* is a a dimorphic filamentous fungus, included in the Ascomycota phylum, being morphologically characterized by the presence of arthroconidia formed in chains by the random fragmentation of hyphae (GENTE *et al*, 2006). Its color is usually cream and an opaque white (ALPER, FRENETTE & LABRIE, 2011). It is described as geotrichosis agent in humans, which is an opportunistic infection that usually manifests itself with low immunity activity. During winter season, *G. candidum* is able to trigger sporadic infections in bovine species (KNUDTSON & KIRKBRIDE, 1992). Despite being a fungus described as a food spoilager, *G. candidum*'s enzymes are responsible for amino acids and fatty acids releasing during cheese ripening, resulting in improvement of sensory characteristics (BOUTROU & GUÉGUEN, 2005; POTTIER *et al*., 2008; ALPER, FRENETTE & LABRIE, 2011; ELISKASES-LECHNER, 2011). Volatile compounds and methyl ketones such as 2-nonanone, 2-undecanone, 2-pentanone and 2-heptanone are produced after the activity of *G. candidum* lipases in several cheese varietes (ELISKASES-LECHNER, 2011).

Li *et al.* (2020) produced a soft soybean cheese using *G. candidum* to improve its sensory characteristics. The fungal activity was responsible for changes in the cheese texture, from a brittle and hard texture observed in soybean cheese to a soft and sticky one. Besides that, *G. candidum* provided a uniform velvety-white coat on the cheese surface, decreasing the beany taste and improving its quality. Several studies have demonstrated the versatility of *G. candidum* and its ability to produce lipases using agroindustrial oily wastes as low-cost substrates, which could represent an alternative to use of conventional substrates (DE MORAIS JUNIOR, 2016; FIBRIANA, UPAICHIT & CHEIRSILP, 2021). De Morais Junior (2016) observed an improved enzymatic acticity for *G. candidum* (11.48 U/mL) cultivated in media supplemented with soybean molasses, a byproduct of soy protein production (DE MORAIS JUNIOR, 2016). The isolate GC-13 showed a similar lipase activity; therefore, it could be used in further studies for optimizing the enzymatic production in an industrial scale.

There is a lack of studies about the lipase activity of *P. steckii*. HUMAID *et al* (2020) evaluated the lipase activity of filamentous fungi from cheese samples produced in Yemen. None of the three *P. steckii* isolates showed lipase activity in Tween 80 agar. The use of a quantitative method to evaluate the lipase activity could be explain the different findings in this

study. Therefore, further studies on the proteolytic and lipolytic activity of this isolate are necessary, in order to evaluated its potential application for the production of industrial enzymes. Regarding to *A. versicolor*, Yadav *et al* (1998) demonstrated its high lipolytic activity on glycerol tributyrate agar plates and in a submerged medium containing olive oil, with higher results than those found in this study (545 U/ml). Besides that, Magan, Jenkins & Howarth (1993) evaluated the enzymatic activity of filamentous fungi isolated from rapeseed and demonstrated the ability of *A. versicolor* to grow and produce lipases in different water activities (a_w) values and temperatures (15 and 25°C) on tributyrin agar (MAGAN, JENKINS & HOWARTH, 1993).

4.3 Mutation induction assay

4.3.1 Conidium core analysis and spore viability

In mutation assays it is important that the fungal isolate is uninucleate in order to improve the selection of mutants (DE BRITO, 1998). In this study, the core staining step was performed using at least 200 conidia and 100% uninucleate conidia frequency. The staining core results are demonstrated in Figure 5. Also, it is possible to observe enlarged conidia with two cores in the microscope images, indicating cell division. Conidia viability is another important data due to its influence on mycelial mass obtaining and metabolite production (DE BRITO, 1998). It was observed a growth of 60% from total seeded conidia after 48 hours of incubation.

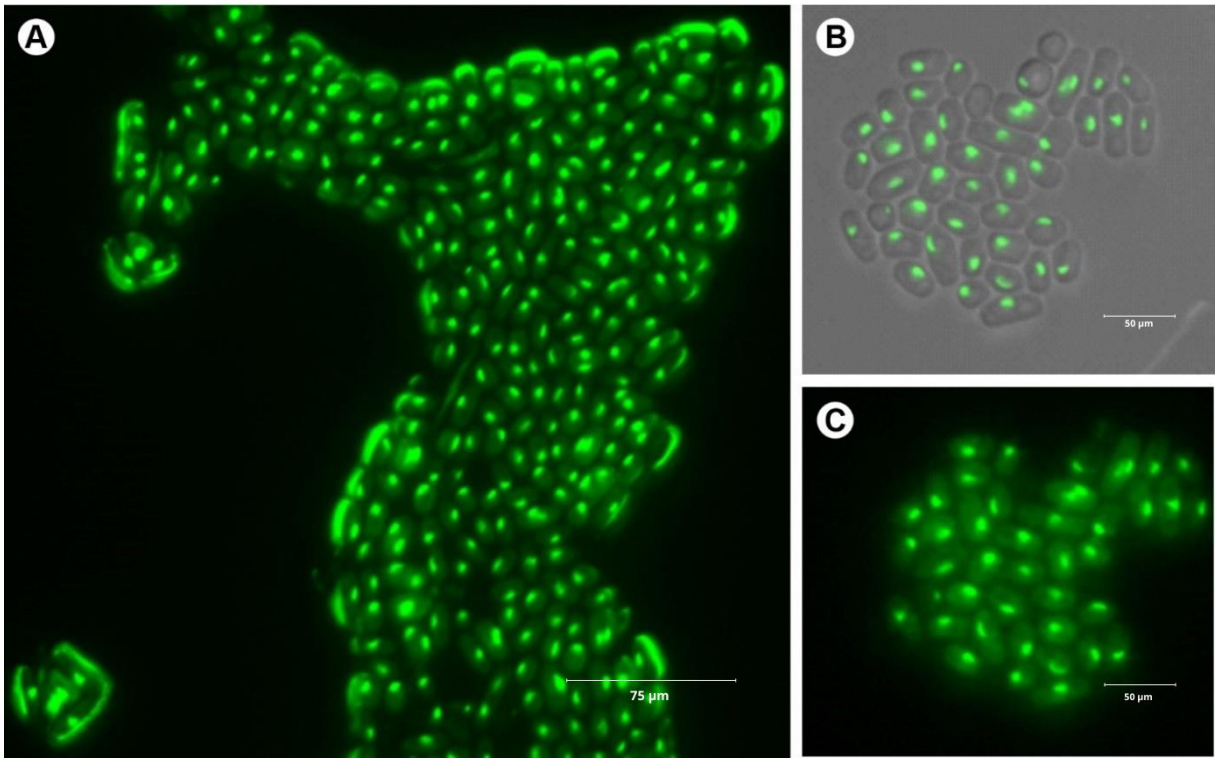

Figure 5. *G. candidum* conidia observed by epifluorescence microscopy: A) Conidia stained with SYBR green exhibiting a fluorescent green coloring (GFP filter) indicating an isolate with uninucleate core (40X); B) Core detail with image overlay, showing the strongest staining in the core (60X) (GPP and TRANS filter); C) Core detail in another microscope focal field (60X) with GFP filter.

4.3.2 UV light survival curve

The curve for UV light conidia survival was performed in 0.85% saline solution in order to test its efficiency. The aim was to reach a UV light exposure whose *G. candidum* survival rate was less than 5%. The *G. candidum* conidia evaluated revealed a rate of survival of 4.35% after 9 minutes of UV exposure (Figure 6).

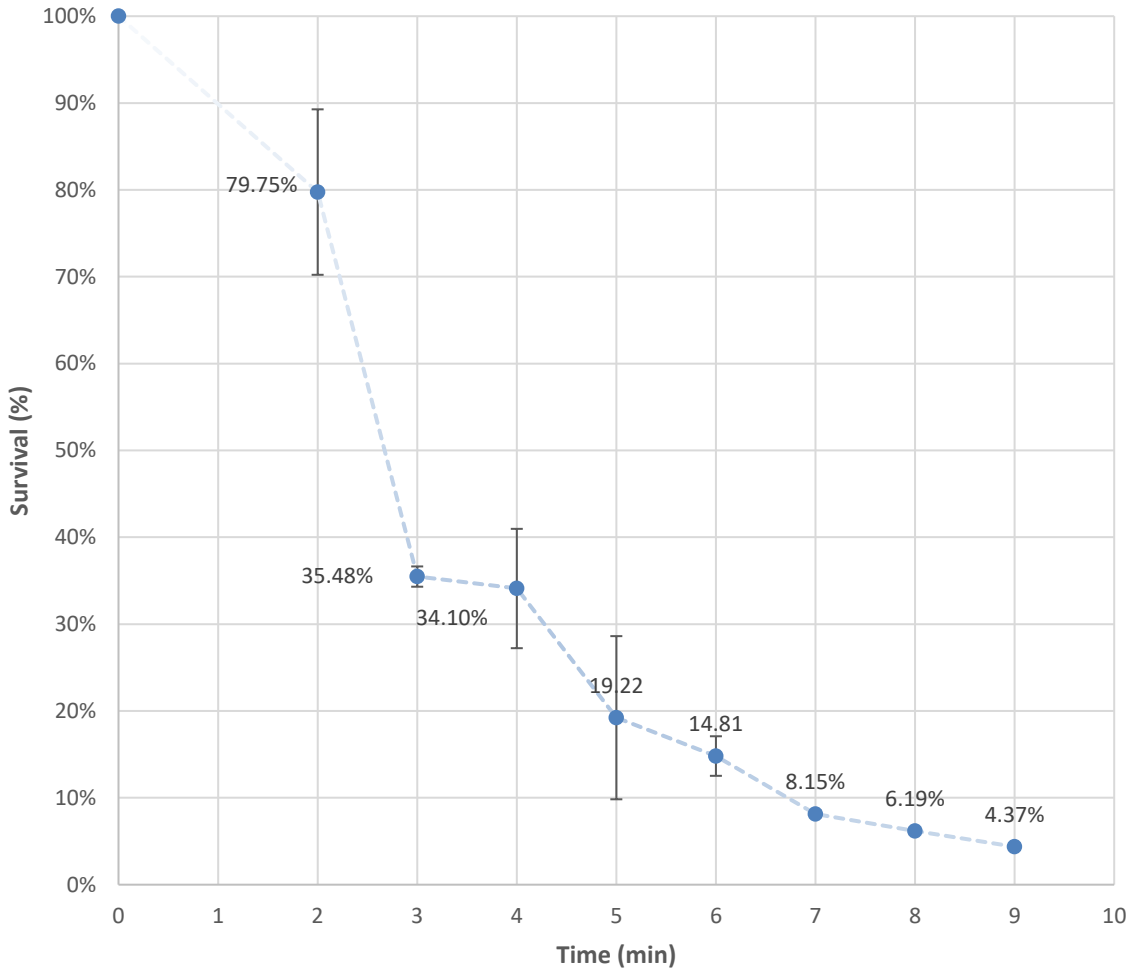

Figure 6. Percentage of *G. candidum* conidia surviving to irradiation with ultraviolet light in liquid medium.

4.3.3 Proteolytic activity of wild *versus* mutant strains

Several strategies can be used to enhance protease activity, such as optimization of fermentation conditions, screening for strains with higher production, genetic engineering or mutation induction (KUMAR *et al*., 2012). Despite being a random method, with no target in DNA, UV mutation is relatively secure and easy, playing an important role in bio-products development (LOC *et al*., 2012). In this study, mutation induced by UV light was applied in orther to reach *G. candidum* strains with improved protease activity. Considering the 18 mutant strains obtained, GC-M5, GC-M6, GC-M8, GC-M9, GC-M10, GC-M11, GC-M12, GC-M14 and GC-M18 isolates showed a statistically significant improvement in their proteolytic activity

evaluated by FS (Figure 7). GC-M10 showed an increasing about 273% (29.96 U/mL) in comparison to the wild type (10,94 U/mL).

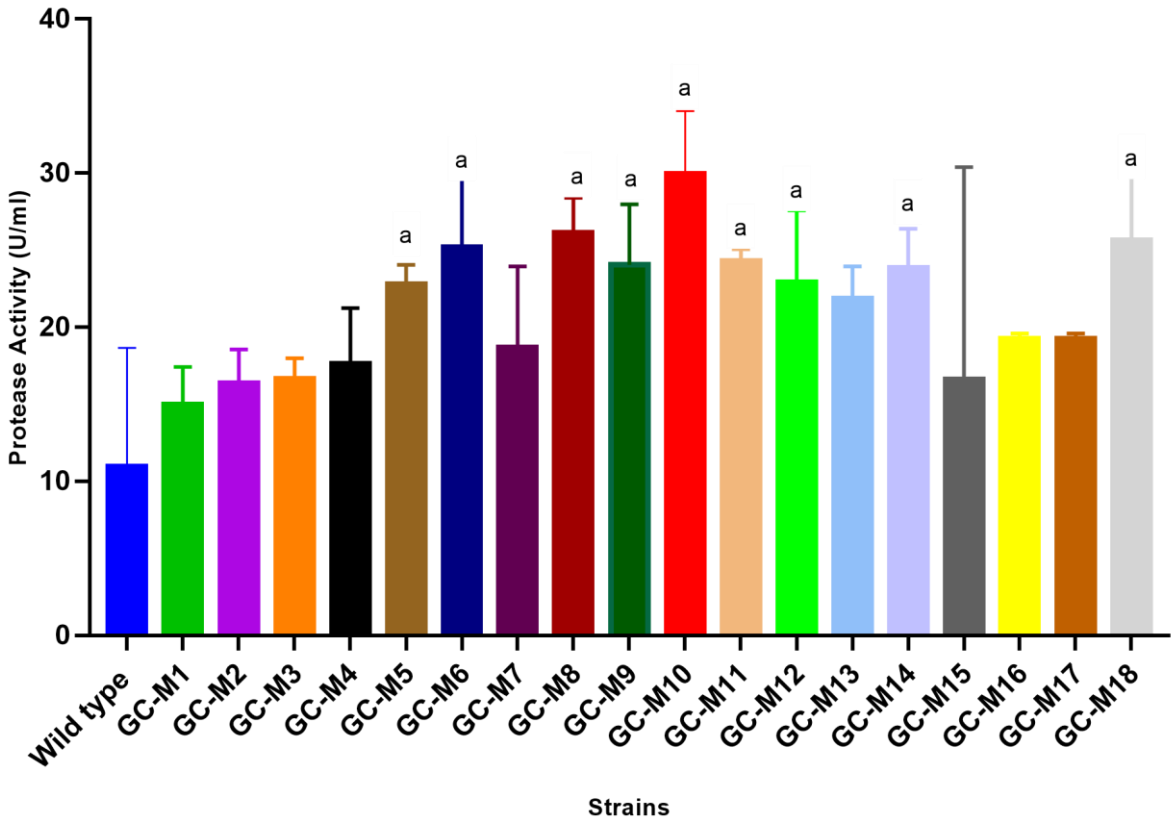

Figure 7: Protease activity of *G. candidum* wild and mutant strains (U/mL). The letter *a* indicates the best results with statistical difference.

Radha *et al*. (2012) demonstrated an increasing in acid protease activity of *A. niger* mutants obtained by UV light. However, sixty minutes of treatment were used, up to 6 times than used in this study (9 minutes). Furthermore, the assays were performed on casein agar and submerged fermentation, with higher halos and higher acid protease production around 2 times for the mutant strains in comparison to the wild type.

As mentioned before, *G. candidum* plays an important role in the ripening of several types of cheeses. At the beginning of the cheese ripening process, the decreasing pH due to LAB activity and the high salt content favors the growth of *Kluyveromyces*, *Saccharomyces* and *G. candidum* on cheese's surface (McSWEENEY, 2007). Besides that, pH on cheese surface progressively increases as lactate is consumed by fungi species; it is responsible for the increase

on activity of endoproteinases from milk (McSWEENEY, 2007; McSWEENEY, 2017). It is well known that *G. candidum* is an important lipase producer whose activity releases volatile compounds involved in aroma and flavor development (ELISKASES-LECHNER, 2011; KUDDUS, 2018; NIMKANDE & BAFANA, 2022). Several studies have isolated *G. candidum* from soft cheeses (Livarot, Limburger and Saint Marcellin), semi-hard cheeses (Reblochon, Saint Nectaire and Armada) and artisanal cheeses (POTTIER, *et al*., 2008; COGAN *et al*., 2014; CESAR, 2019). Moreover, it can be used in combination with *Brevibacterium linens* and *Debaryomyces hansenii* in commercial cultures for the production of washed-rind cheeses (COSTA, JUNIOR & DE PAULA, 2009).

On the other hand, several studies have been developed in order to demonstrate the biotechnological potential of *G. candidum* in the food industry. Its aminopeptidases can reduce bitterness caused by low molecular weight hydrophobic peptides from *Penicillium camemberti* endoprotease activity, acting specifically in beta-casein (MOLIMARD *et al*., 1994; MOLIMARD *et al*., 1995). A *G. candidum* strain GEO CD1 was applied for Camembert production, improving the sensory quality in comparison to traditional Camembert cheese manufactured with only *P. candidum* (DIAS, 2007). Finally, a new protocol for production of soft cheeses with *G. candidum* demonstrated a significant improvement on its sensory and physical-chemical charactheristics (Jaster *et al.,* 2018).

5. CONCLUSIONS

This study investigated the enzymatic activities of filamentous fungi isolated from artisanal cheeses produced in Canastra region. *P. steckii* and *A. versicolor* presented the highest proteolytic activities. Considering that few studies have explored the enzymatic activity of these species, the results obtained could represent a new perspective for their biotechnological exploitation in several fields, such as detergent, leather, pharmaceutical and textile industries. Both, in addition to *G. candidum*, showed the highest lipolytic activities. This findings for *G. candidum* are in accordance with other studies, reinforcing its use in the dairy industry as a secondary culture for cheese ripening. *G. candidum* mutants were obtained from UV light exposure; the method increased its protease activity around 273%. Among 18 mutant strains, 9 had higher proteolytic activity than the wild strain. Therefore, the use of UV light for obtaining *G. candidum* mutant strains with enhanced enzymatic activity can be considered a profitable strategy.

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