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VALORIZAÇÃO DE RESÍDUOS AGROINDUSTRIAIS POR FERMENTAÇÃO EM
ESTADO SÓLIDO: UMA ESTRATÉGIA SUSTENTÁVEL PARA OBTENÇÃO DE
PROTEASES VISANDO APLICAÇÕES BIOTECNOLÓGICAS

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Tese apresentada ao Programa de Pós-Graduação em Engenharia e Ciência de Alimentos da Universidade Estadual do Sudoeste da Bahia em cumprimento dos requisitos para obtenção do título de Doutor (a) em Engenharia e Ciência de Alimentos.

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LISTA DE ABREVIACES

FES - Fermentaco em Estado Slido

FSM - Fermentaco Submersa

PA – Atividade proteoltica

MCA –Atividade de Coagulaco

DPPH – Radical 2,2-difenil-1-picrilhidrazilil

FRAP – Capacidade de reduco do ferro

HPLC - Cromatografia lquida de alta eficincia

MIR – Infravermelho Mdio

ABTS - cido 2,2'-azinobis-(3-etilbenzotiazolina-6-sulfnico)

MSR - Metodologia de Superfcie de Resposta

DH - Grau de hidrlise

NIR – Infravermelho prximo

RESUMO

SILVA, E. C. “Valorização de resíduos agroindustriais por fermentação em estado sólido: Uma estratégia sustentável para obtenção de proteases visando aplicações biotecnológicas”. Itapetinga, BA: UESB, 2026. 141 p. Tese. (Doutorado em Engenharia e Ciência de Alimentos, Área de Concentração em Ciência de Alimentos).

O uso de enzimas microbianas obtidas por processos biotecnológicos tem ganhado destaque na área de alimentos, especialmente como alternativa tecnológica e funcional aos coagulantes e enzimas comerciais tradicionalmente utilizados. Entretanto, ainda há lacuna científica relacionada ao desenvolvimento de biocatalisadores obtidos a partir de resíduos agroindustriais, com aplicação simultânea na geração de compostos bioativos e na indústria de laticínios. Neste contexto, esta tese teve como objetivo produzir, caracterizar e aplicar uma protease aspártica microbiana obtida por fermentação em estado sólido (FES) por *Penicillium roqueforti* para obtenção de peptídeos bioativos a partir de proteínas lácteas, bem como avaliar seu potencial biotecnológico na coagulação do leite de cabra. Inicialmente, a protease foi produzida por FES utilizando combinações de ora-pro-nóbis, mandacaru e farelo de soja, substratos selecionados com base em sua disponibilidade regional no semiárido nordestino e no seu potencial nutricional, sendo o processo otimizado por delineamentos simplex-centroide e Box–Behnken. As melhores condições foram obtidas com 144 h de fermentação, 25 °C e 70% de umidade, empregando a mistura 1:1 de ora-pro-nóbis e farelo de soja, resultando em atividade proteolítica de 4,29 U/mL. O extrato apresentou elevada atividade coagulante do leite ($482 \pm 1,52$ U/mL), alta razão MCA/PA (116,11), atividade ótima em pH 6,0 e 60 °C, estabilidade térmica, ativação por Ca^{2+} e massa molecular estimada de 23 kDa, evidenciando seu potencial como coagulante lácteo. Posteriormente, a protease foi aplicada na hidrólise enzimática de caseínas bovina, caprina e bubalina, visando à obtenção de peptídeos bioativos. Os resultados demonstraram que o grau de hidrólise e o perfil peptídico foram dependentes da origem da caseína e do tempo de reação. Os hidrolisados apresentaram elevada atividade antioxidante pelos métodos DPPH, ABTS, FRAP e β -caroteno-ácido linoleico, com valores máximos em aproximadamente 2 h de hidrólise, quando foram obtidos cerca de 85–88% de inibição no DPPH, 90–95% no ABTS, 1500–1600 μM Fe^{2+} no FRAP e 55–60% de proteção na oxidação, além de expressiva atividade inibitória da α -amilase. As análises por HPLC confirmaram a formação de peptídeos com ampla diversidade estrutural. Por fim, avaliou-se a substituição parcial da quimosina comercial por essa protease aspártica microbiana (PAM) na produção de queijo Minas Frescal de leite de cabra. Formulações contendo 50% ou mais de PAM mantiveram rendimento semelhante ao controle, enquanto o tratamento PAM70% promoveu aumento do teor proteico (17,86%), redução da umidade (60,54%) e maior firmeza do produto. As análises reológicas indicaram comportamento viscoelástico típico de géis proteicos, e os espectros MIR e NIR evidenciaram ausência de alterações químicas globais na matriz do queijo. De forma geral, os resultados demonstram que a protease aspártica de *Penicillium roqueforti* apresenta elevado potencial tecnológico e funcional, tanto na geração de peptídeos bioativos quanto como coagulante alternativo na produção de queijos.

Palavras-chave: Protease aspártica; *Penicillium roqueforti*; Fermentação em estado sólido; Coagulação do leite.

ABSTRACT

SILVA, E. C. “Valorization of agro-industrial residues through solid-state fermentation: a sustainable strategy for obtaining proteases for biotechnological applications”. Itapetinga, BA: UESB, 2026. 141 p. Ph.D. Thesis (Doctorate in Food Engineering and Science, Concentration Area in Food Science).

The use of microbial enzymes obtained through biotechnological processes has gained prominence in the food sector, especially as a technological and functional alternative to traditionally used coagulants and commercial enzymes. However, there is still a scientific gap regarding the development of biocatalysts derived from agro-industrial residues, with simultaneous application in the generation of bioactive compounds and in the dairy industry. In this context, this thesis aimed to produce, characterize, and apply a microbial aspartic protease obtained by solid-state fermentation (SSF) using *Penicillium roqueforti* to obtain bioactive peptides from milk proteins, as well as to evaluate its biotechnological potential in goat milk coagulation. Initially, the protease was produced by SSF using combinations of ora-pro-nóbis, mandacaru, and soybean bran as substrates, selected based on their regional availability in the Brazilian semi-arid region and their nutritional potential. The process was optimized using simplex-centroid and Box–Behnken designs. The best conditions were obtained at 144 h of fermentation, 25 °C, and 70% moisture, using a 1:1 mixture of ora-pro-nóbis and soybean bran, resulting in a proteolytic activity of 4.29 U/mL. The extract showed high milk-clotting activity (482 ± 1.52 U/mL), a high MCA/PA ratio (116.11), optimal activity at pH 6.0 and 60 °C, thermal stability, activation by Ca^{2+} , and an estimated molecular mass of 23 kDa, demonstrating its potential as a milk coagulant. Subsequently, the protease was applied to the enzymatic hydrolysis of bovine, goat, and buffalo caseins to obtain bioactive peptides. The results showed that the degree of hydrolysis and peptide profile depended on the casein source and reaction time. The hydrolysates exhibited high antioxidant activity by DPPH, ABTS, FRAP, and β -carotene–linoleic acid assays, with maximum values observed at approximately 2 h of hydrolysis, reaching about 85–88% inhibition (DPPH), 90–95% (ABTS), 1500–1600 μM Fe^{2+} (FRAP), and 55–60% lipid oxidation inhibition, in addition to significant α -amylase inhibitory activity. HPLC analyses confirmed the formation of peptides with broad structural diversity. Finally, the partial replacement of commercial chymosin by this microbial aspartic protease (PAM) was evaluated in the production of Minas Frescal cheese from goat milk. Formulations containing 50% or more PAM maintained yield similar to the control, while the PAM70% treatment increased protein content (17.86%), reduced moisture (60.54%), and improved firmness. Rheological analyses indicated typical viscoelastic behavior of protein gels, and MIR and NIR spectra showed no global chemical changes in the cheese matrix. Overall, the results demonstrate that the aspartic protease from *Penicillium roqueforti* has high technological and functional potential, both for generating bioactive peptides and as an alternative coagulant in cheese production.

Keywords: Aspart protease; *Penicillium roqueforti*; Solid-state fermentation; Milk coagulation

CAPÍTULO I - REVISÃO BIBLIOGRÁFICA

1. INTRODUÇÃO

Nos últimos anos, o aumento da demanda global por soluções sustentáveis para o manejo de resíduos agrícolas e alimentares tem despertado crescente interesse no uso desses materiais como substratos para a produção de enzimas microbianas via fermentação em estado sólido (FES). Essa abordagem biotecnológica tem se mostrado promissora para o aproveitamento de resíduos orgânicos na produção enzimática, contribuindo para a valorização desses materiais e para a redução dos impactos ambientais associados ao seu descarte inadequado (KhaswaL et al., 2024).

Nos países em desenvolvimento, cuja economia depende predominantemente da agricultura, os resíduos agrícolas são frequentemente descartados por meio de queimadas, descarte indiscriminado ou em aterros não regulamentados. Essas práticas inadequadas podem resultar em contaminação do meio ambiente e provocar danos à vida animal, humana e vegetal (Perwez; Asheh, 2025). Nesse contexto, os resíduos gerados pela indústria agroalimentar representam perdas significativas de biomassa e nutrientes valiosos, os quais poderiam ser reaproveitados na produção de produtos industriais ou como matérias-primas para outros processos (Esparza et al., 2020).

A FES destaca-se como uma estratégia biotecnológica eficiente para o aproveitamento desses resíduos, uma vez que utiliza substratos sólidos umedecidos como suporte e fonte de nutrientes para o crescimento microbiano. Essa técnica apresenta vantagens como maior concentração do produto final, menor geração de efluentes e possibilidade de utilização de substratos de baixo custo, contribuindo para estratégias de valorização de resíduos agroindustriais (Belorkar; Jogaiah, 2022; Mirsalami; Mirsalami, 2025). No entanto, embora a FES seja frequentemente associada à redução de impactos ambientais, sua efetiva inserção em modelos de economia circular ainda depende da integração com outras cadeias produtivas.

Nesse sentido, a biomassa residual gerada após o processo fermentativo ainda representa um desafio. Estudos recentes têm demonstrado o potencial de reaproveitamento desse material em aplicações subsequentes, como a produção de carvão ativado com elevada área superficial, que pode ser utilizado como suporte para imobilização enzimática e síntese de compostos de interesse industrial (Gonçalves et al., 2025). Esses achados evidenciam a possibilidade de integração da FES em estratégias de bioeconomia, com geração de produtos de valor agregado e alinhamento com princípios de consumo e produção responsáveis.

No processo de FES, os resíduos agrícolas funcionam simultaneamente como matriz sólida e fonte de nutrientes que favorecem o desenvolvimento microbiano, especialmente de fungos filamentosos. Esses resíduos apresentam composição rica em carbono, principalmente

na forma de celulose, hemicelulose e açúcares, além de conter nitrogênio, lipídios e minerais, tornando-se meios de cultura adequados para o crescimento fúngico (Singh; Jana, 2023). No contexto do semiárido nordestino, especialmente na Bahia, destaca-se a disponibilidade de espécies como ora-pro-nóbis e mandacaru, além de subprodutos amplamente disponíveis como o farelo de soja. Esses materiais apresentam potencial nutricional relevante, especialmente quanto ao teor proteico, sendo ainda subexplorados como substratos em bioprocessos para produção enzimática.

Os principais microrganismos utilizados na FES incluem fungos filamentosos dos gêneros *Aspergillus*, *Fusarium*, *Penicillium*, *Rhizopus* e *Trichoderma* (Yafetto, 2022). Dentre esses, espécies do gênero *Penicillium* têm se destacado pela capacidade de produção de enzimas extracelulares de interesse industrial. As proteases, classificadas como as principais enzimas hidrolíticas, são amplamente reconhecidas por sua capacidade de clivar ligações peptídicas e por sua versatilidade de aplicação em diferentes setores industriais (Ariaeenejad; Motahar, 2025). As proteases microbianas têm sido amplamente estudadas devido às suas diversas aplicações biotecnológicas, incluindo biomedicina, biorremediação e indústria de alimentos (Ajayi; Lateef, 2025).

Na indústria de alimentos, as proteases desempenham papel fundamental na fabricação de queijos, promovendo a hidrólise específica da κ -caseína e possibilitando a formação da rede proteica responsável pela coagulação do leite (Aguilar; Sato, 2018; Mamo; Assefa, 2018). Além disso, essas enzimas têm sido utilizadas na obtenção de peptídeos bioativos a partir da hidrólise de proteínas lácteas, especialmente a caseína, que apresenta elevada susceptibilidade à ação enzimática e potencial para liberação de compostos com diferentes atividades fisiológicas (Zhou et al., 2025).

Apesar dos avanços na produção de proteases por FES, ainda são limitados os estudos que associam o uso de substratos regionais alternativos à obtenção de enzimas com aplicações simultâneas na geração de peptídeos bioativos e na indústria de laticínios. Nesse sentido, destaca-se a relevância de abordagens que integrem sustentabilidade, valorização de recursos regionais e desenvolvimento de biocatalisadores com potencial aplicação tecnológica.

Este estudo teve como objetivo produzir, caracterizar e aplicar proteases obtidas por fermentação em estado sólido utilizando *Penicillium roqueforti*, cultivado em substratos alternativos de baixo custo, incluindo ora-pro-nóbis, farelo de soja e mandacaru. A proposta integra o aproveitamento de recursos regionais do semiárido com o desenvolvimento de enzimas de interesse industrial, visando aplicações tanto na obtenção de peptídeos bioativos quanto na produção de queijos. O estudo englobou a otimização das condições do processo

fermentativo, visando maximizar a produção enzimática e o aproveitamento desses substratos. O extrato bruto obtido foi aplicado na hidrólise de caseínas bovina, caprina e bubalina, bem como na substituição parcial da quimosina na produção de queijo Minas Frescal a partir de leite de cabra.

2. REVISÃO BIBLIOGRÁFICA

2.1 Resíduos agroindustriais

Atividades agroindustriais geram grandes volumes de resíduos em escala global, impondo desafios à gestão sustentável. O setor agrícola produz quantidades expressivas de cereais como trigo, arroz, milho e cevada, que constituem a base alimentar de bilhões de pessoas. No entanto, o processamento desses grãos por meio de moagem, cervejaria e outras operações agroindustriais resulta na geração significativa de resíduos orgânicos e subprodutos, incluindo cascas, farelo, palha e grãos residuais (Adil et al., 2026). Esses materiais, frequentemente descartados ou subutilizados, apresentam elevado potencial de aproveitamento biotecnológico, especialmente como substratos alternativos em processos fermentativos.

Em razão da elevada geração desses resíduos, especialmente em países em desenvolvimento, a ausência de alternativas sustentáveis para sua recuperação e reaproveitamento favorece práticas inadequadas de descarte, como a incineração e o acúmulo em aterros não planejados. Essas práticas resultam em impactos ambientais e riscos à vida humana e animal, além de contribuírem para o aumento das emissões de gases de efeito estufa e para a perda de potencial energético (Freitas et al., 2021; Perwez; Asheh, 2025). Nesse contexto, por serem ricos em diversos compostos, esses materiais podem ser convertidos, após fermentação, em biogás, biocombustíveis e diferentes metabólitos secundários (Perwez; Asheh, 2025).

A composição química desses resíduos, caracterizada pela presença de açúcares, amido, proteínas e frações fibrosas, como celulose, hemicelulose e lignina, reforça seu potencial como substratos para FES, devido à abundância, baixo custo e ampla disponibilidade (Yafetto, 2022). Além disso, essa composição favorece sua aplicação na produção microbiana de compostos bioquímicos e ingredientes funcionais de alto valor agregado, incluindo antibióticos, vitaminas, enzimas e biossurfactantes (Singh; Jana, 2023).

Dentre os resíduos agroindustriais mais utilizados como substratos para a produção de proteases, destacam-se os farelos (arroz, trigo, aveia, semente de algodão e soja), as tortas vegetais (mostarda, linhaça e coco), além de resíduos como cascas de grama verde, cascas de laranja e cascas de batata (Dubey et al., 2024). Exemplos de resíduos agroindustriais empregados na produção de proteases por fermentação em estado sólido (FES) estão apresentados na Tabela 1.

Tabela 1. Resíduos agroindustriais utilizados na produção de proteases por FES

Resíduo agroindustrial	Microrganismo utilizado	Característica	Referências
Farelo de trigo	<i>A. oryzae</i>	Cisteína	Murthy <i>et al.</i> (2020)
Farelo de trigo	<i>A. oryzae</i>	Carboxipeptidase	Murthy <i>et al.</i> (2019)
Cascas de ervilha	<i>Aspergillus terreus</i>	Aspártica	Sethi <i>et al.</i> (2016)
Resíduos de mandioca	<i>Stenotrophomonas acidaminiphila</i>	Serina	Asitok <i>et al.</i> (2022)
Farelo de trigo	<i>Aspergillus heteromorphus</i>	Serina	Fernandes <i>et al.</i> (2024)
Farelo de trigo	<i>Bacillus halodurans</i>	Serina	Balachandran <i>et al.</i> (2021)
Polpa de café e sabugo de milho	<i>Bacillus</i> sp	Serina	Kandasamy <i>et al.</i> (2016)
Farelo de trigo	<i>Mycothermus thermophilus</i>	Protease (termoestável)	Talhi <i>et al.</i> (2022)
Farelo de trigo	<i>Rhizomucor miehei</i>	Aspártica	Aljammas, Yazji e Azizieh (2022)

No contexto desta pesquisa, destacam-se como substratos potenciais o farelo de soja, o mandacaru (*Cereus jamacaru* DC.) e a ora-pro-nóbis (*Pereskia aculeata* Miller), cujas características de disponibilidade e composição química justificam sua aplicação em processos de fermentação em estado sólido.

O farelo de soja é um dos principais subprodutos da agroindústria brasileira, sendo obtido a partir do processo de extração do óleo de soja. O Brasil figura entre os maiores produtores mundiais dessa oleaginosa, o que assegura ampla disponibilidade desse resíduo para aplicações biotecnológicas. Esse material destaca-se por seu elevado teor proteico, variando entre 30 e 50%, além de apresentar perfil equilibrado de aminoácidos, carboidratos e minerais, características que o tornam altamente favorável à produção de enzimas proteolíticas (Shen *et al.*, 2024). A utilização do farelo de soja em FES tem demonstrado elevada eficiência na conversão biológica do substrato por meio da ação enzimática microbiana, resultando na formação de produtos com maior valor agregado e metabólitos de baixo peso molecular, com elevada digestibilidade e valor nutricional (Dai *et al.*, 2023; Liu *et al.*, 2024).

Além de fontes convencionais, espécies vegetais não convencionais também apresentam grande potencial para aplicação em bioprocessos. Entre elas, destacam-se plantas da família Cactaceae, como o mandacaru e a ora-pro-nóbis, que oferecem oportunidades promissoras para inovação tecnológica, especialmente em regiões semiáridas. A fermentação desses materiais possibilita o aproveitamento de carboidratos fermentáveis e de seus derivados, além de explorar

a diversidade microbiana capaz de metabolizar esses compostos como fontes de carbono (Carpena et al., 2023).

O *Cereus jamacaru* DC., popularmente conhecido como mandacaru, é uma cactácea amplamente distribuída na região semiárida do Brasil, caracterizando-se por apresentar caule colunar ramificado e flores brancas (Mataruco et al., 2023). Essa espécie possui composição nutricional relevante, com abundância de carboidratos estruturais, aminoácidos e elevado teor de fibras alimentares. Destaca-se ainda por seu conteúdo mineral, com predominância de cálcio e potássio, além da presença de compostos fenólicos que conferem atividade antioxidante (Medeiros et al., 2024). Tais características reforçam seu potencial como fonte de carbono e suporte físico em processos fermentativos.

Pereskia aculeata Miller (Cactaceae), popularmente denominada ora-pro-nóbis (OPN), é uma espécie nativa da América do Sul com ampla distribuição no território brasileiro. Suas folhas, tradicionalmente utilizadas na culinária regional, destacam-se por seu elevado valor nutricional, contendo teores significativos de proteínas, minerais (como ferro e cálcio), microelementos, β -caroteno e vitaminas, incluindo ácido ascórbico e ácido fólico (Cruz et al., 2021). Devido à sua rica composição nutricional e propriedades biológicas, as folhas de OPN representam uma alternativa promissora para exploração biotecnológica, contribuindo para a diversificação produtiva e a agregação de valor na agricultura familiar (Torres et al., 2022).

De maneira geral, a valorização de resíduos agroindustriais e de biomassa vegetal subutilizada contribui significativamente para o desenvolvimento de bioprocessos sustentáveis, promovendo a redução de impactos ambientais e a geração de produtos de alto valor agregado. Nesse sentido, a utilização do farelo de soja, do mandacaru e da ora-pro-nóbis como substratos em fermentação em estado sólido apresenta-se como uma estratégia promissora para a produção de proteases microbianas, alinhando viabilidade econômica, sustentabilidade ambiental e inovação tecnológica.

2.2 Processos de fermentação

Os sistemas de fermentação mais utilizados incluem a fermentação submersa (FSM) e a fermentação em estado sólido (FES). A seleção do método adequado para produção de proteases depende de aspectos como o tipo de microrganismo empregado, a estabilidade enzimática e as exigências operacionais (Dubey et al., 2024).

A FSM consiste no cultivo de microrganismos em meio líquido nutritivo. Esse método de fermentação é ideal para bactérias e outros organismos que necessitam de elevada umidade e é comumente utilizado para obtenção de metabólitos secundários em solução, apresentando

como vantagens a homogeneidade do meio e o fácil controle de parâmetros como temperatura, pH e agitação. Contudo, o processo requer grandes volumes de água para preparo do meio, o que eleva os custos de produção e gera resíduos líquidos com alto teor de nutrientes, podendo causar impactos ambientais se descartados sem tratamento adequado (Singh; Jana, 2023; Ali et al., 2025).

Em contraste, a FES corresponde a um processo de biotransformação no qual microrganismos se desenvolvem em condições de baixa disponibilidade de água livre, convertendo substratos sólidos em produtos químicos (Barbosa; Silva; Carvalho, 2021). No processo de FES, o teor de água geralmente se mantém abaixo de 70%, reduzindo ou eliminando, em muitos casos, a necessidade de mecanismos de agitação, embora aeração controlada possa ser necessária dependendo do sistema e do microrganismo utilizado. Com isso, ocorre uma redução significativa nos gastos energéticos associados à transferência de massa, suprimento de oxigênio e etapa de secagem (Lu et al., 2025).

A FSM apresenta maior facilidade de escalonamento e controle do processo, enquanto a FES está associada à maior produção enzimática, especialmente de enzimas nativas naturalmente expressas pelos fungos, em razão de sua adaptação fisiológica a ambientes contendo substratos sólidos (Ahmed et al., 2025). Nesse contexto, a fermentação em estado sólido apresenta-se como uma abordagem particularmente adequada para o aproveitamento de resíduos agroindustriais.

2.2.1 Fermentação em estado sólido (FES)

A FES é um método de fermentação microbiana no qual microrganismos específicos (bactérias, fungos filamentosos e leveduras) são cultivados em um substrato orgânico úmido, sólido e insolúvel, que serve como suporte e fonte de nutrientes para os microrganismos, em condições de ausência ou mínima presença de água livre (Yafetto, 2022). O baixo volume de água na FES impacta positivamente a economia do processo, principalmente devido ao menor tamanho requerido para os biorreatores, à redução do processamento *downstream*, já que os produtos podem ser facilmente recuperados, e aos custos mais baixos de esterilização (Banat et al., 2021).

A FES é realizada em três fases principais: *upstream*, *midstream* e *downstream*. O procedimento *upstream* inclui a preparação do substrato, a esterilização e a preparação do meio de cultura e do inóculo microbiano. A etapa *midstream* corresponde ao processo de FES propriamente dito, com incubação dos microrganismos no substrato e monitoramento das condições operacionais. Já o *downstream* envolve as etapas de extração, recuperação,

purificação e, quando necessário, concentração e formulação das enzimas ou biomoléculas de interesse, culminando na obtenção do produto final (Perwez; Asheh, 2025).

A FES simula as condições naturais de crescimento de diversos microrganismos, especialmente fungos e bolores. Esse processo apresenta menor suscetibilidade à contaminação bacteriana e favorece o aumento da eficiência enzimática. No entanto, fatores como a seleção do substrato, a escolha do microrganismo e as condições do processo fermentativo influenciam diretamente os rendimentos obtidos (Chilakamarry et al., 2022).

Nesse contexto, os fungos filamentosos se destacam como alguns dos microrganismos mais adequados para a FES, devido às condições bióticas favoráveis durante esse processo e por serem menos suscetíveis à contaminação em relação às bactérias (Muñoz-Seijas et al., 2024). Particularmente no caso dos fungos filamentosos, suas características morfológicas únicas, como o crescimento de hifas tubulares por alongamento apical, a ramificação intensa formando uma rede micelial tridimensional e porosa e a capacidade de aderência e colonização eficiente de superfícies sólidas, fazem com que sejam altamente eficientes na FES. O desenvolvimento de hifas a partir de esporos germinados permite a colonização do substrato sólido, favorecendo a absorção de nutrientes e a troca gasosa (Cebrián; Ibarruri, 2023). Além disso, o meio utilizado para a produção de proteases por fungos na FES pode facilitar sua fixação, fornecendo nutrientes e água. Pesquisas indicam que a síntese de proteases depende da disponibilidade de nitrogênio e carbono no meio, sendo que ambos atuam na regulação da produção enzimática (Schuster et al., 2019).

Carbono e nitrogênio constituem nutrientes essenciais para o desenvolvimento fúngico, atuando em funções complementares. O carbono serve como fonte energética e pode ser obtido a partir de monossacarídeos (glicose, frutose) ou de polímeros complexos (amido, celulose). O nitrogênio também tem uma função crucial na biossíntese de biomassa, pois sua concentração e disponibilidade influenciam a atividade e a eficiência do crescimento, considerando que atua como elemento fundamental no desenvolvimento celular e no sustento de elevadas taxas de crescimento microbiano. Assim, a escassez de nitrogênio pode resultar em um menor crescimento dos fungos (Cebrián; Ibarruri, 2023; Zhang et al., 2023).

A produção enzimática via FES tem ganhado relevância devido à utilização de substratos de baixo custo, provenientes de subprodutos agroindustriais, o que contribui para a viabilidade econômica do processo. Os substratos empregados consistem em matérias-primas de baixo custo, provenientes de resíduos agrícolas e das indústrias de processamento de alimentos. Além do custo reduzido, esses materiais possuem nutrientes simples e complexos

indispensáveis para o estímulo ao desenvolvimento microbiano e à produção de enzimas (Belorkar; Jogaiah, 2022).

2.3 Otimização da fermentação

A otimização estatística de processos fermentativos apresenta vantagens significativas em relação ao método convencional de alteração univariada, destacando-se pela redução no número de experimentos e pela possibilidade de avaliar os efeitos de interação entre as variáveis que influenciam as condições de fermentação selecionadas. Para isso, são utilizados métodos estatísticos como delineamentos fatoriais, ortogonais e a metodologia de superfície de resposta (Colla et al., 2016; Sathish et al., 2016).

Nesse contexto, a otimização simultânea de múltiplos parâmetros físico-químicos é fundamental para o estabelecimento de processos produtivos eficientes. Historicamente, delineamentos como o fatorial de três níveis, o Box-Behnken (BBD), o Composto Central (CCD) e o Doehlert (DDD) são utilizados para ajustar modelos polinomiais de segunda ordem por meio do método dos mínimos quadrados. Essas abordagens permitem avaliar a qualidade e a precisão dos modelos ajustados, viabilizando previsões confiáveis com base nos dados experimentais (Dhayalan et al., 2025). Métodos estatísticos baseados em delineamentos experimentais têm sido amplamente empregados para avaliar a interação entre variáveis e maximizar respostas de interesse, destacando-se a Metodologia de Superfície de Resposta (MSR) (Hadrach, 2025). Além disso, a construção de gráficos de contorno e superfícies de resposta tridimensionais possibilita a visualização clara das interações entre os fatores e a identificação das regiões ótimas de operação. Consequentemente, a aplicação dessas ferramentas estatísticas na otimização da produção enzimática tem permitido prever condições ótimas e maximizar o rendimento enzimático (Almanaa et al., 2020).

Assim, no contexto da FES, a definição e a otimização dos parâmetros operacionais tornam-se essenciais, exigindo a análise da relação entre a fisiologia do microrganismo e o produto final desejado. Nesse sentido, é fundamental otimizar parâmetros como a seleção adequada dos microrganismos e da composição do substrato sólido, a definição das condições ideais de umidade e temperatura, além disso, a escolha eficiente dos métodos de extração e purificação tem relação direta com os rendimentos e viabilidade do processo (Postigo et al., 2021).

2.4 Parâmetros que influenciam o crescimento microbiano na FES

A otimização de múltiplas condições de parâmetros críticos para a fermentação, como pH, temperatura, teor de umidade, tipo de microrganismo, concentração de inóculo e composição do substrato, é essencial para a produção enzimática microbiana em FES. As interações entre esses fatores exercem influência significativa no rendimento dos produtos microbianos (Sathish et al., 2016; Srivastava et al., 2019; López-Gómez; Manan; Webb, 2020).

2.4.1 Microrganismo e inóculo

Entre os fatores envolvidos no desenvolvimento de um processo de FES, destaca-se a seleção adequada dos microrganismos e dos substratos. Os fungos filamentosos são considerados particularmente adequados para FES, pois a técnica reproduz seu ambiente natural, permitindo-lhes sintetizar quantidades significativas de enzimas e outros metabólitos (Soccol et al., 2017).

Diversas espécies fúngicas desempenham papel essencial na produção enzimática utilizando biomassa agrícola como substrato. Entre os fungos mais utilizados, destacam-se *Talaromyces* sp., *Aspergillus* sp., *Pleurotus* sp., *Trichoderma* sp., *Coprinus* sp., *Penicillium* sp. e *Trametes* sp., que são amplamente empregados para a síntese de diversas classes de enzimas, incluindo celulasas, α -amilases, β -glucosidases, invertases, fitases, poligalacturonases, lignina peroxidases, lacases e pectina liases (Kukreti; Kumar; Kataria, 2024).

Dentre esses, o *Penicillium roqueforti* é um fungo saprofítico filamentoso, frequentemente isolado de solos ou de matéria orgânica em decomposição. Caracteriza-se por seu rápido crescimento e formação de colônias aveludadas, com coloração verde-escura (Martínez-Rodríguez et al., 2014; Coton; Jany; Coton, 2022).

Penicillium roqueforti destaca-se entre as principais espécies de fungos filamentosos utilizadas na indústria de laticínios, especialmente na maturação de queijos, sendo uma das mais estudadas por suas aplicações tecnológicas. Especificamente, esse microrganismo produz diversas proteases com atividade específica sobre caseínas, catalisando sua hidrólise em pequenos peptídeos e aminoácidos, além de apresentar histórico de uso seguro em alimentos e ser reconhecido como GRAS (*Generally Recognized As Safe*), o que reforça seu potencial para aplicações biotecnológicas na indústria alimentícia (Gillot et al., 2017; Coton et al., 2020; Dogan; Tekiner, 2021).

Além da seleção do microrganismo, a idade do inóculo, o meio de cultivo utilizado e seu estado fisiológico representam fatores cruciais nos processos fermentativos. Quando o inóculo destinado à produção de metabólitos secundários não se encontra no estado fisiológico

adequado, observa-se uma redução significativa na produção. Isso ocorre porque as primeiras horas de fermentação determinam a trajetória futura da cultura (López-Gómez; Manan; Webb, 2020). A duração da fase de latência é influenciada pelo tamanho e pelas condições fisiológicas do inóculo, sendo recomendado que a transferência ocorra quando as células se encontram metabolicamente ativas, preferencialmente na fase logarítmica de crescimento. A idade do inóculo é considerada um fator determinante, uma vez que alterações fisiológicas associadas às fases de crescimento podem impactar o desempenho fermentativo (Kaur et al., 2020). A FES é tradicionalmente iniciada com o uso de inóculos miceliais ou esporulados. Os esporos, em particular, oferecem vantagens significativas, incluindo maior homogeneidade, maior vida útil e facilidade de manipulação (Kumar et al., 2021).

2.4.2 Substratos

Um substrato ideal para FES deve conter todos os nutrientes essenciais para o desenvolvimento microbiano. Entretanto, na prática, a maioria dos substratos naturais requer suplementação nutricional adicional para garantir um crescimento microbiano adequado (López-Gómez; Manan; Webb, 2020). Além da composição nutricional, a escolha do substrato influencia diretamente a viabilidade econômica da produção de proteases. Isso ocorre porque o alto custo dos substratos é considerado o principal fator limitante para a produção de enzimas microbianas em aplicações industriais (Fahmy; El-Deeb, 2023). Esses materiais não atuam apenas como fontes primárias de carbono e nitrogênio para o desenvolvimento microbiano, mas também regulam os processos metabólicos envolvidos na síntese enzimática (Dubey et al., 2024). Portanto, a seleção de um substrato adequado é determinada tanto por sua composição química quanto por fatores econômicos, especialmente o custo e a facilidade de acesso (Chilakamarry et al., 2022).

Os resíduos agroindustriais são tradicionalmente empregados como substratos devido ao seu baixo custo, sustentabilidade ambiental e fácil disponibilidade (Bamidele et al., 2025). Esses materiais possuem composição predominantemente fibrosa, sendo constituídos basicamente por celulose, hemicelulose, lignina, amido, pectina e outras fibras (Soccol et al., 2017). Dentre os resíduos agroindustriais adequados para uso como substratos em FES, incluem-se bagaço de cana-de-açúcar, bagaço de mandioca, farelos de cereais (trigo, arroz, aveia, soja), polpa e cascas de café, cascas e polpas de frutas, sabugos de milho, além de palhas e cascas diversas. A utilização de misturas desses resíduos é uma estratégia frequentemente empregada na FES, uma vez que um único subproduto pode não oferecer todos os nutrientes essenciais para o desenvolvimento microbiano (Leite et al., 2021).

Assim, a prática de utilizar resíduos agroindustriais ricos em proteínas como substratos para a produção de proteases é favorável, tendo como consequência o aumento da produção dessa enzima, devido ao estímulo dos microrganismos para excretá-la no ambiente, a fim de obter carbono ou nitrogênio por meio da hidrólise de proteínas (Schuster et al., 2019).

2.4.3 Preparo do substrato

As características do substrato, como composição química, porosidade e tamanho das partículas, exercem influência determinante no desempenho da FES. As condições naturais desse meio favorecem o desenvolvimento de fungos filamentosos, criando um ambiente ideal para a síntese enzimática (Kukreti; Kumar; Kataria, 2024).

Para maximizar a eficiência antes do processo fermentativo, o substrato pode precisar de algumas etapas de pré-tratamento para ajustar seu tamanho por meio de corte, moagem ou granulação. Também pode ser umedecido ou cozido para melhorar a disponibilidade de nutrientes, ou suplementado com compostos específicos. Além disso, pode ser necessário esterilizar ou pasteurizar o substrato, seja fora ou dentro do biorreator (Cebrián; Ibaruri, 2023).

A moagem tem como intuito reduzir o tamanho das partículas do substrato favorecendo o desenvolvimento adequado dos microrganismos, pois ao romper as estruturas vegetais ocorre a exposição de alguns nutrientes e potencializa a ação microbiana. Além disso essa característica possibilita a transferência correta de calor e massa no meio sólido. A relação superfície-volume das partículas determina a fração do substrato que é efetivamente acessível aos microrganismos, sendo esta determinada pela concentração de empacotamento do microrganismo no substrato (Perwez; Asheh, 2025). Partículas menores oferecem maior relação superfície-volume, proporcionando geometria definida e ampla área para interação microbiana. Contudo, um tamanho de partícula muito pequeno pode levar à aglomeração das partículas, interferindo na respiração microbiana e, conseqüentemente, resultando em um crescimento microbiano insatisfatório. Por outro lado, partículas muito grandes resultam em superfície de contato insuficiente para a ação microbiana (Srivastava et al., 2019).

Após a inoculação do substrato, os microrganismos produtores de enzimas se desenvolvem na superfície e realizam a hidrólise enzimática dos substratos poliméricos primários, tais como polissacarídeos e proteínas. A variedade de técnicas de pré-tratamento é fundamental para superar os obstáculos que afetam a digestibilidade da biomassa, principalmente devido à estrutura compacta formada por celulose, hemicelulose e lignina (Postigo et al., 2021; Kukreti; Kumar; Kataria, 2024).

2.4.4 pH

O pH influencia diretamente a velocidade de crescimento dos microrganismos na FES. As principais alterações no pH ocorrem devido à produção de ácidos orgânicos, como ácido cítrico, láctico e acético, que reduzem o pH, enquanto a assimilação desses ácidos pelo metabolismo microbiano causa aumento do pH, e a hidrólise da ureia leva à alcalinização do meio (Perwez; Asheh, 2025). No entanto, na FES, o controle externo do pH torna-se inviável devido às características físicas do substrato sólido (Srivastava et al., 2019).

Diferentes microrganismos apresentam respostas distintas às variações de pH durante a fermentação. As bactérias desenvolvem-se principalmente em pH neutro; os fungos filamentosos em ampla faixa (pH 2–9), com ótimo entre 3,8–6; e as leveduras em pH 2,5–8,5, com ótimo entre 4–5 (Kumar et al., 2020; Perwez; Asheh, 2025). Quanto ao efeito do pH, *Penicillium roqueforti* é capaz de crescer em uma faixa de 3,0 a 10,5, com pH ótimo em torno de 6,0 (Coton et al., 2020). Assim, a contaminação bacteriana em cultivos de fungos filamentosos e leveduras pode ser reduzida ajustando-se o pH a um valor que não favoreça o crescimento bacteriano (Kumar et al., 2020; Perwez; Asheh, 2025). Entretanto, a prevenção da contaminação por leveduras em cultivos de fungos filamentosos não é garantida apenas pelo controle do pH, visto que ambos apresentam faixas de tolerância semelhantes.

2.4.5 Temperatura

A temperatura é um dos principais fatores que influenciam o bioprocessamento em estado sólido. Seus efeitos podem variar desde a modulação do crescimento microbiano e da atividade enzimática até a supressão da produção de metabólitos específicos ou mesmo a morte celular. Cada microrganismo pode ser categorizado com base na faixa de temperaturas em que pode se desenvolver, além da temperatura ótima de crescimento (López-Gómez; Manan; Webb, 2020). Os fungos filamentosos apresentam capacidade de desenvolvimento em uma ampla faixa, de 20 a 55 °C, sendo que a temperatura ótima para a produção do metabólito pode variar (Chilakamarry et al., 2022). No caso de *Penicillium roqueforti*, o crescimento ótimo ocorre a 25 °C (Coton et al., 2020).

2.4.6 Umidade

Em FES, a umidade exerce papel determinante no processo fermentativo. Níveis muito baixos interrompem o crescimento microbiano e a interação com o substrato, enquanto níveis muito elevados causam aglomeração de partículas e prejudicam a transferência de gases. Com base na cinética do crescimento microbiano, deve-se estabelecer um nível ideal de umidade

para o aprimoramento do processo fermentativo (Srivastava et al., 2019). A FES apresenta níveis de umidade que variam de 30 a 85%. Uma matriz sólida deve possuir, no mínimo, 70% de umidade para bactérias e de 20 a 70% para fungos filamentosos (Chilakamarry et al., 2022).

2.5 Proteases: características e classificações

As enzimas proteolíticas, também denominadas peptidases ou proteases, são catalisadoras de proteínas responsáveis pela hidrólise de uma ligação peptídica (amida) em substratos peptídicos e proteicos. Essas enzimas pertencem ao grupo 3 das hidrolases, subgrupo 4, responsáveis pela hidrólise de ligações peptídicas. Podem ser categorizadas com base na origem do isolamento (animal, vegetal ou microbiana), na posição da clivagem peptídica (endopeptídica ou exopeptídica), na estrutura do sítio ativo, nas propriedades de carga, no tamanho molecular, além da especificidade do substrato (Aguilar; Sato, 2018; Ćwilichowska et al., 2022).

Com base no local de clivagem na cadeia polipeptídica, as proteases podem ser classificadas em exopeptidases e endopeptidases. As exopeptidases clivam as ligações peptídicas no final de uma cadeia de aminoácidos, dividindo-se em aminopeptidases (que se ligam ao terminal N) e carboxipeptidases (que se ligam à extremidade C). Por outro lado, as endopeptidases são responsáveis por quebrar as ligações peptídicas dentro da cadeia de aminoácidos (Shaikh et al., 2023).

Além do critério de localização da clivagem, as proteases podem ser categorizadas de acordo com a estrutura do seu sítio catalítico, sendo estas divididas em aspárticas, cisteínas, glutâmicas, serinas, treoninas, metalopeptidases, peptidases liases de asparagina e peptidases de tipo catalítico misto (Figura 1) (Petushkova; Savvateeva; Zamyatnin, 2022).

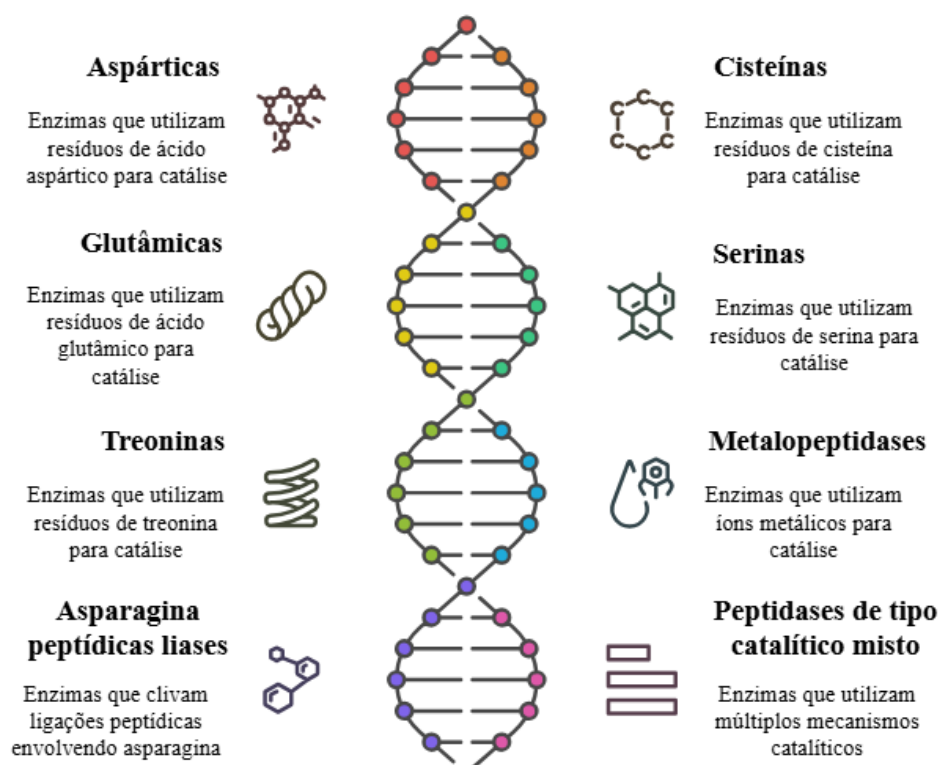


Figura 1: Classificação das proteases em função do sítio ativo

Outra classificação relevante das proteases é estabelecida conforme seu pH ideal de atuação, sendo agrupadas em ácidas, neutras e alcalinas. As proteases ácidas funcionam bem em valores de pH baixos na hidrólise de ligações que envolvem cadeias laterais volumosas de aminoácidos aromáticos. As proteases neutras atuam na faixa de pH de 5 a 8, possuem alta afinidade por aminoácidos hidrofóbicos, e apresentam baixa tolerância ao calor. Por outro lado, as proteases alcalinas operam em valores de pH entre 7 e 11, sendo capazes de hidrolisar ligações que contêm tirosina, fenilalanina, leucina, aspartato, histidina e serina. (Aguilar; Sato, 2018; Spandana; Bhagya, 2025).

Para aplicações industriais, a escolha da protease deve ser criteriosa, uma vez que finalidades distintas exigem enzimas específicas. Assim, a alta especificidade, a estabilidade e as condições econômicas são os principais fatores que influenciam a escolha de proteases comerciais para uma aplicação industrial específica (Sujitha; Shanthi, 2023).

2.6 Proteases microbianas: fontes e propriedades

As proteases microbianas representam uma parcela significativa do mercado mundial de enzimas, correspondendo a cerca de 60% do total comercializado. Microrganismos, especialmente bactérias, destacam-se como fontes ideais para produção em larga escala devido a características como crescimento rápido, elevada diversidade bioquímica, facilidade de

manipulação genética, processos produtivos eficientes e condições de cultivo acessíveis (Neog; Saini; Konwar, 2024).

O cultivo e a manutenção de microrganismos apresentam vantagens relevantes em relação a sistemas baseados em plantas e animais, demandando menor tempo e menor complexidade nos procedimentos. Além disso, em comparação com outras fontes enzimáticas, os microrganismos oferecem benefícios significativos na produção de proteases, destacando-se tanto pelo maior rendimento nos processos de purificação quanto pela diversidade de características bioquímicas e catalíticas (Gomes et al., 2020).

A seleção de microrganismos para produção de proteases oferece algumas vantagens, tais como a possibilidade de otimizar parâmetros para maximizar a produção, a capacidade de cultivar grandes volumes em um curto intervalo de tempo e a aplicação de engenharia genética para potencializar o rendimento (Mankge et al., 2024). Diversos microrganismos, com destaque para fungos filamentosos, além de bactérias e leveduras, são fontes potenciais para o isolamento de proteases (Ashraf et al., 2023). Nesse contexto, destaca-se que fungos filamentosos possuem elevada capacidade de secretar proteases de forma eficiente e funcionalmente ativa, sendo gêneros como *Trichoderma* e *Aspergillus* amplamente empregados na produção industrial de proteases. Outras fontes fúngicas de interesse incluem espécies dos gêneros *Mucor*, *Penicillium* e *Aspergillus clavatus*, além de fungos comestíveis como *Pleurotus citrinopileatus*, reconhecido como fonte relevante de protease alcalina (Gurumallesh et al., 2019).

Na indústria, as proteases ácidas são principalmente derivadas de fungos, enquanto as proteases neutras e alcalinas são frequentemente derivadas de gêneros bacterianos como *Bacillus*, *Halobacillus* e *Pseudomonas* (Majithiya; Ghoghari; Gohel, 2025). Esses microrganismos sintetizam diversas proteases, que podem apresentar localização extracelular ou intracelular. As proteases intracelulares atuam essencialmente na regulação de processos metabólicos e celulares, enquanto as extracelulares exercem função crucial na hidrólise de proteínas no meio externo, facilitando a absorção e utilização dos produtos hidrolíticos pelas células (Limkar; Pawar; Rathod, 2019). Na Tabela 2, podemos destacar exemplos de proteases de origem microbiana.

Tabela 2. Microrganismos produtores de proteases e suas aplicações industriais

Microrganismo	Classe	Tipo de protease	Principais aplicações	Referências
<i>Vagococcus fluvialis</i> FL1	Bactéria	Protease alcalina	Indústria de detergente	Ajayi e Lateef (2025)

<i>Bacillus safensis</i> PRN	Bactéria	Protease alcalina	Indústrias de detergente e couro	Neog, Yadav e Konwar (2023)
<i>Alkalihalobacillus clausii</i>	Bactéria	Protease alcalina	Mercado de ração animal	Fu <i>et al.</i> (2025)
<i>Aspergillus niger</i>	Fungo filamentoso	Protease alcalina	Recuperação de prata	Wahab e Ahmed (2018)
<i>Aspergillus oryzae</i>	Fungo filamentoso	Protease aspártica	Indústria alimentícia	Murthy e Kusumoto (2015)
<i>Penicillium brevicompactum</i>	Fungo filamentoso	Protease aspártica	Indústria de laticínios	Ozturkoglu-Budak <i>et al.</i> (2016)
<i>Rhizopus oryzae</i>	Fungo filamentoso	Protease aspártica	Indústria alimentícia	Hsiao <i>et al.</i> (2014)
<i>Bacilo proteolyticus</i>	Bactéria	Protease alcalina	Atividade antibacteriana	Bhaskar <i>et al.</i> (2007)
<i>Aspergillus oryzae</i>	Fungo filamentoso	Protease aspártica	Indústria alimentícia	Murthy <i>et al.</i> (2020)
<i>Aspergillus oryzae</i>	Fungo filamentoso	Protease aspártica	Indústria alimentícia	Murthy <i>et al.</i> (2019)

No âmbito das aplicações industriais, as proteases microbianas apresentam ampla utilização em diferentes setores, evidenciando sua relevância biotecnológica (Song et al., 2023). Nesse cenário, as proteases bacterianas constituem um dos principais grupos de proteases microbianas empregadas industrialmente, sobretudo em processos que requerem elevada atividade catalítica em condições alcalinas. Essas enzimas, naturalmente alcalinas, são amplamente utilizadas nos setores de couro, alimentos, lavanderia, seda e na indústria de detergentes, em razão de sua eficiência produtiva e estabilidade em pH alcalino (8-12) e em faixas de temperatura entre 50 e 70 °C (Naveed et al., 2020). A maior parte das proteases alcalinas microbianas comercializadas é isolada do gênero *Bacillus* spp., sendo uma parcela significativa direcionada à formulação de detergentes (Mankge et al., 2024).

De modo semelhante, as proteases fúngicas também ocupam posição de destaque em diferentes segmentos, especialmente nos setores alimentício, de bebidas, farmacêutico e biotecnológico (Patyshakuliyeva, 2021). Essa ampla utilização está associada a benefícios técnicos, como a capacidade de produção direta e eficiente de enzimas extracelulares, ampla especificidade de substrato, estabilidade sob diferentes condições operacionais, diversidade enzimática e facilidade de separação do micélio por filtração simples (Ashraf et al., 2023; Nouri; Sadeghi; Marefat, 2024). Para essas proteases, a faixa de pH pode variar de 4 a 11, enquanto a temperatura ótima situa-se entre 30 e 70 °C (Egbune et al., 2023).

Dentre os fungos produtores de proteases, incluem-se diversas espécies do gênero *Aspergillus*, como *A. niger*, *A. clavatus* ES1, *A. flavus*, *A. fumigatus*, *A. melleus*, *A. nidulans* HA-10, *A. sojae*, *A. terreus* e *A. oryzae*, sendo estas as cepas fúngicas mais conhecidas e amplamente utilizadas para a produção de proteases. No entanto, outros microrganismos, como *Myxococcus*, *Neurospora*, *Penicillium*, *Ophiostoma* e *Rhizopus*, também têm demonstrado capacidade relevante para a produção dessas enzimas, conforme indicado em diferentes estudos (Ashraf *et al.*, 2023). Dentre estas novas espécies com potencial relevante para produção de protease, aquelas pertencentes ao gênero *Penicillium* (*P. restrictum*, *P. citrinum*, *P. roqueforti*, *P. camemberti* e *P. griseoroseum*), que vêm ganhando destaque e sendo cada vez mais estudadas (Akram; Asgher; Qamar, 2023).

Diante desse panorama, a produção de proteases fúngicas tem sido amplamente investigada tanto por fermentação submersa (FSM) quanto por fermentação em estado sólido (FES), empregando principalmente gêneros como *Aspergillus*, *Penicillium*, *Rhizopus* e *Mucor*, além de fungos termofílicos, como *Thermoascus* e *Thermomyces*.

2.7 Aplicações industriais das proteases microbianas

Atualmente, as enzimas microbianas desempenham um papel fundamental no desenvolvimento de bioprocessos industriais, apresentando um mercado global predominante e mais de 500 produtos fabricados com sua utilização. A demanda por essas enzimas cresce continuamente, impulsionada pela busca por soluções biotecnológicas sustentáveis em diversos setores industriais (Liu; Kokare, 2023). As proteases, como a principal classe de enzimas hidrolíticas, são amplamente empregadas nos setores de couro, têxtil, agroalimentar, farmacêutico, médico e biotecnológico (Anand *et al.*, 2025).

Entre as proteases, as de origem fúngica se destacam por sua ampla aplicação biotecnológica, especialmente na indústria alimentícia, onde são usadas na fabricação de cerveja, processamento de cereais e panificação, produção de queijos e elaboração de hidrolisados proteicos (Tabela 3) (Patyshakuliyeva, 2021).

Tabela 3. Aplicações das proteases produzidas fungos filamentosos na indústria de alimentos.

Setor industrial	Função da protease	Fonte ou tipo de protease	Benefícios principais
Cervejaria	Hidrolisar proteínas do malte e cevada; melhorar disponibilidade de nitrogênio	Fúngica (geral)	Otimiza fermentação; favorece produção de cerveja sem glúten
Processamento de cereais	Melhorar propriedades reológicas de farinhas sem glúten; modificar o glúten	<i>Aspergillus oryzae</i> , outras fúngicas	Aumenta viscosidade, elasticidade e volume do pão; reduz toxicidade do glúten
Indústria de queijos	Coagulação do leite; amadurecimento; redução da alergenidade	Quimosina (aspártica); microbiana	Formação ideal do coágulo; acelera maturação; reduz proteínas alergênicas do leite
Indústria de panificação	Modificar força do glúten; facilitar manipulação da massa; melhorar características sensoriais	Fúngicas (diversas)	Aumenta volume do pão; melhora maciez, elasticidade, cor e sabor; reduz tempo de processamento
Produção de hidrolisados	Hidrolisar proteínas em peptídeos bioativos com funções funcionais e nutricionais	<i>Aspergillus</i> , <i>Penicillium</i> , <i>Rhizopus</i> ,	Geração de compostos com atividades antioxidante, antimicrobiana, anti-hipertensiva, entre outras

Na fabricação de cerveja, as proteases são essenciais na etapa inicial, facilitando a extração de proteínas da cevada e do malte e regulando a disponibilidade de compostos nitrogenados para o mosto, promovendo um balanço nutricional ideal para a fermentação (Naveed et al., 2020). Durante a mosturação, essas enzimas hidrolisam proteínas de cadeia longa, promovendo a liberação de aminoácidos e peptídeos necessários para a fermentação eficiente (Philipps-Wiemann, 2018). Além disso, as proteases têm mostrado potencial na produção de cervejas sem glúten, reduzindo o teor de glúten no processo (Shetty et al., 2017). No contexto da clarificação de bebidas, proteases aspárticas têm sido propostas como alternativa à utilização de bentonita na remoção de proteínas responsáveis por turbidez e opacidade em sucos de frutas (Guo et al., 2019). Esse efeito está associado à modificação de complexos proteína-polifenol envolvidos na formação de turbidez, por meio da catálise enzimática promovida pelas proteases (Cerreti et al., 2017).

No processamento de cereais, as proteases atuam como aditivos enzimáticos para melhorar as propriedades das farinhas, como a farinha de arroz utilizada em pães sem glúten. O tratamento com proteases promove melhorias na viscosidade, elasticidade e volume da massa, suprimindo limitações naturais das proteínas do arroz na retenção de gases e estruturação da massa, tradicionalmente compensadas por hidrocolóides (Renzetti; Arendt, 2009). Na indústria panificadora, essas enzimas desempenham papel fundamental na modificação estrutural do glúten por meio de hidrólise controlada, responsável pela viscoelasticidade da massa. Essa ação promove a quebra de cadeias peptídicas e a liberação de aminoácidos livres, resultando em melhor homogeneidade, aumento de volume e maciez dos pães, além da redução do tempo de mistura e de manipulação da massa e da minimização do encolhimento dos produtos. Além disso, o uso de proteases pode contribuir para a redução da toxicidade do glúten para indivíduos com sensibilidades, bem como para a melhoria de características sensoriais, como cor e sabor (Aguilar; Sato, 2018; Philipps-Wiemann, 2018; Gu et al., 2022; Dubey et al., 2024).

Na indústria de laticínios, as proteases têm papel crucial na fabricação de queijos, principalmente a quimosina, uma protease aspártica que hidrolisa especificamente a κ -caseína para formar coágulos ideais, sem comprometer as características sensoriais (Sujitha; Shanthi, 2023). Contudo, a substituição crescente da quimosina por proteases microbianas tem sido observada devido a vantagens comerciais e tecnológicas (Aguilar; Sato, 2018). Além disso, diferentes proteases aceleram o amadurecimento dos queijos e modificam as propriedades das proteínas do leite, reduzindo alergênicos para bebês, principalmente por hidrolisar proteínas como a α s1-caseína e as proteínas do soro (Patyshakuliyeva, 2021; Liu; Kokare, 2023). O uso

de extratos proteolíticos também contribui para o desenvolvimento de compostos responsáveis pelo aroma e sabor dos queijos, como os ácidos graxos voláteis, além de promover maior extensão da proteólise das proteínas do leite durante o processo de maturação (Dubey et al., 2024).

Por fim, também são utilizadas na elaboração de hidrolisados proteicos. As proteases são fundamentais para a hidrólise enzimática de proteínas em peptídeos e aminoácidos, produzindo compostos com alto valor nutricional e funcional. Esses hidrolisados, derivados tanto de proteínas animais quanto vegetais, são usados como ingredientes funcionais e realçadores de sabor em alimentos processados, como sopas, molhos e refeições prontas (Patyshakuliyeva, 2021; Ketemepi et al., 2024). Os peptídeos bioativos gerados apresentam diversas atividades benéficas à saúde humana, como propriedades anti-hipertensivas, antioxidantes e imunomoduladoras (Mohanty et al., 2016; Ahangari et al., 2021; Althnaibat et al., 2024). A especificidade das proteases e as condições do processo controlam as características funcionais desses hidrolisados, o que destaca a importância da seleção adequada da enzima e do controle dos parâmetros de hidrólise (Aguilar; Sato, 2018; Philipps-Wiemann, 2018).

Diante da versatilidade de aplicações das proteases nos diversos segmentos industriais, torna-se evidente a necessidade de estudos que busquem fontes alternativas e sustentáveis para sua produção. Nesse contexto, a utilização de resíduos agroindustriais como substratos em processos fermentativos, especialmente por fermentação em estado sólido (FES), representa uma estratégia promissora para a redução de custos de produção e para a valorização de subprodutos abundantes. Além disso, a otimização dos parâmetros fermentativos é essencial para o aumento dos rendimentos enzimáticos e para a viabilização de sua aplicação em escala industrial. Assim, investigações voltadas à produção, caracterização e aplicação de proteases microbianas, como as obtidas a partir de *Penicillium roqueforti*, apresentam elevado apelo biotecnológico, contribuindo para o avanço de processos sustentáveis e para o desenvolvimento de produtos com maior valor agregado.

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3. OBJETIVOS

3.1 Objetivo geral

Avaliar o potencial biotecnológico da protease aspártica de *Penicillium roqueforti* produzida por fermentação em estado sólido, desde sua otimização e caracterização bioquímica até sua aplicação na coagulação de diferentes leites e na geração de peptídeos bioativos.

3.2 Objetivos específicos

- Produzir e otimizar proteases aspárticas por *Penicillium roqueforti* utilizando substratos alternativos, avaliando as condições do processo fermentativo em função da atividade proteolítica.
- Caracterizar bioquimicamente o extrato enzimático, avaliando a influência de temperatura, pH e sais (NaCl e CaCl₂).
- Determinar os parâmetros cinéticos da protease (K_m e V_{max}) utilizando caseína como substrato.
- Estimar o peso molecular da protease por cromatografia líquida de alta eficiência (HPLC).
- Avaliar a eficiência da protease na hidrólise enzimática de caseínas bovina, caprina e bubalina em diferentes tempos de reação.
- Determinar o grau de hidrólise das diferentes caseínas ao longo do tempo e caracterizar o perfil peptídico dos hidrolisados obtidos por HPLC.
- Avaliar a atividade antioxidante *in vitro* dos hidrolisados por diferentes métodos e investigar o potencial inibitório dos hidrolisados sobre a enzima α -amilase.
- Avaliar a viabilidade da substituição parcial da quimosina pela protease fúngica na produção de queijo Minas Frescal de cabra e seu efeito sobre o rendimento tecnológico.
- Analisar o impacto da substituição enzimática sobre a composição físico-química centesimal e sobre as propriedades instrumentais de cor (CIE Lab*) dos queijos.
- Caracterizar o efeito da substituição do coagulante nas propriedades texturais (TPA) e no comportamento reológico (módulos viscoelásticos) da matriz láctea.
- Investigar as alterações nas interações intermoleculares por espectroscopia no infravermelho médio e próximo (MIR e NIR), visando identificar os níveis de substituição que preservem o desempenho tecnológico do queijo.

CHAPTER II

PROTEASE PRODUCTION BY *PENICILLIUM ROQUEFORTI* THROUGH SOLID-STATE FERMENTATION OF ORA-PRO-NÓBIS (*PERESKIA ACULEATA*): OPTIMIZATION AND CHARACTERIZATION

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Protease production by *Penicillium roqueforti* through solid-state fermentation of ora-pro-nóbis (*Pereskia aculeata*): optimization and characterization

ABSTRACT

The demand for sustainable microbial enzymes has increased due to the need for efficient, low-cost alternatives in the food industry. Proteases are essential in cheesemaking and bioactive peptide production. This study aimed to produce and characterize protease from *Penicillium roqueforti* ATCC 10110 via solid-state fermentation (SSF) using alternative substrates and to optimize fermentation yield. Ora-pro-nóbis, mandacaru, and soybean meal were combined, and simplex-centroid and Box–Behnken designs evaluated the impact of substrate proportions, temperature, moisture, and fermentation time on protease production. Best conditions were 144 h, 25 °C, and 70% moisture, with a 1:1 mixture of ora-pro-nóbis and soybean meal yielding the highest activity (4.29 U/mL). The extract showed milk coagulation activity (482 ± 1.52 U/mL), high MCA/PA ratio (116.11), optimal activity at pH 6.0 and 60 °C, thermal stability, Ca^{2+} activation, and a molecular mass of 23 kDa. These results highlight SSF as a sustainable strategy for protease production.

Keywords: Ora-pro-nóbis; microbial protease; milk coagulation; proteolytic activity

1. INTRODUCTION

Proteases, also known as peptidases, are essential enzymes that catalyze the cleavage of peptide bonds in proteins, resulting in the formation of smaller peptides and free amino acids. They account for approximately 60% of the global enzyme market, standing out as one of the most relevant groups in the industrial biotechnology sector, with a wide distribution in nature (Sisa et al., 2023). The increasing demand for industrial enzymes has driven efforts to discover, redesign, or synthesize biocatalysts with improved properties. These enzymes represent sustainable, environmentally safe, and commercially viable alternatives for applications across different sectors (Solanki et al., 2021). Although they can be obtained from animal and plant sources, microorganisms emerge as the main producers of proteases due to their economic and technical advantages, as well as the possibility of genetic manipulation (Dos Santos Aguilar and Sato, 2018).

Among enzyme-producing microorganisms, filamentous fungi play a prominent role. *Penicillium roqueforti* is a classic example, being ecologically versatile and capable of colonizing diverse environmental niches, with populations exhibiting traits of adaptive differentiation. This species is widely recognized for its fundamental technological role in the production of blue cheeses (Crequer et al., 2024). During ripening, *P. roqueforti* strains display high enzymatic capacity, producing distinctive aromatic compounds or their precursors. In terms of proteolytic activity, the fungus secretes multiple proteases involved in casein hydrolysis, releasing bioactive peptides and free amino acids, highlighting its technological potential in the food industry (Gillot et al., 2017). This characteristic makes *P. roqueforti* a promising candidate for enzyme production in solid-state fermentation (SSF).

SSF has been established as an efficient and sustainable strategy for protease production, especially when combined with the use of agro-industrial byproducts as substrates. This approach contributes to cost reduction, valorization of organic waste, and the promotion of a circular economy, aligning with the United Nations Sustainable Development Goals (SDGs), particularly SDGs 9, 12, and 13 (Colla et al., 2023). Moreover, the efficiency of enzyme production via SSF depends on several variables, including substrate selection, fermentation conditions, and the characteristics of the microorganism employed (Sosa-Martínez et al., 2023). To optimize these processes, statistical techniques such as mixture experimental design and response surface methodology have been widely applied, as they allow the systematic evaluation of process variables and their interactions, as well as the development of predictive models, enabling maximization of production while reducing time, energy, and reagent consumption (Muñoz-Seijas et al., 2024; Hadrich, 2025).

In the present study, substrates were selected based on their nutritional potential and regional availability. Soybean meal, the main byproduct of soybean oil extraction, has a balanced nutritional profile, containing 46% protein, 0.5% residual lipids, 7% crude fiber, 6% ash, and 12% moisture (López-Trujillo et al., 2023). As alternative sources, *ora-pro-nóbis* (*Pereskia aculeata* Miller), native to South America and rich in proteins, minerals (iron and calcium), β -carotene, and vitamins (Cruz et al., 2021), and mandacaru (*Cereus jamacaru* DC), typical of the Brazilian semi-arid region and high in carbohydrates, amino acids, and dietary fibers (de Vasconcelos Medeiros et al. 2024), were chosen. The use of these substrates aims to enhance the enzymatic production of *P. roqueforti* in SSF while promoting sustainable and cost-effective solutions.

In the food industry, proteases perform a variety of functions, including meat tenderization, milk coagulation, and improvement of protein functional properties, such as emulsification and solubility (Arya et al., 2022). Specifically in the dairy sector, acidic proteases act as milk-coagulating agents, catalyzing the hydrolysis of specific bonds in casein (Phe105-Met106) and resulting in the formation of κ -casein and macropeptides (Azadi et al., 2022).

In this context, the present study aimed to optimize protease production by *Penicillium roqueforti* ATCC 10110 in SSF using alternative agro-industrial substrates, as well as to characterize its biochemical properties, including stability, enzymatic activity, and milk-clotting potential. The results are expected not only to expand technical and scientific knowledge but also to provide innovative and sustainable solutions for the food industry, contributing to a more resilient and environmentally responsible future.

2. METHODOLOGY

2.1. Materials

This study used bovine casein, tyrosine, and Coomassie brilliant blue, purchased from Sigma Aldrich Brazil. Other reagents will be described throughout the methodology. Soybean meal, ora-pro-nóbis (*Pereskia aculeata* Miller) leaves, and mandacaru (*Cereus jamacaru* DC) stems, purchased from local retailers and from the rural area of Itapetinga, Bahia, Brazil, respectively, were used for fermentation.

2.2 Substrate Characterization

The chemical composition of the substrates was characterized according to the methods described by the AOAC (2000), including: moisture content (method 926.08), total nitrogen by the Kjeldahl method (2001.14), ash (method 935.42), and fat in dry matter (GES) (method 920.125).

2.3 Inoculum preparation for Solid-State Fermentation (SSF)

The filamentous fungus used in this study was *Penicillium roqueforti*, donated by the Oswaldo Cruz Foundation (FIOCRUZ, Rio de Janeiro, RJ, Brazil), with lot number 079840075. The fungus was reactivated in Potato Dextrose Agar and Agar-Agar culture media in an Erlenmeyer flask (500 mL) for 7 days in a bacteriological incubator (SL 101, SOLAB, São Paulo, Brazil) at 25°C. After the incubation period, the spores were recovered in sterile saline using glass beads to scrape the mycelium. All material was previously sterilized in a vertical autoclave at 121°C for 15 min. From the collected suspension, a 0.1 mL aliquot was diluted in a test tube for spore counting, which was performed in a Neubauer chamber and observed under a binocular microscope (L1000; Bioval, São Paulo, Brazil). The spore concentration used was 10⁷ spores/gram of substrate.

2.3.1 Substrate preparation

The ora-pro-nóbis leaves and mandacaru cladodes were washed and sanitized, cut into smaller pieces, and then dried in an oven with air circulation at 65°C for 24 hours. After drying, the materials were ground in an industrial blender until they reached an average particle size of approximately 2 mm. The soybean meal was used directly, as it already had low moisture content and a suitable particle size for the process.

2.3.2 Solid-State Fermentation

Fermentations were carried out in 125 mL Erlenmeyer flasks used as bioreactors. Each flask contained 5 g of substrate previously sterilized in an autoclave at 121°C for 15 min. After sterilization, the material was cooled in a biological safety cabinet under ultraviolet light (Filterlux Vertical Laminar Flow, São Paulo, Brazil). Each flask was then inoculated with a spore suspension at a concentration of 10 spores per gram of substrate, along with sufficient saline solution to adjust the moisture. Fermentations were conducted in a bacteriological incubator at 25°C (SL 101, SOLAB, São Paulo, Brazil).

2.4 Simplex-Centroid mixture design for protease production

To determine the better proportions of substrates (soybean meal, ora-pro-nóbis, and mandacaru) for optimum protease synthesis, a mixture experimental design was applied. The design was based on valid predictive models to estimate the response variable, consisting of six substrate combinations and three replicates of the central point. The design was applied to fermentation at 72, 144, and 216 h of fermentation at 30 °C and 70% moisture. All statistical analyses were performed using Statistica 12 software (StatSoft, Tulsa, OK, USA).

2.5 Optimization of protease production

A Box-Behnken (BBD) experimental design with three factors and three replicates at the central point was used to optimize protease production by SSF and evaluate the influence of variations in temperature (20-35°C), moisture (55-85%), and time (72-216 hour). The experimental matrix, composed of 15 experiments, including three replicates at the central point, allowed estimating experimental error and evaluating linear, quadratic, and interaction effects between variables. Based on the data obtained, a second-order polynomial mathematical model was adjusted. Analysis of variance (ANOVA) was used to assess the statistical significance of the model ($p < 0.05$). Standardized regression coefficients, evaluated by Student's t-test ($p < 0.05$), indicated the importance of each factor and interaction in the model. The coefficient of determination (R^2) and the adjusted coefficient of determination (adjusted R^2) were used to assess the goodness of fit of the model, while the lack-of-fit test verified the goodness of fit of the model to the data. All experiments were repeated in triplicate to assess the reproducibility of the results. Analyses were performed using Statistica software version 12 (STATSOFT, USA).

2.6 Obtaining the crude enzyme extract

After the respective fermentation time, 25 mL of autoclaved saline solution was added to each treatment to obtain the crude enzymatic extract. The suspensions were shaken in a shaker (SL 222, SOLAB, São Paulo, Brazil) at 25 °C, 160 rpm, for 30 min. Subsequently, the suspended solids were removed by mechanically pressing through gauze to separate the solids from the enzymatic extract. The filtrate was collected in a Falcon tube and centrifuged (CIENITEC 6000R, Fortaleza, Brazil) at 6000 rpm for 15 min. The supernatant was used to quantify the enzymatic activity.

2.6.1 Protein concentration determination

Protein concentration was determined according to the methodology proposed by Bradford (1976) at 595 nm, in a spectrophotometer (UV-Vis Quimis - Brazil), using bovine serum albumin as the standard for the calibration curve.

2.6.2 Determination of proteolytic activity

Enzymatic activity was determined according to a methodology adapted from Kunitz (1947), using casein as a substrate. The enzyme extract (0.150 mL) was added to a 1.0% casein solution (1.0 mL) in 100 mM potassium phosphate buffer, pH 6.0. The mixture was incubated at 37 °C in a thermostatic bath (Tecnal, model Te-184) for 10 min. A 10% trichloroacetic acid solution (1.0 mL) was used to stop the reaction, and the solution was centrifuged at $5000 \times g$ for 15 min. The amount of amino acid released was determined by measuring the absorbance of the supernatant at 280 nm, using tyrosine as a standard to construct the calibration curve.

One unit of enzyme activity (U) (Equation 1) was defined as the amount of enzyme required to produce 1.0 μmol of tyrosine in 1 mL of reaction medium per minute.

$$AP (U) = \text{Tyrosine} \left(\frac{\mu\text{mol}}{\text{mL}} \right) \times \left(\frac{\text{Dilution Factor}}{\text{Enzyme Volume}} \right) \times \left(\frac{\text{Total Extract Volume}}{\text{Incubation Time}} \right) \quad (1)$$

2.6.3 Determination of coagulating activity

Milk clotting activity (MCA) was determined in triplicate, according to Arima, Yu, and Iwasaki (1970), with modifications. Briefly, skim milk powder was dissolved in 10 mM CaCl_2 to a final concentration of 10%, and the pH was adjusted to 6.5. This substrate (1.0 mL) was preincubated for 5 min at 37°C, and 100 μL of the enzyme extract was added. The test tubes were rotated by hand until discrete visible particles appeared. Milk clotting activity (MCA) is

defined as the amount of protein that coagulated 10 mL of the substrate in 40 min at 37°C, using the formula:

$$\text{MCA (U/mL)} = (2400 / \text{clotting time (s)}) \times \text{dilution factor} \quad (2)$$

2.7 Biochemical Characterization

2.7.1 Effect of temperature on protease activity and stability

The optimum temperature for protease activity was determined by estimating activity at different temperatures ranging from 30 to 80°C. The assay was performed in 0.1 M potassium phosphate buffer (pH 6.0). The maximum activity observed under any of the reaction conditions was defined as 100%, and relative activities were calculated as a fraction of this value. To analyze thermostability, the enzyme extracts were incubated at 50, 60, and 70°C for 0 to 60 min for the indicated time intervals, and then the residual protease activity was analyzed.

2.7.2 Effect of pH on protease activity and stability

To determine the optimal pH and stability of the protease, enzyme activity was evaluated in different buffers (0.5 M sodium acetate, pH 5.0; 0.5 M sodium phosphate, pH 5.5, 6.0, and 6.5; 0.5 M Tris-HCl, pH 7.0 and 8.0) at 37°C. Stability was determined by incubating the enzyme extract in the different buffers for 60 min under the same conditions and measuring residual activity.

2.7.3 Effect of NaCl and CaCl₂ on enzymatic activity

The effect of NaCl and CaCl₂ on the enzymatic activity of casein was evaluated at pH 6.5 and 37 °C. Different concentrations of NaCl (100, 200, 400, 600, 800, and 1000 mmol) and CaCl₂ (10, 20, 40, 60, 80, and 100 mmol) were added to the reaction mixture. The highest activity obtained was taken as 100% and expressed as % residual activity.

2.7.4 Effect of temperature, NaCl, and CaCl₂ on coagulation activity (MCA)

The optimum temperature for coagulation activity was determined by evaluating activity at different temperatures, ranging from 30 to 80°C, in a thermostatic bath (Tecnal, model TE-184, São Paulo, Brazil). To investigate the effect of CaCl₂ and NaCl concentrations, different concentrations of NaCl (100, 200, 400, 600, 800, and 1000 mmol) and CaCl₂ (10, 20,

40, 60, 80, and 100 mmol) were tested, added to the substrate. Maximum coagulation activity (MCA) was measured at pH 6.5 and a temperature of 37°C.

2.7.5 Determination of kinetic parameters

The protease kinetic parameters were determined using Michaelis–Menten kinetics, using casein as a substrate at different concentrations (0.4 to 2.0 mg/mL) under standard assay conditions. The assays were performed at pH 6.0 in 100 mM potassium phosphate buffer and at a temperature of 37 °C, following the same conditions used for the determination of proteolytic activity. The kinetic rate constants, K_m and V_{max} , were determined using the Michaelis–Menten equation.

2.7.6 Molecular weight estimation by gel filtration chromatography

Molecular weight distributions were determined according to (Purushothaman et al., 2019) using a high-performance liquid chromatography (HPLC) system on an SRT-C SEC 150 Å size exclusion column (Delaware Technology Park, USA) (4.6 × 300 mm, 5 µm) coupled to a pre-column (ZORBAX SB-C 18, 4.6 mm inner diameter (ID) × 12.5 mm, 5 µm). Twenty microliters of the sample were injected and eluted at a flow rate of 0.4 mL/min at 30 °C. Proteins were separated by isocratic elution with 20 mmol sodium phosphate (pH 7.0). Protein detection was performed at 215 nm using a UV detector. Molecular weight was calculated by plotting the logarithmic molecular weight versus V_0 obtained from gel filtration chromatography using standards: bovine serum albumin (BSA) (66 kDa), lipase (29 kDa), pepsin (35 kDa), bromelain (31 kDa), and lysozyme (14.6 kDa).

2.8 Statistical analysis

Statistical analyses related to the experimental design and process optimization were carried out using Statistica software (version 12, StatSoft Inc., USA). The software was used for analysis of variance (ANOVA), estimation of model coefficients, evaluation of the statistical significance of linear, quadratic, and interaction effects, as well as assessment of model adequacy through determination coefficients and lack-of-fit tests.

The experiments were performed in triplicate, and the data obtained were subjected to analysis of variance (ANOVA). To compare means, Tukey's test was applied at a 5% significance level ($p < 0.05$) using Statistical Analysis System (SAS) software. Graphs were created using Origin 2018 64Bit software (OriginLab Corporation, Northampton, MA, USA).

3. RESULTS AND DISCUSSION

3.1 Characterization of the substrates

The proximate compositions of the agro-industrial by-products used as substrates in SSF for protease production are presented in Table 1.

Table 1. Proximate composition of agro-industrial by-products used as substrates in SSF*

Component	Ora-pro-nóbis	Mandacaru	Soybean meal
Protein (%)	17.92 ± 0.15 ^a	5.78 ± 0.54 ^b	47.18 ± 0.53 ^c
Moisture (%)	5.84 ± 0.10 ^a	8.44 ± 0.30 ^b	8.87 ± 0.07 ^b
Ash (%)	22.08 ± 0.04 ^a	18.22 ± 0.15 ^b	7.03 ± 0.06 ^c
Lipids (%)	2.94 ± 0.01 ^a	1.44 ± 0.17 ^b	0.76 ± 0.12 ^c
Carbohydrates* (%)	51.19 ± 0.04 ^a	66.10 ± 0.58 ^b	36.14 ± 0.58 ^c

Carbohydrates calculated by difference.

Mean values ± standard deviation. Means followed by the same letter in the same row do not differ significantly according to Tukey's test ($p < 0.05$).

The centesimal analysis revealed significant differences in the nutritional composition of the substrates evaluated, with emphasis on protein content, a key parameter for protease production in SSF. The results demonstrated that soybean meal had the highest protein content (47.18% ± 0.53), followed by ora-pro-nóbis (17.92% ± 0.15) and mandacaru (5.78% ± 0.54). This difference is particularly relevant since organic nitrogen sources offer a more complex nutritional profile compared to inorganic sources, providing not only nitrogen but also amino acids, peptides, vitamins, and various growth factors. This comprehensive nutritional composition creates more favorable conditions for fungal development, enhancing growth, reproduction, and metabolism (Cao et al., 2025). In the specific case of proteases, the inclusion of protein sources in the medium can stimulate microorganisms to secrete the enzyme.

In addition to protein content, other chemical properties of substrates can also influence protease production, such as the structure and availability of components, the types and proportions of organic materials, and nutrient content (Aljammal et al., 2022a).

When comparing these data with the literature, consistency in the obtained values is observed. Liu et al. (2024) reported 46.59% protein, 1.65% fat, 7.24% ash, and 3.26% moisture in soybean meal, values similar to those obtained in the present study. For *Pereskia aculeata* (ora-pro-nóbis), the protein and ash contents observed (17.92% and 22.08%, respectively) are also close to those reported by Mendes et al. (2024), who found 20.8 ± 0.2% protein and 14.0 ± 2.0% ash.

As for mandacaru, its low protein content (5.78%) suggests more limited potential as a source of nitrogen for protease production. However, its high carbohydrate concentration can act as a fundamental component in the fermentation process, contributing to cell construction, polysaccharide synthesis, and energy supply for metabolic activities (Oliveira et al., 2021).

Thus, the substrates evaluated have distinct nutritional characteristics that can influence fermentation performance and protease production. The high protein content in soybean meal and ora-pro-nobis can be used as a source of nitrogen for fungal growth during fermentation. On the other hand, mandacaru can act as a carbon source for the energy demand required by the fungus in its development. This characteristic suggests that mandacaru can play a complementary role when associated with high-protein substrates, supplying the energy demand of the fungus in the early stages of growth and enzyme synthesis. Therefore, the selection and strategic combination of substrates with different nutritional profiles are fundamental to optimizing protease production in SSF.

3.2 Synergistic and antagonistic effects of substrates on protease production

To evaluate the combined effects of ora-pro-nobis, mandacaru, and soybean meal substrates on protease production by *P. roqueforti*, a simplex-centroid experimental design was applied (Table 2). The variation in enzyme activity over time (72, 144, and 216 h) was represented by two-dimensional ternary contour graphs (Fig. 1), in which the vertices correspond to the isolated substrates, the midpoints to the binary combinations, and the centroid to the ternary mixtures.

Table 2. Simplex-centroid mixture design matrix for protease production by *P. roqueforti* under SSF and results for protease activities at 72, 144, and 216 h of fermentation

Experiment	Ora-pro-nobis (g)	Mandacaru (g)	Soybean Meal (g)	Enzyme Activity (U/mL)		
	A	B	C	72 h	144 h	216 h
1	5	0	0	1.54± 0.00	3.09± 0.16	2.24± 0.34
2	0	5	0	1.51± 0.13	1.42± 0.15	1.67± 0.15
3	0	0	5	1.53± 0.13	2.98± 0.51	2.97± 0.48
4	2.5	2.5	0	1.63± 0.16	1.77± 0.10	1.78± 0.10
5	2.5	0	2.5	1.75± 0.05	3.39± 0.08	3.37± 0.13
6	0	2.5	2.5	1.92± 0.09	1.83± 0.09	1.60± 0.21

7*	1.67	1.67	1.67	2.14± 0.10	2.48± 0.06	2.21± 0.14
8*	1.67	1.67	1.67	1.96± 0.08	2.13± 0.13	2.29± 0.05
9*	1.67	1.67	1.67	2.05± 0.01	2.19± 0.27	2.42± 0.26

* Central point

At 72 h of fermentation, a synergistic effect was observed in the three binary formulations (runs 4 to 6). The medium composed of ora-pro-nóbis (A) and mandacaru (B) (trial 4) showed increases of 5.73% for ora-pro-nóbis and 7.99% for mandacaru in protease production, compared to the individual substrates (runs 1 and 2, respectively). In the medium containing ora-pro-nóbis (A) and soybean meal (C) (trial 5), protease production increased by 13.42% for ora-pro-nóbis and 14.39% for soybean meal, compared to runs 1 to 3 using the A and C column. The formulation with mandacaru (B) and soybean meal (C) (trial 6) resulted in increases of 27.64% and 26.04%, respectively. The ternary mixtures (trials 7, 8, and 9) also exhibited a positive effect, with increases ranging from 39.07% to 42.04%.

The influence of the different formulations on protease enzymatic activity after 72, 144, and 216 h is illustrated by two-dimensional ternary contour plots (Fig. 1).

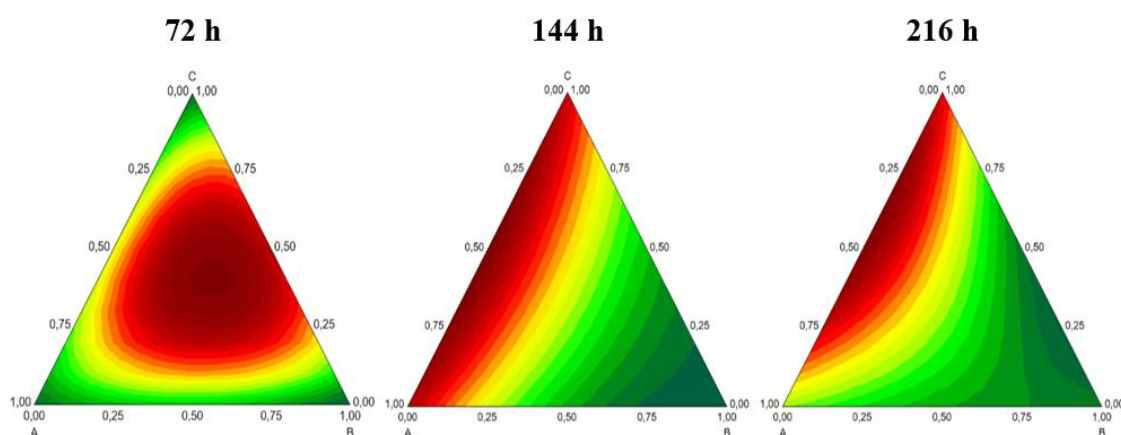


Fig. 1 - Mixture contour plots for the enzymatic activity of aspartic protease produced by SSF after 72, 144, and 216 h

After 144 and 216 h, most interactions began to show antagonistic behavior, except the binary formulation composed of ora-pro-nobis (A) and soybean meal (C) (trial 5), which maintained superior performance. In this treatment, increases of 9.97% for ora-pro-nobis and 14.07% for soybean meal were observed at 144 h, reaching peak enzymatic activity (3.40 U/mL). At 216 h, the increase reached 50.3% for ora-pro-nobis and 13.3% for soybean meal.

The performance of trial 5 suggests that the combination of the two substrates provided optimal physicochemical conditions for the growth of *P. roqueforti* and protease synthesis. In SSF, substrate composition is crucial for the synthesis of enzymes and microbial metabolites. For proteases, the presence of protein fractions in the medium is essential to induce and sustain enzymatic secretion (Colla et al., 2023). Soybean meal, with a high protein content (47.18%) and good availability of peptides and amino acids, acts as a potent enzymatic inducer, in addition to providing bioactive compounds such as flavonoids and phenolics (Huang et al., 2023). Ora-pro-nóbis, despite its lower protein content (17.92%), has a diverse nutritional profile, including fibers, vitamins, and minerals such as calcium, zinc, and iron, as well as antioxidant compounds (Silveira et al., 2020). The fibers present in ora-pro-nóbis play a fundamental structural role in SSF, promoting moisture retention and the formation of a porous matrix that enhances aeration and gas exchange, creating microenvironments that prolong the metabolic activity of the fungus (Ban et al., 2024).

Thus, the combination of the high nitrogen availability from soybean meal and the functional and structural components of ora-pro-nóbis likely created an optimized microenvironment, allowing the fungus to maintain a high growth rate and enzymatic secretion even during prolonged incubation periods. This observation is consistent with the literature, which emphasizes the importance of a balanced availability of carbon and nitrogen sources for microbial growth and the maintenance of metabolic activity. Substrates with higher nutritional value, particularly those rich in proteins and carbohydrates, promote both cellular adaptation and protease secretion in SSF (Londoño-Hernández et al., 2024). Therefore, the binary formulation of trial 5 was selected for the optimization stage.

For the statistical evaluation of the models, an analysis of variance (ANOVA) was performed, and the results are presented in Table 3.

Table 3. Analysis of variance of the regression models applied to enzyme activity at 72, 144, and 216 h

Time	Model	F-calculated	F-tabulated	p-value	R ²
72 h	Special Cubic	32.63	19.00	0.030	0.989
144 h	Quadratic	30.73	5.53	0.009	0.980
216 h	Quadratic	56.48	5.14	0.004	0.989

*Confidence level of 95% (p = 0.05)

The mathematical regression models for each fermentation time were fitted as follows, where the letters represent the mixture components: ora-pro-nóbis (A), mandacaru (B), and

soybean meal (C).

$$Y_{72h}: 1.54A + 1.51B + 1.53C + 0.42AB + 0.86AC + 1.64BC + 4.56ABC \quad (3)$$

$$Y_{144h}: 3.09A+1.43B+2.98C-0.98AB+1.44AC-1.52BC \quad (4)$$

$$Y_{216h}: 2.23A+1.66B+2.97C-0.52AB+3.22AC-2.72BC \quad (5)$$

Where A, B, and C are the coded levels of ora-pro-nóbis, mandacaru, and soybean meal amounts, respectively.

The coefficient of determination (R^2) and the F-test (analysis of variance - ANOVA) were used to evaluate the quality of the models. The high R^2 values indicate that the response functions fit the experimental data well. In addition, the models proved to be effective for predictive purposes in the production of protease by *P. roqueforti* during SSF, using different combinations of raw materials, especially after 144 hours of fermentation. At this point, the production reached its maximum value, and the predictive model showed a high coefficient of determination ($R^2 = 0.980$).

The p-value ($p < 0.05$) indicates that the effects of the independent variables are statistically significant. Thus, the models used proved effective in predicting protease production by *Penicillium roqueforti* in SSF.

3.3 Optimization of protease production using response surface methodology

In the present study, after selecting soybean meal and ora-pro-nobis as the most promising substrates, experiments were conducted to evaluate the effects of fermentation time (72, 144, and 216 h), initial moisture (55, 70, and 85%), and incubation temperature (20, 27.5, and 35 °C) on protease activity. For this purpose, a Box-Behnken experimental design was used, totaling 15 trials with different combinations of these variables. The experimental results obtained from the Box-Behnken design are presented in Table 4. Data analysis was performed using multiple regression, and the fitted quadratic model was evaluated by analysis of variance (ANOVA) (Table 5).

Table 4. Matrix for the Box-Behnken design for the independent variables fermentation time (h), initial moisture content (%) and incubation temperature (°C), and considering the dependent variable (response) as the protease enzymatic activity (U/mL) by *P. roqueforti*

Treatment	Time (h) X_1	Moisture (%) X_2	Temperature (°C) X_3	Experimental Response
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				(U/mL)
1	72	70	20	0.9407±0.03
2	216	70	20	1.9754±0.33
3	72	70	35	2.7437±0.09
4	216	70	35	2.8666±0.46
5	72	55	27.5	0.9889±0.18
6	216	55	27.5	2.0937±0.05
7	72	85	27.5	0.6372±0.03
8	216	85	27.5	1.2780±0.04
9	144	55	20	0.7836±0.04
10	144	55	35	2.1700±0.02
11	144	85	20	0.6454±0.03
12	144	85	35	1.9012±0.18
13	144	70	27.5	4.2918±0.31
14	144	70	27.5	3.8497±0.01
15	144	70	27.5	3.9425±0.53

The significance of the quadratic model was evaluated by ANOVA (Table 5). Terms with p-values ≥ 0.05 were considered not significant, while p-values < 0.05 indicated statistical significance. Fisher's F-test was used to assess the overall significance of the model, calculated as the ratio between the model mean square and the residual mean square. For the protease production model, the F and p-values were 53.50 and < 0.001 , respectively. The coefficient of determination (R^2) was 0.999, indicating that the model explains 99.9% of the variation in protease production. Furthermore, the non-significant lack-of-fit value ($p > 0.05$) suggests that the model is adequate to predict and explain variations in enzymatic activity.

Table 5. Analysis of variance for the quadratic model

Source of variation	SS	Df	MS	F-calculated	p-value	R^2
Regression	21.30	9	2.37	53.50	0.001	0.999
Lack of fit	0.11	3	0.04	0.70	0.634	
Pure error	0.11	2	0.05			
Total	21.52	14				

Abbreviations: Sum of squares (SS), degrees of freedom (Df), mean square (MS), coefficient of determination (R^2).

$$\text{Model: } -55.99 + 0.08X_1 + 1.11X_2 + 1.04X_3 - 0.001X_1X_3 - 0.001X_1X_2 - 0.001X_3X_2 - 0.01X_1^2 - 0.008X_2^2 - 0.016X_3^2 \quad (6)$$

Where X_1 , X_2 , and X_3 are the coded levels of time, moisture, and temperature, respectively.

The statistical significance of the linear, quadratic, and interaction (linear $\times$ linear) terms was illustrated using a Pareto chart (Fig. 2), which shows the magnitude and importance of each factor in the experimental response.

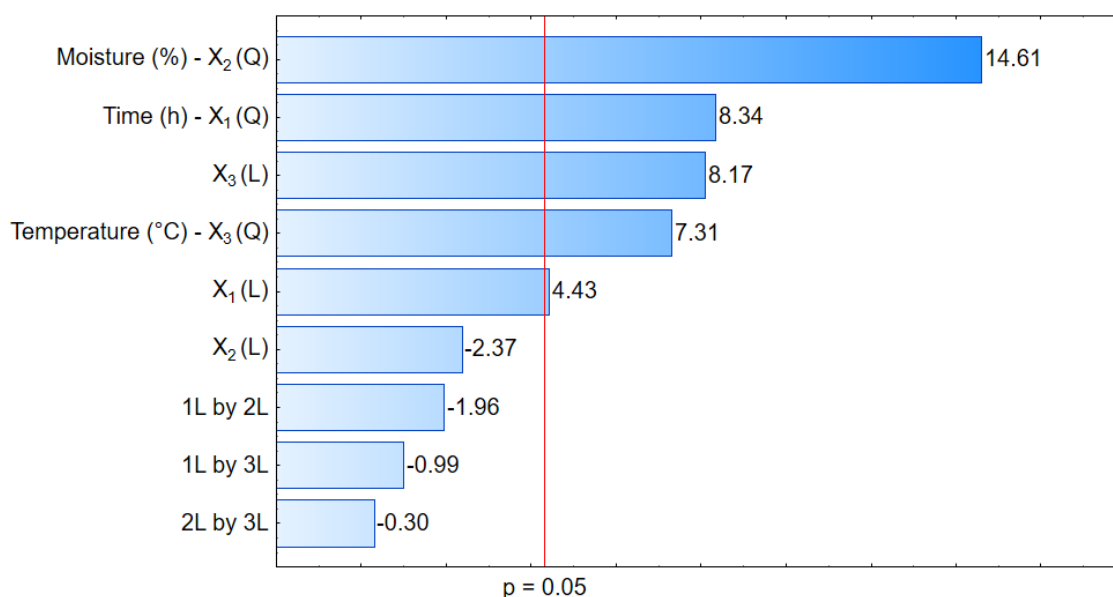


Fig. 2 - Standardized effects of the investigated factors (Pareto chart)

Considering the 95% confidence level, the interactions between time and temperature, time and moisture, and temperature and moisture were not statistically significant ($p > 0.05$), as was the linear effect of moisture. On the other hand, the quadratic terms and linear terms of time and temperature showed statistical significance ($p < 0.05$), indicating that the three variables are critical to the process. Among them, the quadratic effect of moisture stood out as the most significant in the model.

The results indicate that the adjusted model accurately describes the behavior of the experimental response, presenting high reliability ($R^2 = 0.999$). Thus, it can be used to optimize process conditions and maximize protease production based on the levels of the variables investigated.

3.3.1 Response surface plot

The response surface plot represents the optimal set of independent variables and serves as a useful tool to predict the relationship between the responses and the test variables.

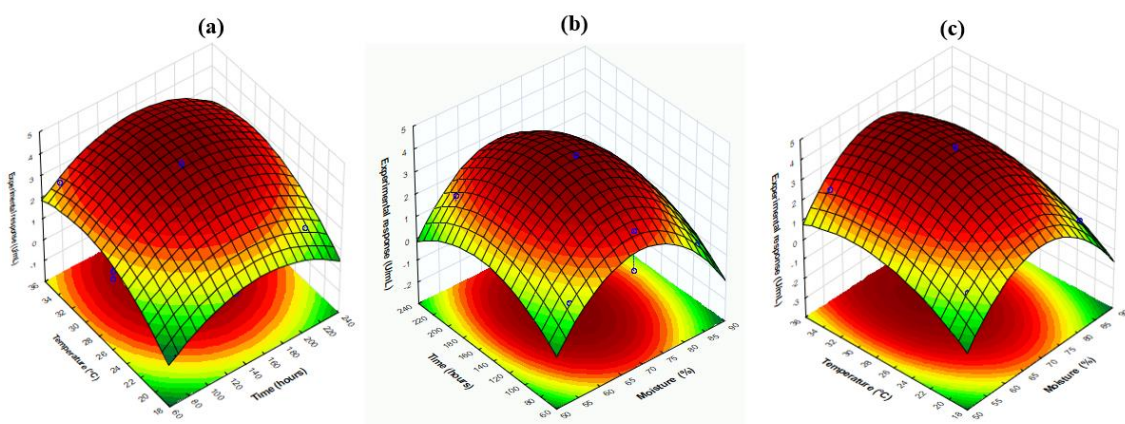


Fig. 3 - Response surface plots representing interactions between variables in relation to protease activity. (a) Interaction between incubation temperature (T , $^{\circ}\text{C}$) and fermentation time (t , h) (initial moisture kept constant at 70%). (b) Interaction between initial moisture (M , %) and fermentation time (t , h) (incubation temperature kept constant at 27.5°C). (c) Interaction between initial moisture (M , %) and incubation temperature (T , $^{\circ}\text{C}$) (fermentation time kept constant at 144 h)

In each response plot of Figure 3, the proportional effects of two independent parameters are illustrated, while the third variable is fixed at its central point level.

Regarding the temperature variable, an optimal region for protease production was observed around the central level (27.5°C), followed by a decrease in activity at higher temperature values. The culture medium temperature significantly influences microbial growth and enzyme production. Higher temperatures can alter the structure of the cell membrane and cause protein degradation, reducing enzymatic production. Conversely, lower temperatures slow microbial growth, increasing the time required for enzyme production (Baskaran and Krishnan, 2020).

Protease production by *Penicillium roqueforti* has been explored in recent studies. Nogueira et al. (2021) optimized the production of a thermostable trypsin-like protease via SSF using cocoa husk as a substrate, achieving the best performance at 25°C . Studies with other filamentous fungi reinforce that incubation temperature has a significant impact on protease production and stability. For example, Qasim et al. (2024) reported that *Mucor racemosus* showed the highest aspartic protease activity at 20°C after optimization of cultivation conditions. Similarly, Ugbede et al. (2023) observed that the protease produced by *Aspergillus flavus* reached maximum activity at 30°C . These results are consistent with those obtained in

the present study, where the highest protease activity with *P. roqueforti* also occurred in the 27–30 °C range.

An increase in fermentation time up to approximately 144 hours resulted in higher protease activity, at which point enzymatic activity reached its maximum value. After this period, production tends to stabilize or decrease, which may be related to the depletion of available nutrients in the substrate, compromising cell viability and enzymatic synthesis capacity (Sun et al., 2023). In general, the optimal duration for protease production by bacteria or fungi can range from 48 to 216 hours, depending on the microorganism and cultivation conditions (Sharma et al., 2017). Ugbede et al. (2023) found that extending the fermentation period led to a progressive increase in protease activity produced by *Aspergillus flavus*, with maximum enzyme production achieved at 120 hours. Aljammas, et al. (2022a) identified an optimal fermentation time of approximately 81.21 hours for protease production by the *Rhizomucor miehei* Rm4 isolate. In recent studies, Sosa-Martínez et al. (2023) investigated the production of hydrolytic enzymes using *Aspergillus* sp. The authors concluded that the optimal fermentation time for maximum enzymatic activity was 96 hours. Similarly, Nogueira et al. (2021) observed that trypsin-like protease production by *Penicillium roqueforti* peaked at around 144 hours of SSF using cocoa husk as a substrate.

Another critical parameter in enzymatic production by SSF is the substrate moisture content. In this study, an initial moisture of 70% resulted in the highest enzymatic activity, while significantly lower or higher levels reduced protease production. Moisture content during SSF directly influences microbial growth and metabolism. Low moisture levels can limit nutrient availability, reducing enzyme yield, whereas high levels decrease particle porosity and increase material stickiness, promoting substrate clumping (Muñoz-Seijas et al., 2024). The selection of the ideal moisture level is determined both by the characteristics of the microorganism used and the nature of the employed substrate (Pranay et al., 2019). For example, Aljammas et al. (2022a) identified 80% as the ideal moisture content for protease production by the *Rhizomucor miehei* Rm4 isolate, while Muñoz-Seijas et al. (2024) observed 61.8% as ideal for *Aspergillus uvarum* during SSF using brewery bagasse.

3.3.2 Model validation

The model was validated by conducting an experiment under the predicted optimal conditions: 150 h fermentation time, 30 °C temperature, and 70% moisture. The obtained enzymatic activity values (experimental: 4.14 ± 0.30 U/mL; predicted: 4.17 U/mL) showed high

agreement, with only a marginal difference between them. The percentage error was calculated using the following formula:

$$\text{Error (\%)} = \frac{|\text{Predicted}|}{|\text{Predicted} - \text{Experimental}|} * 100$$

In this case, the error was approximately 0.72%, below 5%, indicating high model accuracy. These results confirm the adequacy and predictive capability of the proposed model.

3.4 Biochemical characterization of the crude extract

The parameters MCA (milk coagulation activity), PA (proteolytic activity), and protein content were determined in the crude protease extract obtained under optimal fermentation conditions. The results showed values of 482 ± 1.52 U/mL for MCA, 4.14 ± 0.01 U/mL for PA, and 3.4 ± 0.01 mg/g for protein content. The MCA/PA ratio obtained was 116.11 ± 0.62 , indicating that the crude extract has good selectivity for milk coagulation.

Coagulating enzymes act catalytically, promoting the hydrolysis of the bond between Phe 105 and Met 106 residues of κ -casein, located on the micelle surface. The cleavage of this bond causes the destabilization of casein micelles, which, in the presence of Ca^{2+} , aggregate and form the curd (McSweeney, 2022). The hydrolysis of κ -casein directly influences the coagulation process during cheese production, significantly affecting milk-clotting activity (MCA) (Mohsin et al., 2024).

To predict the efficiency of the coagulation process and the sensory characteristics of cheese, it is essential to consider the MCA/PA ratio. MCA is related to the enzyme's specificity in hydrolyzing κ -casein, whereas PA corresponds to the progressive degradation of curd proteins (α s1-, α s2-, β -, and κ -casein), a process that intensifies during ripening and can influence the development of residual flavors (Nicosia et al., 2022). High MCA/PA ratios indicate more selective and efficient clotting activity, associated with higher cheese yield and lower bitterness, while low ratios may compromise curd firmness and intensify undesirable flavors (Afsharnezhad et al., 2019).

Commercial chymosin exhibits an MCA/PA ratio of approximately 243.20, reflecting its high selectivity in cleaving κ -casein and efficiency in the coagulation process (Nicosia et al., 2022). In comparison, the crude *P. roqueforti* extract used in this study showed a ratio of 116.11 ± 0.62 , indicating a promising profile, although still lower than that of chymosin. This result suggests that, despite its lower specificity compared to chymosin, the microbial enzyme has potential for use in cheeses with different sensory profiles and ripening characteristics.

Therefore, the results obtained in this study demonstrate that crude protease extract, with high milk coagulation activity and low proteolytic activity, has a promising profile for application in cheese manufacturing and can be exploited in processes that value specific sensory characteristics or prolonged maturation.

3.4.1 Effect of temperature on protease activity and stability

The effect of temperature on protease activity was determined by assaying the enzymatic activity at different temperatures, ranging from 30 to 80 °C, for 10 minutes at pH 6.0 (Fig. 4a).

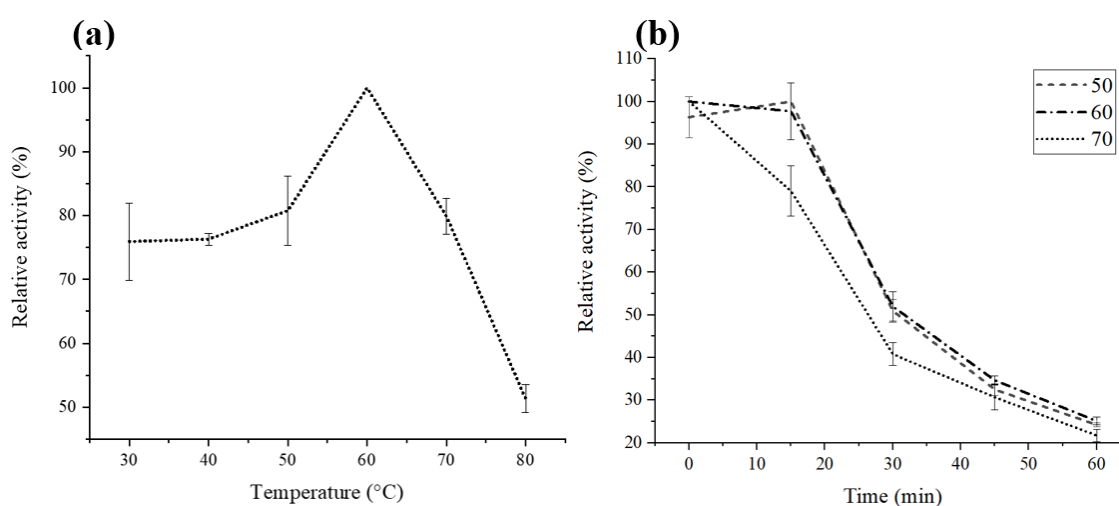


Fig. 4 - Effect of temperature on the proteolytic activity (a) and thermal stability (b) of the crude protease extract. In the stability assay, the enzyme was pre-incubated for 60 min at different temperatures and residual activity was measured. Results are expressed as relative activity (%), normalized to the highest activity (100%). Values are shown as mean $\pm$ standard deviation ($n = 3$). The reference point (100%) corresponds to the temperature of maximum activity and does not display error bars due to normalization.

The results showed that the relative activity of the protease increased with temperature ($p < 0.05$), reaching its maximum value at 60 °C, followed by a decline above this value, indicating that temperatures higher than 60 °C may have caused protease denaturation. This value is consistent with the findings of Zheng et al. (2020), who determined an optimal temperature of 55 °C for the alkaline protease from *Neurospora crassa*, with significantly higher activity in the 50–60 °C range. In addition, the authors observed that protease activity decreased almost linearly at temperatures below 55 °C and above 60 °C. According to Afsharnezhad et al. (2019), protein unfolding above certain temperatures can lead to the sudden

loss of its activity. This occurs because unfolding exposes hydrophobic residues, which promote immediate aggregation and inactivate the protein. Purushothaman et al. (2019) determined that the optimal temperature values for the activity of the aspartic protease from *Aspergillus niger* were between 60 and 65 °C. Enzyme activity dropped rapidly to zero at 75 °C.

The thermostability results (Fig. 4b) indicated that the protease retained significant stability at 50 and 60 °C, preserving at least 50% of its relative activity after 30 minutes of incubation. However, at 70 °C, a gradual decrease in proteolytic activity was observed, with more than 50% loss of the initial activity, probably due to thermal denaturation. These results suggest that the protease exhibits moderate heat resistance, but the maintenance of its functionality depends on both temperature and exposure time.

3.4.2 Effect of pH on protease activity and stability

The effect of pH was evaluated within the range of 5 to 8, as illustrated in Fig. 5a. The maximum protease activity was observed at pH 6 ($p < 0.05$), reaching 100% of its relative activity. In addition, the enzyme exhibited high stability, maintaining more than 90% of its activity between pH 5.5 and 6.5. The retention of proteolytic activity at pH 6.5 is particularly relevant for technological applications, such as milk coagulation, whose natural pH ranges between 6.5 and 6.7, and in which coagulation preferably occurs between 6.3 and 6.6 (Afsharnezhad et al., 2019).

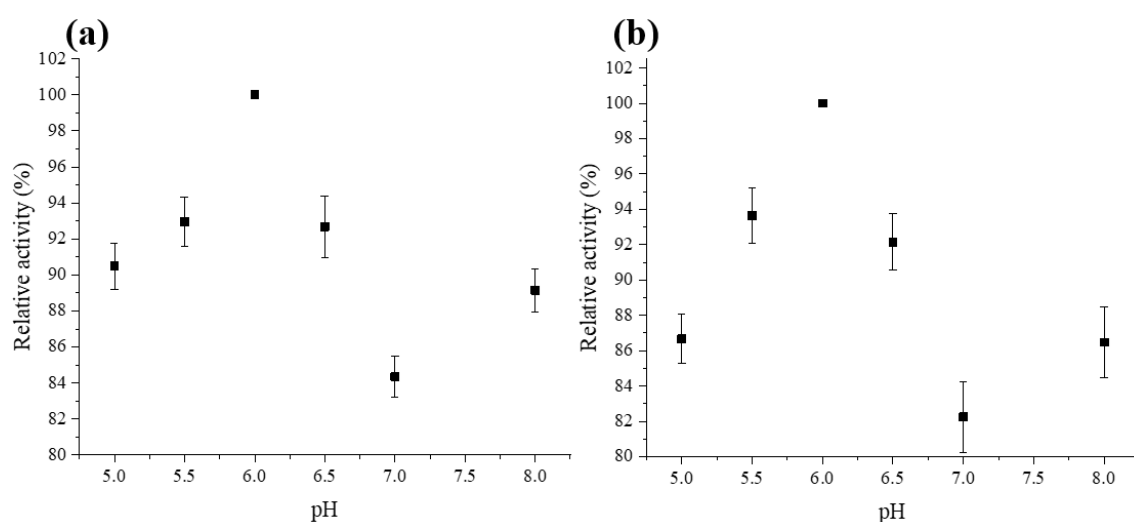


Fig. 5 - Effect of pH on the proteolytic activity (a) and pH stability (b) of the crude protease extract. In the stability assay, the enzyme was pre-incubated for 60 min at 37 °C at different pH values and residual activity was measured. Results are expressed as relative activity (%),

normalized to the highest activity (100%). Values are shown as mean $\pm$ standard deviation (n = 3). The reference point (100%) corresponds to the pH of maximum activity and does not display error bars due to normalization.

The influence of pH on enzymatic activity is related to the ionization state of the amino acid residues that compose the protein structure, which is directly affected by the hydrogen ion concentration in the medium (Oliveira et al., 2021). Since ionic bonds are fundamental for stabilizing the active conformation, changes in pH may alter the charge of essential functional groups, affecting the structural arrangement of the protein and compromising its catalytic activity (Artifon et al., 2023). More pronounced variations can lead to enzyme denaturation, resulting in the loss of its function. Thus, each enzyme exhibits an optimal pH for activity, with a progressive reduction in activity under conditions above or below this range (Thakur et al., 2018). Although the optimum pH of the protease was observed at pH 6.0, a higher relative activity was detected at pH 8.0 compared to pH 7.0. Small variations in pH can influence the ionization state of amino acid residues involved in substrate binding and catalysis, leading to subtle conformational changes that may favor enzyme–substrate interactions under slightly alkaline conditions (Fernandes et al., 2024). Furthermore, since a crude enzyme extract was used, the contribution of other proteolytic enzymes or isoenzymes with activity under alkaline pH conditions cannot be excluded, as previously reported for fungal proteases (Novelli, Barros & Fleuri, 2016).

As observed by Ben Mefteh et al. (2019), an acidic protease produced by *Penicillium bilaiae* exhibited maximum activity at pH 6, indicating behavior similar to that observed in the present study for the protease from *P. roqueforti*. These results reinforce that the protease from *P. roqueforti* exhibits stability and suitable performance under mildly acidic conditions, consistent with industrial applications in cheese and other dairy products.

The highest stability of the protease was observed under slightly acidic conditions (pH 5.5, 6.0, and 6.5), retaining more than 90% of its enzymatic activity after 1 h of incubation at 37 °C (Fig. 5b). This behavior is consistent with previous reports on microbial proteases, reinforcing its potential applicability in industrial processes involving dairy products (Zheng et al., 2020).

3.4.3 Effect of NaCl and CaCl₂ concentrations on enzymatic activity

Sodium and calcium ions play a crucial role in the cheese-making process (Salehi et al., 2017). Therefore, their effects on enzymatic activity were investigated. Considering their broad

application in the dairy industry, it is noteworthy that calcium contributes to the stability of casein micelles and protein–protein interactions, influencing the functional, textural, and rheological properties of the products. Sodium chloride, in turn, acts in controlling microbial growth, regulating curd firmness, aiding in the removal of excess whey, and directly affecting the textural characteristics of the final product (Barone et al., 2023).

In the present study, protease enzyme activity increased with NaCl concentration ($p < 0.05$), reaching 100% relative activity at 400 mmol (Fig. 6a). This behavior can be explained by the salt-in effect, in which the addition of salt at low concentrations promotes greater protein solubility. Salt ions interact with opposite charges on the protein surface, increasing the hydration of the molecule (Maurer, Sandler and Lenhoff, 2011; Sinha and Khare, 2014). Concentrations above 400 mmol caused a gradual decrease in activity, reaching 82% at 1000 mmol, possibly due to the salt-out effect. In this case, competition between ions and proteins for water molecules reduces the hydration layer, favoring denaturation and compromising catalytic activity (Mokashe et al., 2018).

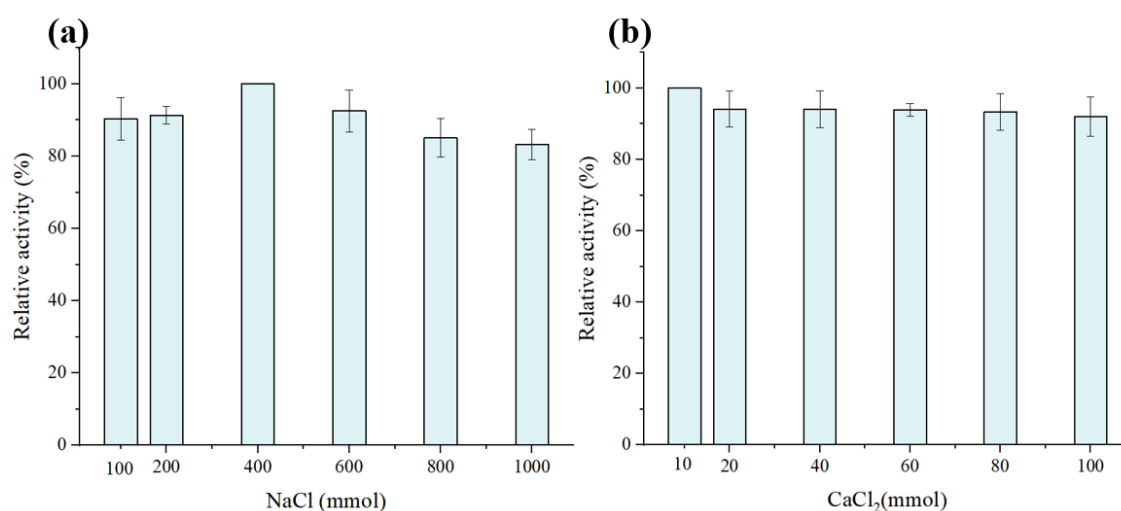


Fig. 6 - Effect of different concentrations of (a) NaCl and (b) CaCl₂ on the proteolytic activity of the crude protease extract. Results are expressed as relative activity (%), normalized to the highest activity (100%). Values are shown as mean $\pm$ standard deviation ($n = 3$). The reference point (100%) corresponds to the salt concentration that resulted in maximum activity and does not display error bars due to normalization.

Regarding CaCl₂, enzyme activity peaked at 10 mmol (100% relative activity) and showed a slight reduction to 94% from 20 mmol (Fig. 5b). According to Souza et al. (2017), the increase in enzyme activity promoted by divalent metal ions, especially Ca²⁺, can be

attributed to their role in stabilizing the ternary structures of enzymes. In addition, metal ions can interact with some amino acid residues in proteases, inducing conformational changes in the enzyme structure that alter its catalytic activity (Song et al., 2020). Similar results were observed by Fernandes et al. (2024), who reported increased microbial protease activity in the presence of Na^+ and Ca^{2+} , and by Salehi et al. (2017), who showed reduced activity at excessive salt concentrations.

The results indicate that moderate concentrations of Na^+ and Ca^{2+} favor protease activity, suggesting that ion strength optimization can be used to maximize enzyme performance in industrial applications.

3.4.5 Effect of temperature, NaCl, and CaCl_2 on milk-clotting activity (MCA)

Figure 7a illustrates the dependence of MCA on CaCl_2 concentration, showing a significant gradual increase ($p < 0.05$) with rising concentration. Calcium directly influences the firmness of milk gels by neutralizing negative charges on the surface of casein micelles and forming calcium bridges (Lucey, 2022). These interactions facilitate binding to para- κ -casein, acting as bridges between micelles and promoting the formation of firm curds during the second phase of coagulation, in addition to accelerating the coagulation rate (Hang et al., 2016). The positive effect of CaCl_2 is also associated with its role in facilitating enzyme-substrate binding, acting as a catalytic cofactor, and contributing to the reduction of the medium's pH (Bauland et al., 2020). The CaCl_2 concentration range was limited to 100 mmol due to its technological relevance in dairy processing, since higher concentrations may lead to non-specific physicochemical effects on milk proteins, such as excessive aggregation of casein micelles, which could interfere with the interpretation of milk-clotting activity.

It is important to note that milk-clotting activity and proteolytic activity represent distinct enzymatic responses. While proteolytic activity reflects global protein hydrolysis, milk clotting depends on the specific cleavage of κ -casein and on calcium-mediated aggregation of casein micelles, which explains the different responses observed in the presence of CaCl_2 and NaCl. The effect of pH on milk-clotting activity was also evaluated; however, no coagulation was observed in the tested range (data not shown).

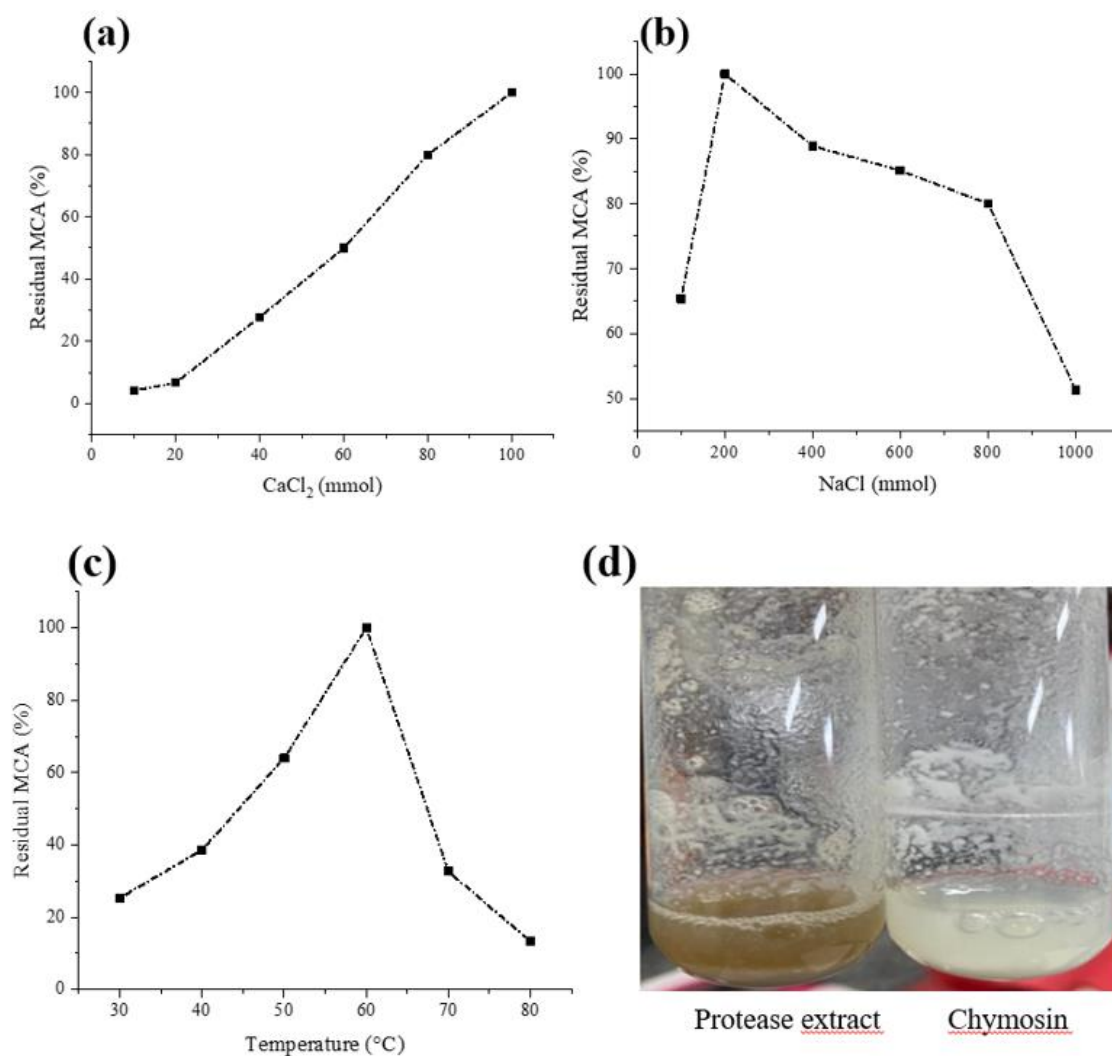


Fig. 7 - Effect of temperature (c) and CaCl₂ (a) and NaCl (b) concentrations on milk-clotting activity. Comparison of the visual appearance of milk curds obtained with the enzymatic extract and with commercial chymosin (d).

As for NaCl, the optimal concentration for coagulating activity was 200 mmol, with a gradual decrease at higher concentrations ($p < 0.05$; Fig. 7b). Although sodium chloride increases the stability of casein micelles, high concentrations can promote enzymatic denaturation and loss of activity (Artifon et al., 2023). The reduction in coagulating activity at high NaCl concentrations can be attributed to the inhibition of electrostatic interactions essential for the formation of the protease- κ -casein complex (Aljammam et al., 2023b).

Regarding temperature, MCA showed a progressive increase as a function of temperature elevation, reaching a maximum at 60°C (Fig. 7c). Above this temperature, a significant reduction was observed ($p < 0.05$). The increase in coagulation at high temperatures is associated with the structural instability of milk proteins, where preheating facilitates the breakdown of peptide chains by enzymes (Zhang et al., 2024). However, temperatures above

70 °C impair the clotting ability due to reduced enzymatic hydrolysis of κ -casein, possibly by decreasing accessibility to the Phe105–Met106 bond and diminishing micellar aggregation capacity (Lucey, 2022). Enzymatic coagulation of casein micelles is strongly temperature-dependent, and it does not occur in bovine milk below 18 °C without increasing the Ca^{2+} concentration (Bensmail et al., 2023).

Studies report similar behavior for fungal proteases. Mamo et al. (2021) observed maximum milk-clotting activity at 60 °C for *Aspergillus oryzae* protease. Bensmail et al. (2023) reported stimulation of *Mucor circinelloides* clotting activity by CaCl_2 (15–25 mM) at the same temperature, highlighting the synergy between moderate temperature and calcium ions for microbial proteases.

The proteolytic action on milk peptide bonds triggers coagulation, which is technologically relevant for the food industry (Reddy et al., 2022). As shown in Fig. 7d, the crude protease extract exhibited coagulation capacity similar to commercial chymosin, indicating its potential for applications in the food industry.

3.4.6 Kinetic parameters

The kinetic parameters of the protease produced by *Penicillium roqueforti* were estimated from experimental data using the Michaelis–Menten model, with variation in the initial casein concentration. The results indicated that the K_m and V_{max} values for the protease were 1.34 mg/mL and 2.32 U/mL, respectively. The kinetic parameter K_m reflects the strength of the interaction between the enzyme and its substrate. A high K_m value suggests that the dissociation rate of the enzyme–substrate complex is greater than the binding rate, indicating lower enzyme affinity for the substrate. Conversely, lower K_m values are associated with higher enzyme affinity for the substrate. The V_{max} parameter represents the maximum rate of the enzymatic reaction, being directly influenced by the concentration of enzyme available in the reaction (Fernandes et al., 2024). For comparison purposes, Zheng et al. (2020) determined the kinetic parameters of the alkaline protease produced by *Neurospora crassa*, obtaining $K_m = 2.18$ mg/mL and $V_{max} = 36.36$ $\mu\text{g/mL/min}$, whereas Souza et al. (2017) characterized the thermostable aspartic protease from *Aspergillus foetidus*, with $K_m = 0.81$ mM and $V_{max} = 90.0$ U/mL. These comparisons highlight the variability of kinetic parameters among different microbial proteases, considering differences in substrates and experimental conditions used (Artifon et al., 2023).

3.4.7 Molecular weight estimation by gel filtration chromatography

The molecular weight of the protease was estimated at 23.0 ± 1.0 kDa by gel filtration chromatography, based on the log (molecular weight) versus V_0 plot (Fig. 8). Microbial proteases generally exhibit molecular weights between 15 and 40 kDa (Dorra et al., 2018), placing the studied enzyme within the typical range for this enzyme group. Similar values have been reported for bacterial proteases, such as 21.8 kDa for *Bacillus cereus* (Lakshmi et al., 2018), and 28-44 kDa for *Bacillus amyloliquefaciens* (Pham et al., 2021), showing some convergence with the results obtained in this study. Similarly, studies with filamentous fungi also show comparable variation, such as the protease from *Volvariella volvacea* with 24 kDa (Xu et al., 2025) and the extract from *Rhizopus oryzae* with 39 kDa (Hsiao et al., 2014), reinforcing that the molecular weight of *Penicillium roqueforti* protease falls within the typical range for microbial enzymes.

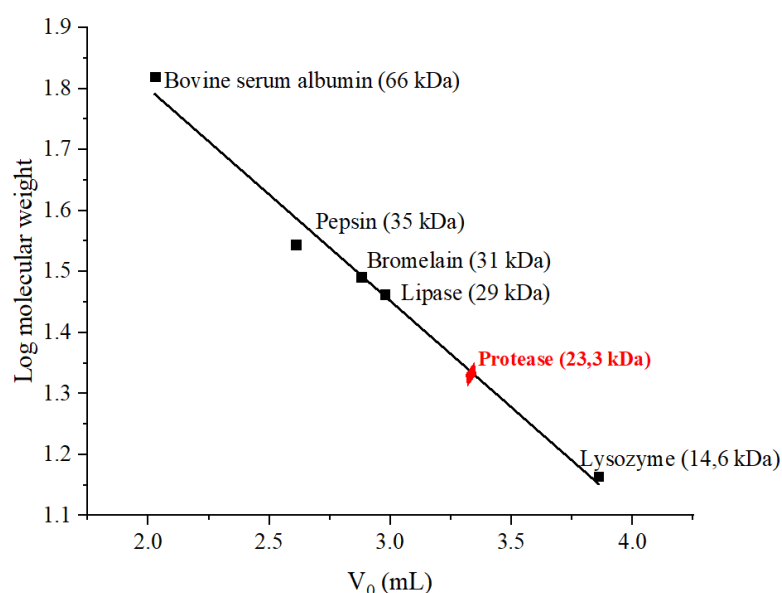


Fig. 8 - Standard plot for molecular weight estimation of the protease enzymatic extract. The molecular weight markers used were bovine serum albumin (66 kDa), pepsin (35 kDa), bromelain (31 kDa), lipase (29 kDa), and lysozyme (14.6 kDa). The molecular weight of the protease enzymatic extract was found to be approximately 23.3 kDa.

CONCLUSIONS

This study demonstrated the feasibility of protease production by *Penicillium roqueforti* ATCC 10110 in SSF using a combination of alternative plant-based substrates. The application of simplex-centroid and Box-Behnken designs proved strategic for defining the optimal fermentation conditions, highlighting the efficiency of statistical tools in enhancing enzymatic biotechnology. The combination of ora-pro-nóbis and soybean meal emerged as the most favorable formulation, balancing nitrogen availability and the structural properties of the medium, which enabled higher enzymatic yield.

The obtained protease exhibited desirable biochemical characteristics, with optimal activity at slightly acidic pH and a temperature of 60 °C. Moreover, the high ratio between milk coagulation activity (MCA) and proteolytic activity (PA) ($MCA/PA = 116.11$) indicates that the produced enzymatic extract has potential for use as a coagulant in cheese production, serving as an alternative to traditional rennet.

Thus, the results not only confirm the possibility of obtaining functional protease from *P. roqueforti* using low-cost plant substrates but also demonstrate the relevance of this approach for the development of more sustainable and innovative processes, aligned with current demands in the food industry.

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CHAPTER III

BIOACTIVE PEPTIDES FROM BOVINE, CAPRINE AND BUFFALO CASEINS HYDROLYZED BY *P. ROQUEFORTI* PROTEASE: ANTIOXIDANT AND ANTIDIABETIC POTENTIAL

Bioactive peptides from bovine, caprine and buffalo caseins hydrolyzed by *P. roqueforti* protease: Antioxidant and antidiabetic potential

ABSTRACT

Enzymatic hydrolysis of milk proteins represents a promising strategy for generating bioactive peptides with nutraceutical and technological applications. This study evaluated the ability of an aspartic protease produced by *Penicillium roqueforti* to hydrolyze bovine, caprine, and buffalo caseins, as well as the structural and functional properties of the resulting hydrolysates. Samples were hydrolyzed for up to 4 h and characterized in terms of degree of hydrolysis (DH), chromatographic profile by HPLC, in vitro antioxidant activity (DPPH, ABTS, FRAP, and β -carotene–linoleic acid system), and α -amylase inhibitory potential. The highest DH was observed for caprine casein, reaching approximately 15% after 2 h, whereas bovine casein achieved about 13% after 3 h and buffalo casein 13% after 4 h. In antioxidant assays, buffalo casein hydrolysates showed the highest DPPH (88%) and ABTS (96%) scavenging activities, while the greatest reducing power was observed for caprine casein, with FRAP values of approximately 1600 $\mu\text{M Fe}^{2+}$. Lipid oxidation protection ranged from 53% to 60%. Regarding α -amylase inhibition, bovine casein hydrolysates exhibited the highest activity, reaching up to 100% inhibition. These findings indicate significant nutraceutical and techno-functional potential, supporting their application as natural food ingredients and possible substitutes for synthetic antioxidants. Overall, this study demonstrates the biotechnological feasibility of using *P. roqueforti* proteases to produce high-value bioactive peptides.

Keywords: Microbial aspartic protease; Protein hydrolysates; Bioactive potential; Antioxidant activity; α -amylase inhibition.

1. INTRODUCTION

Milk proteins are among the most nutritionally valuable ingredients and are widely applied in the formulation of functional foods (Balivo, D'Errico & Genovese, 2024). Casein constitutes the major protein fraction in milk and is recognized for its high nutritional value as well as its broad spectrum of bioactivities (Han et al., 2026). This protein is composed of four main phosphoproteins, namely α s1-, α s2-, β -, and κ -casein, which are classified according to the homology of their primary amino acid sequences (Yang et al., 2026). The major caseins are found in all milk types as self-organized colloidal particles, known as casein micelles, which vary among animal species in terms of size, degree of hydration, mineral composition, and the relative proportion of casein subfractions (Roy et al., 2020). Differences in the primary structure of milk proteins across species may directly influence the profile and potential bioactivities of the peptides released (Tagliazucchi et al., 2018).

In this context, enzymatic hydrolysis stands out as one of the most promising biotechnological approaches for the release of bioactive peptides, since enzymes exhibit specificity toward well-defined cleavage sites. The catalytic selectivity of the protease determines the cleavage pattern and, consequently, the molecular weight and bioactivity of the resulting fragments. For this reason, several classes of enzymes have been employed in the production of hydrolysates and functional peptides derived from protein sources, with a predominance of commercial enzymes (Jiang et al., 2024; Castañeda-Valbuena et al., 2022; Mora & Toldrá, 2023).

Proteases are responsible for protein hydrolysis, promoting the release of bioactive peptides. These enzymes may be obtained from animal, plant, or microbial sources. Among them, microbial proteases stand out due to their relevant operational advantages, including lower production costs, high stability, and greater catalytic specificity, characteristics that favor their application in industrial processes (Ren et al., 2024). Among microbial sources, the fungus *Penicillium roqueforti* is widely recognized as a producer of a diverse set of enzymes, particularly proteases, which exhibit high affinity for milk proteins. These proteolytic enzymes act predominantly on the hydrolysis of the main casein fractions, including α s1-, β -, and κ -caseins (Coton et al., 2020).

As a result of this targeted cleavage, protein hydrolysates or isolated peptides generally exhibit bioactivities that are often superior to those of their precursor proteins, due to reduced molecular weight and increased structural flexibility. Evidence from both in vitro and in vivo studies has demonstrated that bioactive peptides may modulate specific physiological functions in the body, including immunomodulatory, antibacterial, antioxidant, antithrombotic,

hypocholesterolemic, or angiotensin-converting enzyme (ACE)-inhibitory activities, which are strongly dependent on amino acid composition, sequence, and structural conformation of the peptides (Gong et al., 2025; Han et al., 2026).

Although many bioactive peptides derived from casein have already been described in the literature, most studies aimed at their production employ bovine milk as the primary protein substrate. However, more recent investigations have highlighted other milk types, particularly goat milk as a promising source of bioactive peptides, as well as buffalo milk (Santos et al., 2023; He et al., 2025). Furthermore, it is observed that a large proportion of these studies employ commercial proteases in the production of protein hydrolysates and bioactive peptides, with emphasis on microbial-derived enzymes such as Flavorzyme, Neutrase, and Alcalase (Ai et al., 2019). For example, An et al. (2025) used Flavorzyme in the hydrolysis of buffalo casein, Mudgil et al. (2022) applied Alcalase and Pronase E to bovine and camel milk caseins, while Magalhães et al. (2022) evaluated the use of the commercial proteases Brazyn®, Alcalase®, and Flavorzyme® in the hydrolysis of fresh goat milk.

Despite these advances, studies investigating the application of microbial proteases produced by *Penicillium roqueforti* in the hydrolysis of different caseins, as well as the characterization of the peptides generated and their functional potential, remain scarce.

In light of this context, the present study aimed to employ a microbial protease produced by *Penicillium roqueforti* in the hydrolysis of bovine, goat, and buffalo caseins, with the objective of obtaining and characterizing bioactive peptides. In addition, a comparative analysis of the resulting peptide profiles was performed in order to evaluate the influence of milk species of origin on peptide release and their functional potential.

2. METHODOLOGY

2.1 Materials

Bovine casein (purity $\geq 90\%$) was purchased from Sigma-Aldrich (USA). Goat and buffalo milk caseins used in this study were previously obtained according to the methodology described by Souza Junior et al. (2025). In addition, a crude enzymatic extract containing aspartic protease produced by *Penicillium roqueforti* through solid-state fermentation using agro-industrial substrates was employed, with an activity of 4.29 U/mL, as reported in a previous study (Silva et al., 2026).

2.2 Preparation and hydrolysis of caseins

Buffalo, goat, and bovine caseins were subjected to enzymatic hydrolysis using the protease extract produced by *Penicillium roqueforti*. Initially, the caseins were dissolved in 100 mM potassium phosphate buffer (pH 6.0) at a concentration of 1% (w/v), in a final volume of 5.0 mL, under continuous stirring for 30 min until complete solubilization. Subsequently, 0.75 mL of the enzymatic extract was added to the casein solutions. Hydrolysis reactions were carried out in a thermostatic water bath (Tecnal, model TE-184) for up to 4 h. Samples were collected at 0.5, 1, 2, 3, and 4 h. The reactions were terminated by the addition of 5.0 mL of 10% (w/v) trichloroacetic acid solution, followed by centrifugation at $5000 \times g$ for 15 min. The supernatant, containing soluble peptides, was collected and used for subsequent analyses.

2.3 Determination of degree of hydrolysis (DH)

The degree of hydrolysis (DH) of the different hydrolyzed caseins was determined by the spectrophotometric *o*-phthalaldehyde (OPA) assay, according to the methodology described by Mirzaei et al. (2015). The OPA reagent solution was freshly prepared on the day of analysis by mixing 25 mL of sodium tetraborate (100 mM), 2.5 mL of 20% (w/v) sodium dodecyl sulfate (SDS), 40 mg of OPA previously dissolved in 1 mL of methanol, and 100 μ L of β -mercaptoethanol, with the final volume adjusted to 50 mL using deionized water. For the hydrolysis assay, the different caseins were previously subjected to the action of the aspartic protease produced by *Penicillium roqueforti*, obtained in a previous study (Silva et al., 2026). Aliquots of the hydrolyzed samples (10-50 μ L, corresponding to 5-100 μ g of protein) were added to 1 mL of OPA reagent in test tubes. The mixtures were homogenized by vortexing and incubated for 2 min at room temperature (25 °C). Subsequently, absorbance readings were recorded at 340 nm using a UV-Vis spectrophotometer. The concentration of free amino groups

released during hydrolysis was determined from a standard curve prepared with L-leucine (0–4 mg·mL⁻¹). The degree of hydrolysis (DH) was calculated according to Equation 1:

$$DH = \frac{L_1 - L_0}{L_{max} - L_0} \times 100 \quad (\text{Equation 1})$$

where L_1 corresponds to the amount of free amino groups released after hydrolysis, L_0 represents the amount of free amino groups initially present in the caseins prior to the enzymatic hydrolysis process, and L_{max} refers to the total amount of free amino groups, determined after complete acid hydrolysis of the caseins with 6 M HCl at 120 °C for 24 h.

2.4 Characterization of casein hydrolysates by reverse-phase high-performance liquid chromatography

The characterization of peptides obtained from casein hydrolysis was carried out by reverse-phase high-performance liquid chromatography (RP-HPLC), as described by Silva et al. (2023). A C18 column coupled to a pre-column was used, connected to a Shimadzu DGU-20A5R HPLC system equipped with a UV-Vis diode-array detector (DAD). Two mobile phases were employed: phase A, consisting of acetonitrile (5.0% v/v) containing trifluoroacetic acid (0.1% v/v), and phase B, consisting of acetonitrile (50% v/v) containing trifluoroacetic acid (0.1% v/v). A sample volume of 20 µL was injected into the column under a flow rate of 1.0 mL·min⁻¹, with a total run time of 70 min. The elution gradient was initiated with 100% phase A, which was linearly reduced over 60 min until reaching 100% phase B. Subsequently, between 60 and 65 min, the gradient was adjusted to return to 100% phase A, and this condition was maintained until the end of the analysis. The column oven temperature was maintained at 30 °C, and peptide detection was performed at a wavelength of 215 nm.

2.5 Determination of antioxidant activity

The *in vitro* antioxidant activity of the peptides obtained from casein hydrolysis was evaluated through DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging assays, ferric reducing antioxidant power (FRAP), and inhibition of oxidation in the β-carotene–linoleic acid system.

2.5.1 ABTS radical scavenging activity

The antioxidant activity of the samples was determined by the ABTS radical scavenging method, as described by Re et al. (1999), with adaptations. Initially, the ABTS radical stock solution was prepared by reacting 10 mL of ABTS (7 mmol/L) with 5 mL of potassium

persulfate ($\text{K}_2\text{S}_2\text{O}_8$, 2.45 mmol/L), and the mixture was allowed to stand for 12–16 h at room temperature in the dark. After this period, the solution was diluted with ethanol to obtain an absorbance of approximately 0.700 at 734 nm. For antioxidant activity determination, 2.0 mL of the ABTS solution was mixed with 100 μL of the sample, and the mixtures were kept in the dark for 6 min. Subsequently, absorbance was measured at 734 nm. The ABTS solution without sample addition was used as the control. Antioxidant activity was expressed as the percentage inhibition of the ABTS radical.

2.5.2 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging capacity

The antioxidant capacity of the casein hydrolysates was evaluated through the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, as described by Sousa et al. (2024), with adaptations. The method is based on the ability of antioxidant compounds to donate electrons or hydrogen atoms to the DPPH radical, promoting its reduction and the consequent decrease in absorbance. An ethanolic DPPH solution (0.06 mM) was previously prepared, with its initial absorbance adjusted to the range of 0.6–0.7 at 515 nm. For the assay, 500 μL of the DPPH solution was added to 500 μL of the sample in test tubes protected from light. The mixtures were homogenized and allowed to stand for 30 min in the dark. Subsequently, absorbance was measured at 515 nm using a spectrophotometer. Antioxidant activity was expressed as the percentage inhibition of the DPPH radical, calculated according to Equation (2):

$$\% \text{DPPH inhibition} = \left[\frac{A_c - A_s}{A_c} \right] * 100 \quad \text{Equation (2)}$$

where A_c and A_s correspond to the absorbance readings of the control and the sample, respectively.

2.5.3 Ferric reducing antioxidant power (FRAP)

The antioxidant potential of the casein hydrolysates was determined using the FRAP assay, as described by Liu et al. (2019), with adaptations. This method is based on the reduction of the Fe^{3+} -TPTZ complex to Fe^{2+} -TPTZ, forming an intense blue-colored complex in the presence of antioxidant compounds capable of donating electrons. The FRAP reagent was prepared immediately before analysis by mixing 0.3 M acetate buffer (pH 3.6), 20 mM ferric chloride solution ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), and 10 mM TPTZ solution in 40 mM HCl, at a volumetric ratio of 10:1:1 (v/v/v), respectively. In test tubes previously protected from light, 50 μL of the sample

was mixed with 150 μL of deionized water and 1.5 mL of the FRAP reagent. The mixtures were homogenized by vortexing and incubated in a water bath at 37 °C for 30 min. After incubation, absorbance readings were performed at 595 nm using a spectrophotometer, with the FRAP reagent as the blank. Quantification of antioxidant potential was carried out using a standard curve of ferrous sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), prepared at concentrations ranging from 50 to 2000 μM under the same assay conditions. The results were calculated from the linear regression equation obtained from the standard curve and expressed as μM Fe (II) equivalents per μL of sample. All analyses were performed in triplicate.

2.5.4 Inhibition of β -carotene/linoleic acid oxidation

The antioxidant activity of the casein hydrolysates was evaluated based on their ability to inhibit β -carotene bleaching in the β -carotene–linoleic acid system, as described by Aminjafari et al. (2016), with modifications. The method is based on the oxidation of linoleic acid during incubation at 50 °C, leading to the formation of peroxy radicals, which in turn promote β -carotene oxidation and the loss of its yellow coloration. In the presence of antioxidant compounds, these radicals are neutralized, thereby delaying β -carotene bleaching. The β -carotene–linoleic acid emulsion was prepared by dissolving 0.2 mg of β -carotene in 1 mL of chloroform, followed by the addition of 20 mg of linoleic acid and 200 mg of Tween 20 in a round-bottom flask. The chloroform was removed under a nitrogen stream, after which 50 mL of distilled water was added. The mixture was homogenized under constant stirring for 30 min. For the assay, 2.5 mL aliquots of the emulsion were transferred into test tubes containing 250 μL of the samples. The control was prepared by replacing the sample with chloroform. Absorbance readings were taken at 470 nm immediately after preparation of the mixtures (time zero) and after incubation for 2 h in a water bath at 50 °C. Antioxidant activity was expressed as the percentage inhibition of β -carotene bleaching (AA%), according to Equation 3.

$$\% \text{ Protection} = \left[1 - \left(\frac{\text{Abs}_{0\text{sample}} - \text{Abs}_{120\text{sample}}}{\text{Abs}_{0\text{control}} - \text{Abs}_{120\text{control}}} \right) \right] * 100 \quad (\text{Equation 3})$$

2.6 Amylase inhibition assay

The α -amylase inhibition assay was performed based on the methodology described by Apostolidis, Kwon, and Shetty (2007), with adaptations. Thermostable α -amylase (EC 3.2.1.1) was purchased from Sigma Chemical. A volume of 50 μL of peptides from each casein and 50 μL of 0.1 M sodium phosphate buffer (pH 7.0) containing α -amylase solution (2 U/mL) were incubated at 37 °C for 10 min. After pre-incubation, 100 μL of a 1% starch solution in 0.1 M sodium phosphate buffer (pH 6.9) was added to each tube. The reaction mixtures were then

incubated at 37 °C for 10 min. The reaction was terminated by adding 200 µL of dinitrosalicylic acid (DNS) color reagent. Subsequently, the test tubes were incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted by adding 3 mL of distilled water, and absorbance was measured at 540 nm. The inhibitory activity was calculated according to Equation 4:

$$\% \text{ Inhibition} = \left(\left[\frac{A_{\text{Control}} - A_{\text{Peptides}}}{A_{\text{Control}}} \right] \right) \times 100 \quad (\text{Equation 4})$$

2.7 Statistical analysis

All experiments were performed in triplicate, and the obtained data were subjected to analysis of variance (ANOVA). Mean comparisons were conducted using Tukey's test at a significance level of 5% ($p < 0.05$) with the Statistical Analysis System (SAS) software. Graphs were generated using Origin 2018 64-bit software (OriginLab Corporation, Northampton, MA, USA).

3. RESULTS AND DISCUSSION

3.1 Degree of hydrolysis

The degree of hydrolysis (DH) is a crucial parameter for evaluating protein hydrolysis, as it may directly influence the functional characteristics and biological activities of protein hydrolysates (Zheng et al., 2019). In general, the increase in DH over time can be explained by the greater exposure of reactive sites following denaturation or unfolding of native proteins, which enhances enzymatic catalytic efficiency and results in the cleavage of a larger number of peptide bonds (Mudgil et al., 2021). In contrast, the decline in hydrolysis rate is mainly attributed to the reduction in available hydrolyzable sites, enzyme autodigestion, or enzymatic inhibition by products formed during the process (Lin et al., 2017).

Figure 1 presents the degree of hydrolysis (DH) of bovine, goat, and buffalo caseins over the reaction time (0.5 - 4 h) when subjected to the action of the aspartic protease produced by *Penicillium roqueforti*. Overall, a significant variation in DH was observed over time for each type of casein evaluated ($p < 0.05$).

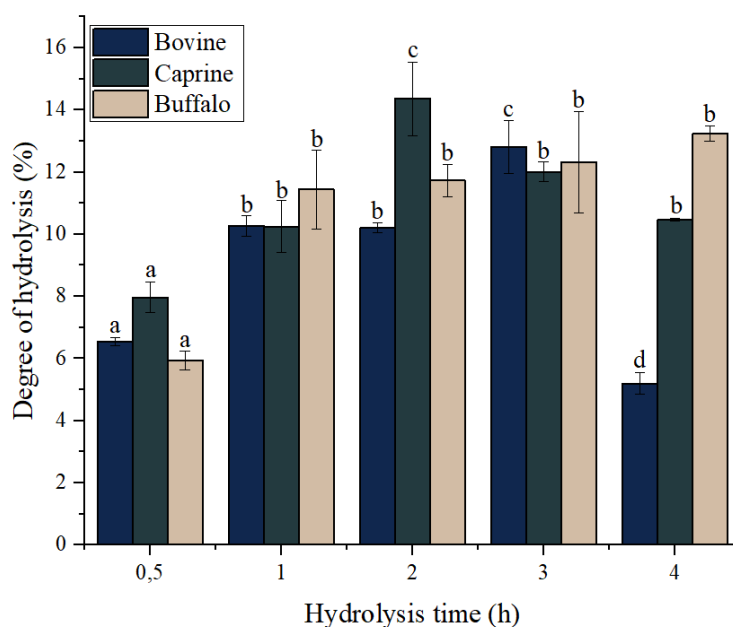


Figure 1. Degree of hydrolysis (%) of bovine, goat, and buffalo caseins subjected to enzymatic hydrolysis by the protease produced by *Penicillium roqueforti* at different reaction times (0.5 - 4 h). Values represent mean $\pm$ standard deviation. Different letters indicate significant differences among hydrolysis times for the same casein ($p < 0.05$).

For bovine casein, a progressive increase in the degree of hydrolysis (DH) was observed between 0.5 and 3 h of reaction, reaching a maximum value of 12.81% at 3 h. However, after 4 h, a marked reduction in DH (5.20%) was detected, which may be associated with the reduced availability of peptide bonds susceptible to protease action as hydrolysis progresses (Garg et al., 2024). Prolonged hydrolysis time favors reaction progression up to a certain limit; however, the hydrolysis rate does not remain constant and tends to decrease over time due to factors such as partial enzyme denaturation, possible product inhibition, or changes in substrate accessibility (Mora & Toldrá, 2023). In addition, the extent of hydrolysis directly influences the properties of the hydrolysates. Hydrolysates obtained through limited hydrolysis ($DH < 10\%$) are generally associated with changes in functional properties, whereas higher hydrolysis levels favor the release of bioactive peptides. Nevertheless, excessive hydrolysis may result in secondary degradation of these peptides, compromising their biological activities (Tu et al., 2018).

For buffalo casein, an increase in DH was observed up to intermediate reaction times, after which no statistically significant differences were detected among the evaluated times ($p > 0.05$), indicating a stabilization tendency in the process. This behavior may be associated with the structural particularities of buffalo milk, which, according to An et al. (2025), presents higher concentrations of α_2 -casein and κ -casein compared to bovine milk, in addition to a casein organization predominantly in larger micelles. These structural characteristics may hinder enzyme penetration and reduce accessibility to cleavage sites, thereby making the hydrolysis process slower and more progressive, with lower variation in DH over time.

Goat casein exhibited a distinct behavior compared to the other caseins, with a significant increase in DH up to 2 h of reaction, at which point it reached the highest DH observed among all samples (14.36%, $p < 0.05$). Higher DH values indicate greater release of amino groups from the native protein, which may influence the functional and bioactive profile of the hydrolysates (Hau et al., 2025). The shorter time required to achieve maximum DH suggests greater susceptibility of goat milk proteins to proteolytic action, possibly related to the structural particularities of this matrix, such as smaller micelle size and a higher relative proportion of β -casein, characteristics that favor exposure of cleavage sites and increase protease accessibility to peptide bonds (Roy et al., 2020). After this period, a reduction in DH was observed at longer times, indicating that the most accessible protein fractions had already been hydrolyzed and that the system was approaching a dynamic equilibrium state (Wang et al., 2026). Thus, goat casein stood out by presenting the highest degree of hydrolysis (14.36%) within the shortest reaction time (2 h), demonstrating greater ease of hydrolysis compared to

the other matrices, which may be directly associated with its simpler structural organization and enhanced enzymatic accessibility.

The DH values obtained in the present study were consistent with those reported in the literature for different casein types hydrolyzed by widely used proteases. Similar results have been observed in the hydrolysis of buffalo casein using the commercial enzyme Flavorzyme (An et al., 2025), goat casein using Alcalase (Magalhães et al., 2022), and bovine casein hydrolyzed by chymotrypsin, trypsin, and pepsin (Wang et al., 2026). Therefore, the results indicate that the protease produced by *Penicillium roqueforti* exhibits efficiency comparable to that of commercial proteases employed in the hydrolysis of different caseins.

3.2 HPLC chromatographic profile of bovine, goat, and buffalo caseins and their hydrolysates

The chromatographic profiles obtained by HPLC for the hydrolysates of the three caseins produced by the action of the aspartic protease from *Penicillium roqueforti* revealed the formation of numerous peaks distributed throughout the elution gradient, with retention times ranging approximately from 5 to 50 min. The predominance of signals at shorter elution times, compared to the native proteins, confirms the occurrence of enzymatic degradation and the consequent generation of lower-molecular-weight peptides, exhibiting different degrees of polarity and hydrophobicity (Dopazo et al., 2023).

The non-hydrolyzed caseins, in turn, showed peaks concentrated at higher retention times, around 64-65 min, a behavior consistent with the elution of native proteins or high-molecular-weight fragments, characterized by greater hydrophobicity and stronger interaction with the stationary phase of the C18 column.

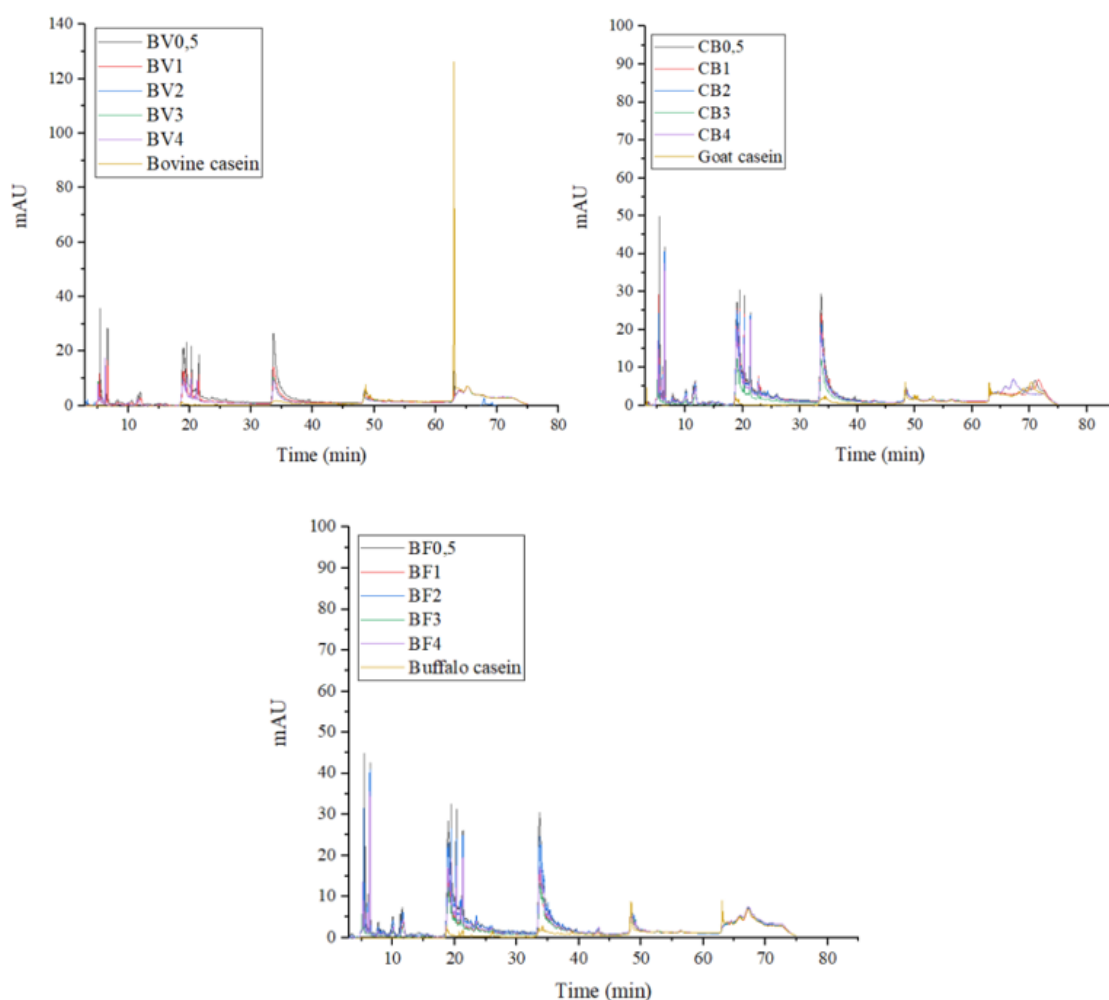


Figure 2. HPLC chromatographic profiles of bovine, goat, and buffalo caseins and their respective hydrolysates obtained by the action of the *Penicillium roqueforti* protease at different hydrolysis times (0.5 - 4 h). Chromatograms were recorded using a UV detector, with the y-axis expressed in milli-absorbance units (mAU) and the x-axis corresponding to retention time (min). The curves referring to non-hydrolyzed caseins are indicated as “bovine casein,” “goat casein,” and “buffalo casein,” whereas the hydrolysates are identified according to the protein matrix and reaction time.

3.3 In vitro antioxidant potential of peptides generated by the enzymatic hydrolysis of different caseins

The casein hydrolysates obtained through the action of the *Penicillium roqueforti* protease exhibited significant antioxidant activity across the different methods evaluated, with behavior dependent on both hydrolysis time and casein type. In general, assays based on free radical scavenging (DPPH and ABTS) and reducing power (FRAP) were more sensitive to the

structural variations of the hydrolysates over reaction time, whereas the β -carotene/linoleic acid system showed more stable responses.

The DPPH radical scavenging assay broadly evaluates the ability of peptides to act as hydrogen donors, providing a direct measure of their antioxidant potential (Li et al., 2026). As illustrated in Figure 3A, DPPH radical inhibition values varied over hydrolysis time for the three caseins evaluated, demonstrating a clear dependence on reaction time, with greater efficiency observed at intermediate times, followed by a reduction during prolonged periods. In general, the highest percentages of DPPH inhibition were observed for hydrolysates obtained between 1 and 2 h of hydrolysis, suggesting that this interval favored the generation of peptides with structural characteristics more suitable for neutralizing this radical. Among the evaluated samples, the buffalo casein hydrolysate obtained after 2 h of hydrolysis showed the highest performance, reaching approximately 86.3% DPPH radical inhibition, a value significantly higher than those observed at the initial and final time points. For goat and bovine hydrolysates, the greatest antioxidant potential was recorded for the hydrolysates obtained after 1 h of reaction, with approximately 75.9% and 72.6% inhibition, respectively. These results indicate that, although the optimal time varied slightly among protein matrices, the maximum antioxidant potential was achieved before the longest reaction times in all evaluated hydrolysates.

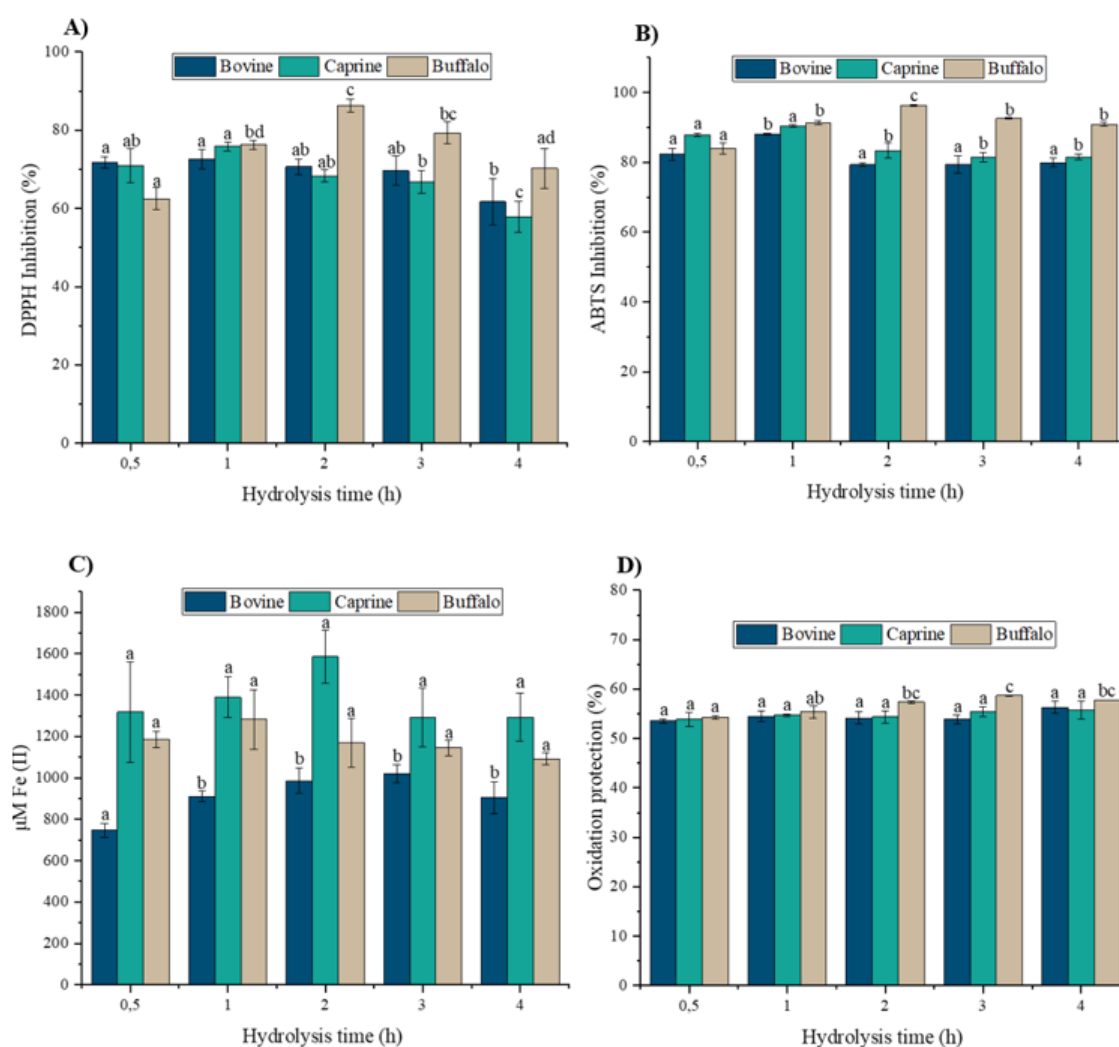


Figure 3. Antioxidant activity of casein hydrolysates obtained by enzymatic hydrolysis using the *Penicillium roqueforti* protease at different hydrolysis times (0.5 - 4 h): (A) DPPH radical scavenging activity, (B) ABTS radical scavenging activity, (C) ferric reducing antioxidant power (FRAP), and (D) inhibition of β -carotene-linoleic acid oxidation. Hydrolysates of bovine, goat, and buffalo caseins are represented by different colors. Values are expressed as mean $\pm$ standard error ($n = 3$). Different lowercase letters indicate significant differences among hydrolysis times for the same type of casein ($p < 0.05$).

The reduction or stabilization of DPPH radical scavenging activity observed in hydrolysates obtained at more advanced hydrolysis times may be associated with the increase in the degree of hydrolysis and the consequent formation of lower-molecular-weight peptides and free amino acids, predominantly hydrophilic in nature. According to Shahi, Sayyed-Alangi, and Najafian (2020), the high polarity of these compounds reduces their affinity for the DPPH radical, resulting in lower antioxidant activity in this assay, even as hydrolysis progresses. Thus,

the results suggest that an intermediate degree of hydrolysis is more effective in maximizing antioxidant activity as measured by the DPPH assay, since it preserves peptide sequences containing hydrophobic residues in the hydrolysates. In this same context, the presence of these residues in peptides formed at intermediate hydrolysis times is a determining factor for enhanced antioxidant activity, as they favor interaction with the DPPH radical in a relatively apolar medium (Devi et al., 2020).

Consistent with the results observed in the DPPH assay, the antioxidant activity evaluated by the ABTS method also showed dependence on hydrolysis time, with the best results obtained for hydrolysates at intermediate reaction times (Figure 3B). The ABTS assay is based on the ability of antioxidant compounds to act as electron or hydrogen donors, promoting the reduction of the ABTS radical cation, and is recognized for its applicability to both hydrophilic and lipophilic antioxidants (Garg et al., 2024). Thus, even considering the greater versatility of the ABTS assay regarding the polarity of the evaluated compounds, the obtained results suggest that the antioxidant activity of the hydrolysates was strongly influenced by the structural characteristics of the peptides formed at intermediate hydrolysis times, similarly to what was observed in the DPPH assay. It was observed that the highest ABTS radical inhibition value was obtained for buffalo casein hydrolysates at 2 h of hydrolysis (96-97%), whereas goat and bovine casein hydrolysates reached their maximum values at 1 h of reaction (90-92% and 88-89%, respectively). These findings reinforce that, although the optimal time varies among matrices, buffalo casein hydrolysates exhibit greater overall efficiency in free radical neutralization. The radical scavenging capacities of protein hydrolysates may be attributed to their amino acid composition and molecular size (Koirala, Prathumpai, & Anal, 2021). The composition of amino acids susceptible to oxidation reactions and the proportion of hydrophobic residues (including aromatic and basic amino acids) are directly related to their free radical scavenging capacity (Shazly et al., 2017).

The ability of antioxidants to donate electrons to free radicals, thereby reducing ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}), was evaluated using the FRAP assay (Figure 3C) (Amarasekara et al., 2025). For the hydrolysates of the three evaluated caseins, high reducing power values were observed, with variations dependent on hydrolysis time, particularly for goat and buffalo casein hydrolysates, which exhibited the highest values between 1 and 2 h of hydrolysis. The highest reducing power value was observed for goat casein hydrolysates at 2 h of hydrolysis ($1600 \mu\text{M Fe}^{2+}$), indicating greater efficiency in electron transfer under this condition, whereas buffalo casein hydrolysates showed elevated values mainly between 0.5 and 1 h, although lower than the maximum observed for goat casein hydrolysates. The increase in

ferric reducing power observed in protein hydrolysates at intermediate hydrolysis times may be related to the cleavage of protein chains, resulting in the formation of smaller peptides with greater availability of functional groups capable of acting as electron donors. According to the literature, enzymatic hydrolysis may promote the release or enhanced accessibility of amino acids associated with reducing activity, such as tryptophan, methionine, lysine, histidine, and tyrosine, which participate in electron transfer mechanisms evaluated by the FRAP assay (Shazly et al., 2019; Shahi, Sayyed-Alangi, & Najafian, 2020).

In the β -carotene/linoleic acid system (Figure 3D), the hydrolysates exhibited relatively constant inhibition percentages throughout the hydrolysis time, with values above 50% for all evaluated caseins. This behavior indicates that, for this antioxidant system, hydrolysis time exerted less influence on the observed activity, suggesting that the peptides formed during the process maintained a similar capacity to protect against lipid oxidation, possibly due to the presence of hydrophobic residues. The highest inhibition values were observed for buffalo casein hydrolysates between 3 and 4 h of hydrolysis (57-58%). Peptides with a high content of hydrophobic amino acids exhibit antioxidant activity due to the ability of their phenolic groups to act as hydrogen donors, thereby inhibiting the propagation of radical-mediated lipid peroxidation. In addition, specific residues, particularly proline, valine, phenylalanine, and histidine, located at the N- and C-terminal ends, play a crucial role in antioxidant activity, as they display greater interaction with fatty acids and a high capacity to scavenge lipid free radicals (Magalhães et al., 2022).

Thus, the results confirm that the antioxidant efficiency of casein hydrolysates is directly related to the specific action of the *Penicillium roqueforti* protease, since the enzyme determines the cleavage sites and the size of the peptides formed, which are decisive factors for the bioactivity of these compounds (Hau et al., 2025). Overall, the combined analysis of the different antioxidant methods indicates that the best performances were achieved at intermediate hydrolysis times (1-2 h), with particular emphasis on buffalo casein hydrolysates at 2 h, which exhibited the highest overall values in the DPPH and ABTS assays, and on goat casein hydrolysates at 2 h, which showed the greatest reducing power in the FRAP assay. These findings demonstrate that the 2 h hydrolysis condition is the most favorable for maximizing the antioxidant potential of the hydrolysates, with buffalo casein hydrolysates showing the best overall performance in free radical neutralization, while goat casein hydrolysates stand out in terms of reducing capacity.

3.4 α -Amylase inhibitory activity

The casein hydrolysates obtained through the action of the *Penicillium roqueforti* protease exhibited high α -amylase inhibitory activity under all evaluated conditions, with inhibition percentages above 80% for the three protein matrices in hydrolysates produced at different hydrolysis times (Figure 4). Although the hydrolysates were obtained at different hydrolysis times, no marked variation in inhibitory activity was observed among them, indicating that hydrolysis time exerted limited influence on this activity. In general, bovine casein hydrolysates showed the highest α -amylase inhibition percentages, maintaining elevated values above 95% in hydrolysates obtained at different hydrolysis times. Goat casein hydrolysates also demonstrated high inhibitory activity, with percentages above 90%, whereas buffalo casein hydrolysates exhibited values above 80% inhibition. Statistically significant differences were observed among hydrolysates from different protein origins at each hydrolysis time ($p < 0.05$). The greater inhibitory potential observed in bovine casein hydrolysates may be related to the generation of peptides with higher affinity for the active site of α -amylase (Hau et al., 2025).

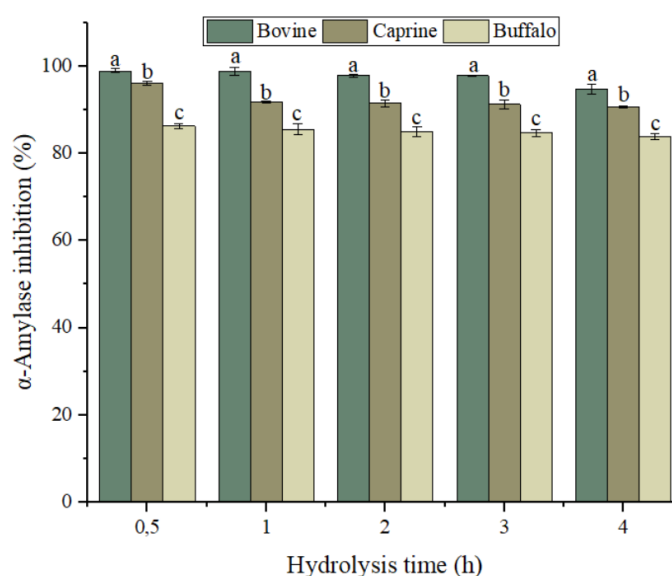


Figure 4. α -Amylase inhibitory activity (%) of bovine, goat, and buffalo casein hydrolysates obtained at different hydrolysis times (0.5 - 4 h). Bars represent mean $\pm$ standard deviation. Different letters indicate significant differences among hydrolysates from different protein origins at the same hydrolysis time ($p < 0.05$).

It is well established that α -amylase catalyzes the hydrolysis of starch into smaller oligosaccharides, which are subsequently cleaved by α -glucosidases into glucose units. In this

context, α -amylase inhibition delays carbohydrate digestion, thereby regulating glycemic levels by reducing the rate of glucose release into the bloodstream (Mudgil et al., 2023). Bioactive peptides may act as competitive inhibitors, competing with the substrate for the enzyme's active site, or as non-competitive inhibitors, binding to distinct regions of the enzyme and interfering with the catalytic process. In addition, mixed inhibition mechanisms have also been described, in which peptides affect both substrate binding and the catalytic step of the reaction, resulting in a decrease in the hydrolysis rate (Li et al., 2022). These interactions reduce the ability of α -amylase to convert starch into sugars available for absorption, thereby contributing to postprandial glycemic control (Su et al., 2024).

Studies have reported that amino acids with different chemical characteristics, including hydrophilic residues (such as Cys and Arg), hydrophobic residues (such as Leu, Phe, Pro, and Gly), and aromatic residues (Trp and Tyr), can interact with α -amylase through hydrophobic interactions and hydrogen bonding, resulting in inhibition of its catalytic activity (Fan et al., 2024). Therefore, the results obtained in this study indicate that α -amylase inhibitory activity was associated with the origin of the casein, with bovine casein standing out as presenting greater *in vitro* antidiabetic potential compared to goat and buffalo caseins.

The findings highlight the potential of casein hydrolysates as functional ingredients with promising applications in the development of foods aimed at glycemic control. Although *in vitro* assays provide relevant preliminary evidence, *in vivo* studies are recommended to confirm the biological efficacy of these peptides under physiological conditions and to consolidate their technological and nutraceutical applicability.

CONCLUSIONS

The microbial aspartic protease produced by *Penicillium roqueforti* demonstrated high efficiency in the hydrolysis of bovine, goat, and buffalo caseins, promoting the release of bioactive peptides with functional characteristics. The degree of hydrolysis and chromatographic profiles confirmed the degradation of intact proteins and the formation of lower-molecular-weight peptides distributed across a broad range of hydrophobicity. The hydrolysates exhibited significant antioxidant activity in the different systems evaluated, with greater efficiency, in general, at intermediate hydrolysis times, indicating that moderate degrees of cleavage favored the generation of more active peptides. In addition, all hydrolysates displayed high α -amylase inhibitory activity, with emphasis on those derived from bovine casein, highlighting their *in vitro* antidiabetic potential.

Overall, the results demonstrate that the catalytic specificity of the *Penicillium roqueforti* aspartic protease enables the production of protein hydrolysates with dual functionality, namely antioxidant and antidiabetic activities, from different dairy matrices. In summary, the use of this microbial protease represents a viable biotechnological strategy for the valorization of milk proteins and for the development of natural ingredients with high application potential in the functional food and nutraceutical industries, fulfilling the biotechnological prospecting objectives proposed in this study.

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CHAPTER IV

USE OF *PENICILLIUM ROQUEFORTI* ASPARTIC PROTEASE AS AN ALTERNATIVE COAGULANT IN GOAT MILK MINAS FRESCAL CHEESE

Use of *Penicillium roqueforti* Aspartic Protease as an Alternative Coagulant in Goat Milk Minas Frescal Cheese

ABSTRACT

Although goat milk has high nutritional value, its compositional particularities challenge enzymatic coagulation, impacting cheese yield and texture. In this study, the potential of a novel aspartic protease from *Penicillium roqueforti* was evaluated as an alternative to commercial chymosin in the production of goat Minas Frescal cheese. Different formulations were prepared using *P. roqueforti* protease in combination with increasing levels of microbial aspartic protease (0 to 70%), with proportional replacement of recombinant chymosin, in order to determine the ideal balance between enzymes for the goat milk matrix. The formulations were evaluated for yield, physicochemical profile, textural, and rheological properties. The structural integrity of the protein matrix was monitored by mid-infrared (MIR) and near-infrared (NIR) spectroscopy. Partial replacement of chymosin promoted changes in the composition and texture of goat Minas Frescal cheeses. The formulation with 70% aspartic protease (PAM70) showed the highest protein content (17.86%) and the lowest moisture (60.54%), as well as greater hardness and gumminess. Formulations with up to 50% replacement maintained yield comparable to the control, demonstrating the technological feasibility of substitution. Rheological analyses revealed a viscoelastic behavior typical of protein gels, with predominance of the elastic character. MIR and NIR spectra showed similar profiles among treatments, suggesting that coagulant substitution did not promote global chemical changes in the cheese matrix, being mainly associated with adjustments in the intermolecular interactions of caseins. It is concluded that the aspartic protease produced by *P. roqueforti* presents potential as an alternative or complementary coagulant to chymosin in the production of goat milk Minas Frescal cheese.

Keywords: Aspartic protease; Minas Frescal cheese; Goat milk; Enzymatic coagulation; Infrared spectroscopy.

1. INTRODUCTION

Goat milk has attracted increasing scientific and industrial interest due to its nutritional and functional properties, such as greater digestibility, associated with the reduced size of fat globules and the lower compactness of casein micelles, as well as its higher content of short- and medium-chain fatty acids and lower allergenicity compared to cow milk (Santos et al., 2023; Feitosa et al., 2025). However, these particularities impose significant technological challenges during processing, especially in the enzymatic coagulation stage, since the predominance of β -casein over α s1-casein, the lower protein and fat content, the reduced κ -casein fraction, and the larger size of casein micelles are associated with weaker protein interactions, resulting in the formation of softer and more fragile curds and, consequently, lower cheese yield (Kotsiou et al., 2025). These characteristics confer a distinct coagulation behavior to goat milk, reinforcing the need for alternative strategies to improve its technological performance.

The efficiency of chymosin can be significantly affected by the particularities of goat milk. Compared to cow milk, goat milk exhibits a shorter coagulation time and a weaker curd consistency during chymosin-induced coagulation, reflecting lower enzymatic efficiency in the formation of a cohesive protein network (Yang et al., 2024). These technological limitations directly impact the yield, texture, and sensory quality of goat cheeses.

Enzymatic milk coagulation, achieved through the addition of chymosin, also known as rennet, is one of the most widely used methods for the production of various types of cheese (Barros et al., 2025). This enzyme (EC 3.4.23.4) is an aspartic protease responsible for milk coagulation in cheese production, acting through the cleavage of the peptide bond in κ -casein between the amino acids Phe105 and Met106 (Ersöz & İnan, 2025). The enzymes that best meet the requirements of cheese manufacturing are microbial enzymes, recombinant enzymes, and calf rennet (Türkmen & Güler, 2022). The use of alternative microbial proteases, obtained through fermentative processes, represents a promising strategy to optimize goat milk coagulation. In addition, the adoption of these proteases may significantly influence the development of cheese flavors and textures, expanding both the diversity and the quality of products that can be introduced into emerging markets (Niero et al., 2024; Sibono et al., 2026).

Microbial proteases obtained through fermentation processes have gained broad acceptance in cheese production (Wiśniewski et al., 2025). Among these, fungal-derived aspartic proteases produced by solid-state fermentation (SSF) stand out for their significant milk-clotting activity and their technological potential as alternatives or complements to commercial rennet.

Several studies have evaluated the effect of different rennet sources on the physicochemical and textural properties of various cheese types, predominantly using cow milk as the matrix (Türkmen & Güler, 2022; García-Gómez et al., 2019; Niero et al., 2024; D’Incecco et al., 2022; Akbar et al., 2025). However, to date, there are no reports assessing the effect of combining commercial recombinant chymosin with microbial aspartic proteases in the production of cheese made from goat milk.

In this context, the present study aimed to evaluate the partial replacement of commercial chymosin with an aspartic protease produced by *Penicillium roqueforti* in the production of Minas Frescal cheese from goat milk, using Minas Frescal cheese as an experimental model due to its high moisture content and textural sensitivity. To this end, the impacts of substitution on yield, physicochemical composition, and the textural and rheological properties of the final product were investigated.

2. METHODOLOGY

2.1 Milk coagulant preparation

The protease extract evaluated as a partial substitute for chymosin was previously obtained by solid-state fermentation, using soybean bran and *ora-pro-nóbis* as substrates and the fungus *Penicillium roqueforti*, according to the methodology described by Silva et al. (2026). The crude extract presented a proteolytic activity of 4.29 U/mL and a milk-clotting activity of 482 ± 1.52 U/mL (MCA). In this study, this extract was designated as PAM (microbial aspartic protease). The commercial microbial rennet (*Aspergillus niger* var. *awamori*) (HÁ-LA®, Brazil, Chr. Hansen; strength 1:3000) was used at different concentrations in combination with PAM, according to the experimental design.

2.2 Experimental design and production of goat cheeses

The experiment was conducted by varying a single factor, corresponding to the different percentages of partial replacement of commercial chymosin with microbial aspartic protease (PAM) in the formulation. The treatments were identified according to the proportion of PAM used in the coagulant mixture and were designated as PAM0, PAM10, PAM20, PAM30, PAM40, PAM50, PAM60, and PAM70, corresponding, respectively, to 0, 10, 20, 30, 40, 50, 60, and 70% PAM replacing commercial chymosin. For the experiment, 1 L of goat milk was used for each treatment. The milk was subjected to filtration and pasteurization at 64 ± 1 °C for 30 min, followed by cooling to 36 °C. Calcium chloride was then added at a concentration of 0.4 mL/L (Coalhopar, Coalhos Bio Paraná LTDA, Alto Piquiri, PR, Brazil).

Coagulation was carried out by adding 0.9 mL/L of the coagulant mixture, composed of different proportions of PAM and commercial chymosin (HA-LA®, Chr. Hansen, Brazil; strength 150 IMCU L⁻¹), according to each treatment. After the addition of the coagulants, the milk was left undisturbed until curd formation, with an average coagulation time of approximately 50 min (Jesus et al., 2024). The formed curd was cut into cubes of approximately 2 cm² and left to rest for 20 min for partial whey drainage. Subsequently, the curd mass was heated to 36 ± 1 °C under continuous stirring for 20 min. After this period, the whey was drained, and the curd was pressed and molded into molds of 10 cm in diameter. The cheeses were then unmolded, vacuum-packaged in sterile polyethylene bags (BS 320, R. Baião, Vila Casal, Ubá, MG, Brazil), and stored under freezing conditions until further analyses.

2.3 Physicochemical analyses

Analytical determinations were performed in triplicate according to the methodologies described by AOAC (2016). The physicochemical composition of the cheeses was determined by quantifying moisture content (AOAC 926.08), ash (AOAC 935.42), fat (AOAC 920.125), and protein (AOAC 2001.14). pH analyses were also carried out using a digital pH meter (Hanna Instruments, São Paulo, Brazil), and titratable acidity was determined according to AOAC method 920.124. Water activity (A_w) was measured using a benchtop water activity analyzer, model Aqualab Mode Series 3TE (Decagon Devices Inc., Pullman, Washington, USA), with measurements conducted at 25 °C. Yield (Y) was expressed as the mass of cheese obtained from 100 g of milk used in its production, as described by García-Gómez et al. (2019).

2.4 Instrumental color analysis

Instrumental color evaluation was performed using a portable colorimeter (Shenzhen Linshang Technology Co., Ltd., China). Measurements were carried out based on the CIE Lab* color system, in which L^* represents lightness, a^* indicates the variation between green (–) and red/magenta (+) tones, and b^* expresses the variation between blue (–) and yellow (+). The analyses were conducted under the standard illuminant D65 (daylight), in accordance with the recommendations of the International Commission on Illumination (CIE, 1996).

2.5 Texture Profile Analysis (TPA)

Texture profile analyses (TPA) were performed using a TA.HD Plus texture analyzer (Stable Micro Systems Ltd., Godalming, United Kingdom). Samples measuring 25 × 20 mm (height × diameter) were subjected to double compression using a 100 mm stainless steel parallel plate. The deformation rate was 1.0 mm/s, with a maximum compression of 35%, as described by Jesus et al. (2024). Test programming and data acquisition were carried out using the software *Texture Expert for Windows 1.20* (Stable Micro Systems, United Kingdom). Automatic processing of the experimental data, calculation of textural parameters, and report generation were performed using *Brookfield Texture Pro CT V1.4* software. The evaluated parameters included hardness, cohesiveness, springiness, resilience, gumminess, and chewiness. All analyses were performed in seven replicates for each cheese sample.

2.6 Determination of cheese rheology

Rheological measurements were performed using a TA Instruments Discovery® HR-20 rheometer coupled to a Solid State cooling system. A cone-plate geometry was employed, with a diameter of 50 mm and a gap of 0.104 mm. Samples were allowed to rest for 5 min for stabilization prior to the beginning of the assays. Initially, controlled-stress tests were conducted by varying the shear rate from 0.01 to 1 s⁻¹. Subsequently, strain sweep tests were carried out at a constant frequency in order to determine the linear viscoelastic region (LVR). Based on these results, frequency sweep analyses were performed over the range of 0.1 to 100 rad/s, maintaining a constant strain of 0.1%, corresponding to the LVR, for the determination of storage modulus (G'), loss modulus (G''), and loss tangent ($\tan \delta$). The equipment simultaneously records torque and angular displacement, converting them into shear stress and strain. In oscillatory mode, the viscoelastic parameters G' , G'' , and $\tan \delta$ were automatically calculated using TRIOS software.

2.7 Mid-infrared spectroscopy (MIR)

Cheese samples were analyzed by Mid-Infrared Spectroscopy (MIR) using FTIR-ATR (Fourier Transform Infrared Spectroscopy with Attenuated Total Reflectance) on a Cary® 630 FTIR instrument (Agilent Technologies Inc., Santa Clara, CA, USA). Spectra were acquired in the mid-infrared region, within the wavenumber range of 4000 to 600 cm⁻¹, with a resolution of 4 cm⁻¹, 64 scans per sample, and readings performed through a diamond crystal. The spectra were recorded in absorbance mode, with crystal background verification (blank) before each sample. All analyses were carried out at room temperature (25 ± 1 °C).

2.8 Near-infrared spectroscopy (NIR)

Cheese samples were analyzed by Near-Infrared Spectroscopy (NIR), with ambient temperature maintained at 25 °C during measurements. Spectra were collected using a Spectra Star 2500XL NIR spectrometer (Unity Scientific, Brookfield, CT, USA), equipped with a tungsten-halogen lamp as the light source and an indium gallium arsenide (InGaAs) detector. Signals were acquired in reflectance mode (%R) and converted into absorbance using the log 1/R relationship. Samples were placed in the instrument compartment and scanned over the range of 1100 to 2500 nm, at 1 nm intervals. Each data point represented the average of 64 scans, resulting in 1400 points per spectrum. Instrument setup, control, and data acquisition were performed using *Unity InfoStar V3.11.3* software.

2.9 Statistical analysis

Data are presented as analytical triplicates ($n = 3$) for each treatment. The results were subjected to one-way analysis of variance (ANOVA) to evaluate the effects of different percentages of commercial chymosin replacement on the analyzed variables. When significant differences were observed, means were compared using Tukey's test at a 5% significance level ($p < 0.05$). Statistical analyses were performed using the *Statistical Analysis System* (SAS) software. Graphs were generated using *Origin 2018* (OriginLab Corporation, Northampton, MA, USA).

3. RESULTS AND DISCUSSION

3.1 Analysis of the chemical composition of cheeses

The partial replacement of commercial chymosin by microbial aspartic protease (PAM) produced by *Penicillium roqueforti* significantly influenced ($p < 0.05$) some physicochemical composition parameters of Minas Frescal cheeses produced from goat milk (Table 1).

Table 1. Mean values ($\pm$ SD) of chemical composition (g/100 g) of Minas Frescal cheese produced from goat milk using different percentages of microbial aspartic protease (0–70%) as a partial substitute for commercial chymosin.

Treatments	Variable				
	Protein (%)	Moisture (%)	Lipids (%)	Ash (%)	Carbohydrates*
PAM0%	15.02 $\pm$ 0.24 ^b	64.66 $\pm$ 1.39 ^b	17.20 $\pm$ 0.75 ^{ab}	1.43 $\pm$ 0.06 ^c	1.68 $\pm$ 0.01 ^a
PAM10%	15.96 $\pm$ 0.13 ^{ab}	63.19 $\pm$ 0.37 ^{ab}	17.76 $\pm$ 0.83 ^{ab}	1.51 $\pm$ 0.04 ^c	1.58 $\pm$ 0.01 ^a
PAM20%	15.45 $\pm$ 0.79 ^b	63.17 $\pm$ 0.46 ^{ab}	18.07 $\pm$ 0.30 ^{ab}	1.57 $\pm$ 0.04 ^{cd}	1.74 $\pm$ 0.01 ^a
PAM30%	14.87 $\pm$ 0.76 ^b	64.84 $\pm$ 0.39 ^b	15.77 $\pm$ 0.59 ^b	1.48 $\pm$ 0.03 ^c	3.05 $\pm$ 0.22 ^b
PAM40%	15.24 $\pm$ 0.61 ^b	62.42 $\pm$ 0.90 ^{ab}	17.94 $\pm$ 0.10 ^{ab}	1.72 $\pm$ 0.05 ^{abd}	2.68 $\pm$ 0.12 ^b
PAM50%	15.06 $\pm$ 0.55 ^b	62.20 $\pm$ 1.31 ^{ab}	18.10 $\pm$ 0.79 ^{ab}	1.79 $\pm$ 0.06 ^{ab}	2.85 $\pm$ 0.04 ^b
PAM60%	14.93 $\pm$ 0.44 ^b	62.98 $\pm$ 0.97 ^{ab}	17.43 $\pm$ 0.68 ^{ab}	1.61 $\pm$ 0.04 ^{bcd}	3.05 $\pm$ 0.21 ^b
PAM70%	17.86 $\pm$ 0.48 ^a	60.54 $\pm$ 1.50 ^a	18.24 $\pm$ 0.56 ^a	1.85 $\pm$ 0.07 ^a	1.51 $\pm$ 0.07 ^a

PAM = percentage of microbial aspartic protease in the formulation.

Different lowercase superscript letters within the same column indicate significant differences ($p < 0.05$) among the values obtained for the different treatments, according to Tukey's test.

Treatments with higher proportions of microbial aspartic protease (PAM) showed lower moisture content and higher protein concentration, suggesting greater efficiency of the syneresis process and consequent whey expulsion. Protein content ranged from 14.87 to 17.86%, with the highest value observed in treatment PAM70%, while the remaining treatments

showed statistically similar values. Similarly, a lower moisture content was observed in treatment PAM70%, which may be related to the action of aspartic protease on different casein fractions, unlike chymosin, whose activity is predominantly specific to κ -casein. This broader cleavage may favor structural rearrangements and greater contraction of the protein network, resulting in a less hydrated matrix and enhanced whey release (Mohsin et al., 2024; Cai et al., 2023). In addition, increased protein–protein interactions or greater formation of cross-links between protein chains within the gel network may also contribute to this behavior, promoting whey release depending on the type of coagulant used (Türkmen and Güler, 2022). In this context, cheese moisture not only affects product yield but is also a determining factor for its quality characteristics (Jia et al., 2021). The moisture content of Minas Frescal cheeses produced from goat milk ranged from 60 to 64% (Table 1), with all treatments remaining within the standards of identity and quality established for very high-moisture cheeses, a characteristic typical of Minas Frescal cheese according to Brazilian legislation (Brazil, 2004).

Lipid contents ranged from 15.77 to 18.24%, remaining high across all treatments and, in most formulations, exceeding protein levels. This behavior indicates high lipid retention efficiency within the cheese matrix, a characteristic commonly observed in fresh cheeses produced from goat milk and without pressing. The elevated lipid retention in goat cheeses is partly attributed to the particularities of goat milk, such as the smaller size of fat globules, which favors their incorporation into the casein network formed during coagulation (Stocco et al., 2022).

The differences observed in ash content may be mainly associated with the concentration effect of solids in the final product, since treatment PAM70% showed lower moisture content. As the cheeses were produced without the addition of sodium chloride, ionic exchange between sodium and calcium in the paracasein matrix does not occur, which influences mineral distribution between the colloidal and soluble phases without directly interfering with the intrinsic mineral composition of the product (Gomes et al., 2011; Matera et al., 2018).

Total carbohydrate content ranged from 1.51% to 3.05%. This behavior may be related to the greater participation of the protein fraction in the cheese matrix, since an increase in protein content tends to reduce the relative proportion of other constituents such as carbohydrates (Acevedo-Correa et al., 2025).

Regarding yield, treatment PAM50% showed a statistically similar value to the control PAM0% (Table 2), indicating that the partial replacement of commercial chymosin by microbial aspartic protease up to this level does not compromise cheese yield.

Table 2. Yield, water activity (Aw), pH, and titratable acidity of Minas Frescal cheeses produced from goat milk with increasing addition of microbial aspartic protease (0–70%) and proportional reduction of commercial chymosin.

Treatments	Variable			
	Yield	Aw	pH	Acidity
PAM0%	22.70±0.05 ^c	0.995±0.0 ^a	6.70±0.00 ^{ab}	0.3±0.0 ^a
PAM10%	23.30±0.16 ^{ed}	0.995±0.0 ^a	6.71±0.02 ^{ab}	0.3±0.0 ^a
PAM20%	23.43±0.13 ^e	0.993±0.0 ^a	6.67±0.02 ^a	0.3±0.0 ^a
PAM30%	22.89±0.20 ^{cd}	0.994±0.0 ^a	6.78±0.04 ^b	0.35±0.07 ^a
PAM40%	22.64±0.16 ^c	0.993±0.0 ^a	6.70±0.03 ^{ab}	0.3±0.0 ^a
PAM50%	22.44±0.11 ^c	0.996±0.0 ^a	6.67±0.02 ^a	0.35±0.07 ^a
PAM60%	21.03±0.13 ^b	0.994±0.0 ^a	6.78±0.04 ^b	0.35±0.07 ^a
PAM70%	20.07±0.05 ^a	0.994±0.0 ^a	6.76±0.02 ^{ab}	0.4±0.0 ^a

PAM = percentage of microbial aspartic protease in the formulation.

Different lowercase superscript letters within the same column indicate significant differences ($p < 0.05$) among the values obtained for the different treatments, according to Tukey's test.

This result is relevant, since microbial proteases exhibit cleavage specificity toward κ -casein similar to that of chymosin, although they may induce greater nonspecific proteolytic activity during cheese processing (Stocco et al., 2025). In this context, it is important to note that rennet substitutes should present relatively weak proteolytic activity in order to preserve product quality, as excessive proteolysis may cause yield losses during manufacture due to κ -casein denaturation in whey, in addition to promoting the development of bitter flavor in the final product (García-Gómez et al., 2019). Thus, the partial replacement of chymosin up to the level of 50% microbial aspartic protease did not compromise cheese yield, highlighting the possibility of its use without technological drawbacks to the product.

pH varied among treatments, although values remained within the characteristic range of Minas Frescal cheese. Similar values were reported by Azorín et al. (2025), who observed pH values of 6.85 and 6.80 in fresh cheeses produced from goat milk. It should be emphasized that pH is a fundamental parameter, as it directly influences the structural and rheological characteristics of cheese, since variations in this parameter may alter protein–water interactions, affecting the firmness and cohesion of the final product matrix (Difonzo et al., 2024).

Regarding water activity and titratable acidity, no differences were observed among treatments, suggesting similar physicochemical behavior across formulations.

3.2 Effect on color parameters

The color parameters of goat Minas Frescal cheeses are presented in Table 3, while the visual appearance of the samples can be observed in Figure 1. A statistically significant difference was observed for the lightness parameter (L^*) ($p < 0.05$), with values ranging from 83.32 to 90.62, indicating predominantly light coloration in all treatments, a characteristic typical of this type of cheese.

Table 3. Color parameters (L^* , a^* , and b^*) of Minas Frescal cheeses produced from goat milk with different concentrations of microbial aspartic protease (0–70%) and proportional reduction of commercial chymosin.

Treatments	Color parameter		
	L	a	b
PAM0%	87.94± 0.42 ^g	-3.66± 0.05 ^d	7.39± 0.23 ^a
PAM10%	90.62± 0.13 ^{df}	-3.76± 0.03 ^{cd}	7.47± 0.18 ^a
PAM20%	90.60± 0.14 ^d	-3.78± 0.01 ^{cd}	7.45± 0.07 ^a
PAM30%	89.79± 0.34 ^{de}	-3.84± 0.04 ^c	7.43± 0.08 ^a
PAM40%	86.19± 0.36 ^c	-3.76± 0.03 ^{cd}	7.45± 0.09 ^a
PAM50%	85.61± 0.27 ^c	-3.56± 0.06 ^{bd}	7.43± 0.08 ^a
PAM60%	84.45± 0.27 ^b	-3.45± 0.03 ^b	7.35± 0.19 ^a
PAM70%	83.32± 0.30 ^a	-3.20± 0.07 ^a	7.28± 0.08 ^a

PAM = percentage of microbial aspartic protease in the formulation.

Different lowercase superscript letters within the same column indicate significant differences ($p < 0.05$) among the values obtained for the different treatments, according to Tukey's test.

L^* represents color lightness ($L^* = 0$ corresponds to black and $L^* = 100$ indicates diffuse white), a^* represents its position between red/magenta and green (negative values indicate green, whereas positive values indicate

magenta), and b^* represents its position between yellow and blue (negative values indicate blue, whereas positive values indicate yellow).

Color is a crucial attribute that directly impacts consumer perception of product quality. Together with parameters such as flavor and texture, it exerts a determining influence on the evaluation of overall product quality (Akbar et al., 2025). Goat cheeses usually present a white coloration due to the smaller size of fat globules and the nearly complete conversion of β -carotene into vitamin A (Miloradovic et al., 2018a; Verruck, Dantas, and Prudencio, 2019). Similar L^* results have been widely reported for fresh goat milk cheeses in the literature (Trejo-López et al., 2021; Wang et al., 2023; Muñoz-Bas et al., 2024).

Cheese lightness is influenced by the presence of colloidal particles in milk, such as fat globules and casein micelles, which promote light scattering in the visible spectrum. The white coloration of cheese is associated with the protein matrix formed during coagulation, which disperses visible light in multiple directions, resulting in a characteristic whitening effect of the product (Singh et al., 2023). Thus, the differences observed among treatments may be related to subtle changes in the structural organization of the protein matrix, promoted by the different levels of microbial aspartic protease used in the formulation.

The values of parameter a^* were negative in all treatments, indicating a greenish coloration, with statistically significant differences among them ($p < 0.05$), ranging from -3.20 to -3.84 . Regarding parameter b^* , no statistically significant differences were observed among treatments ($p > 0.05$), with mean values between 7.3 and 7.5 , indicating maintenance of the yellowish tone characteristic of goat milk Minas Frescal cheese.



Figure 1. Visual appearance of Minas Frescal cheeses produced from goat milk with different concentrations of microbial aspartic protease (0–70%) and proportional reduction of commercial chymosin.

3.3 Texture Profile Analysis (TPA)

The effect of different concentrations of microbial aspartic protease and the proportional reduction of commercial chymosin on the texture of Minas Frescal cheeses was evaluated through texture profile analysis, whose mean values and standard deviations are presented in Table 4. Texture is a fundamental characteristic that defines cheese identity and directly influences consumer acceptance (Kondyli et al., 2023), being strongly dependent on composition, internal structure, and intermolecular forces present in the product matrix (Akbar et al., 2025). The cheeses were evaluated for the parameters of hardness, cohesiveness, springiness, resilience, and gumminess.

Table 4. Texture profile analysis (TPA) parameters measured in Minas Frescal cheeses produced from goat milk with different concentrations of microbial aspartic protease (0–70%) and proportional reduction of commercial chymosin.

Treatments	Texture variable				
	Hardness (N)	Cohesiveness	Springiness	Resilience	Gumminess (N)
PAM0%	1.80± 0.27 ^b	0.39± 0.03 ^a	3.82± 0.04 ^a	0.17± 0.02 ^b	0.67± 0.16 ^b
PAM10%	1.83± 0.18 ^{ab}	0.40± 0.04 ^a	3.56± 0.19 ^a	0.20± 0.01 ^{ab}	0.72± 0.15 ^{ab}
PAM20%	2.46± 0.59 ^{ab}	0.43± 0.04 ^a	3.71± 0.33 ^a	0.20± 0.01 ^{ab}	1.04± 0.24 ^{ab}
PAM30%	2.59± 0.52 ^{ab}	0.45± 0.02 ^a	3.83± 0.28 ^a	0.20± 0.01 ^{ab}	1.12± 0.22 ^{ab}
PAM40%	2.60± 0.79 ^{ab}	0.45± 0.05 ^a	3.83± 0.84 ^a	0.21± 0.03 ^{ab}	1.12± 0.23 ^{ab}
PAM50%	2.61± 0.68 ^{ab}	0.44± 0.01 ^a	3.83± 0.40 ^a	0.21± 0.01 ^{ab}	1.13± 0.32 ^{ab}
PAM60%	2.82± 0.50 ^{ab}	0.44± 0.03 ^a	3.92± 0.28 ^a	0.22± 0.02 ^a	1.20± 0.36 ^{ab}
PAM70%	3.06± 0.53 ^a	0.44± 0.04 ^a	4.26± 0.24 ^a	0.23± 0.01 ^a	1.34± 0.36 ^a

PAM = percentage of microbial aspartic protease in the formulation.

Different lowercase superscript letters within the same column indicate significant differences ($p < 0.05$) among the values obtained for the different treatments, according to Tukey's test.

As presented in Table 4, the PAM70% treatment, containing the highest concentration of aspartic protease, showed the highest hardness value (3.06 N), whereas the control treatment PAM0%, produced exclusively with commercial chymosin, exhibited the lowest value (1.80 N), with statistically significant differences among treatments. This behavior may be associated with the higher concentration of aspartic protease, which may favor increased protein–protein interactions and the formation of a more rigid protein matrix, in addition to the lower moisture retention observed in this treatment. Increased moisture in cheese reduces the hydrophobic interactions responsible for the stability of the casein matrix and promotes greater spacing between proteins, thereby weakening the gel network, making the matrix less compact, and resulting in lower hardness and greater softness of the product (Wu et al., 2025; Trejo-López et al., 2021), as observed in the PAM0% treatment. Despite this increase in hardness, the observed values remained consistent with the expected characteristics of Minas Frescal cheese, which is known for its soft texture and moderate resistance to compression, without compromising the product identity.

The cohesiveness and springiness parameters did not show statistically significant differences among treatments, indicating that, despite the variations observed in the rigidity of the protein matrix, the structural integrity of the gel was not significantly affected by the different concentrations of aspartic protease. Cohesiveness, which expresses the ability of the material to withstand successive deformations without rupture, is related to the strength of the internal bonds within the protein matrix (Moraes et al., 2018; Akbar et al., 2025), whereas springiness is defined as the rate at which a deformed sample returns to its original state after removal of the applied force, serving as an indicator of its viscoelastic behavior (Costa et al., 2024). The absence of variation in these parameters suggests that the protein bonds formed during coagulation exhibited a similar structural organization among treatments (Miloradovic et al., 2018b).

Resilience, which refers to the ability of cheese to recover part of its original shape after being subjected to a first compression cycle and is associated with the initial elastic behavior of the matrix (Muñoz-Bas et al., 2024), showed a significantly higher value in the PAM70% treatment, reflecting a greater capacity for immediate structural recovery after deformation, which is consistent with the formation of a more compact protein matrix.

Gumminess followed the same trend as hardness, presenting a higher value in the PAM70% treatment, associated with the greater structural rigidity observed in this formulation, since this parameter results from the interaction between product hardness and cohesiveness (Kondyli et al., 2023). Gumminess corresponds to the amount of energy required to disintegrate

a semisolid food to a consistency suitable for swallowing and is directly related to the mechanical resistance of the protein matrix (Najjar et al., 2025). It has been reported that the structural organization of the network formed by casein micelles plays a determinant role in the textural properties of cheese (Wang et al., 2023). In this context, although higher protease levels promoted greater resistance to mechanical deformation, the formulations maintained characteristics consistent with the technological standard of Minas Frescal cheese.

3.4 Rheological properties of cheeses

Figure 2 presents the results of the frequency sweep analysis of Minas Frescal cheeses produced with different concentrations of microbial aspartic protease and proportional reduction of commercial chymosin, expressed in terms of storage modulus (G'), loss modulus (G''), and loss tangent ($\tan \delta$). In all treatments, G' values were higher than G'' values throughout the entire evaluated frequency range, indicating a viscoelastic behavior with predominance of the elastic response, which is characteristic of protein gels (Alinovi; Mucchetti, 2020; Moghiseh et al., 2021).

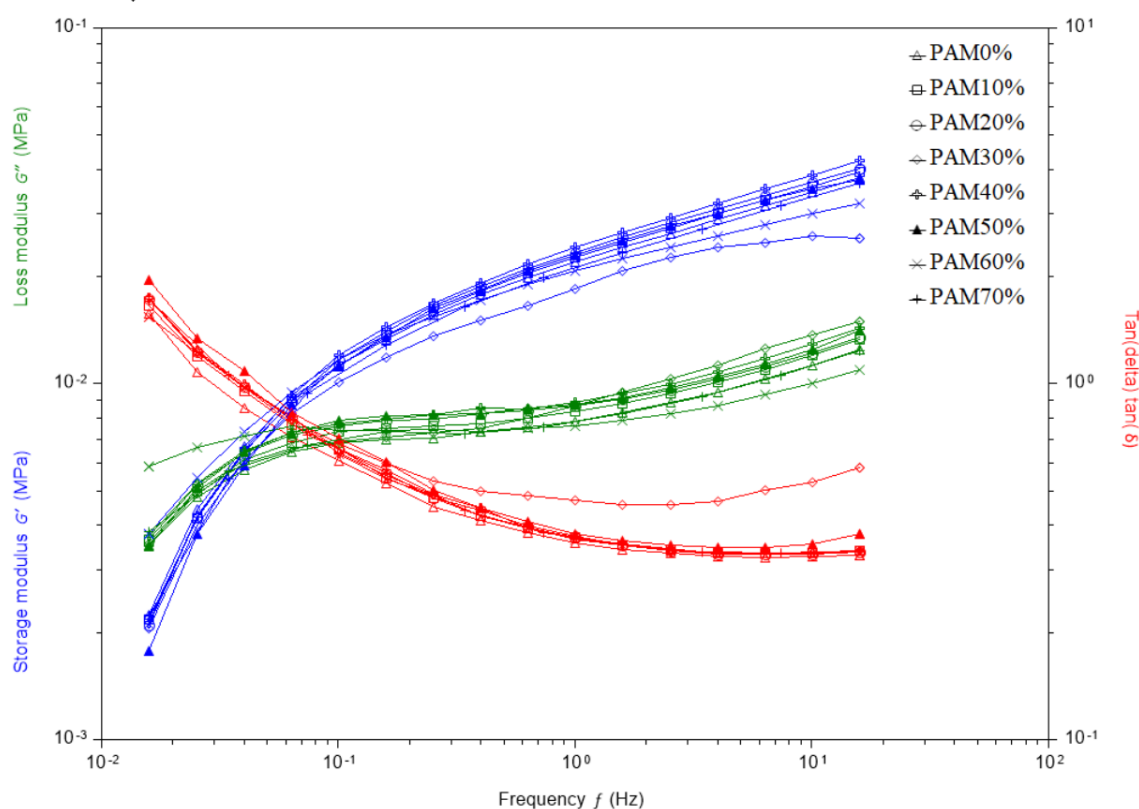


Figure 2. Viscoelastic behavior of Minas Frescal cheeses produced from goat milk with different concentrations of microbial aspartic protease (0–70%) and proportional reduction of

commercial chymosin, expressed by the storage modulus (G'), loss modulus (G''), and loss tangent ($\tan \delta$) as a function of frequency, obtained at 25 °C within the linear viscoelastic region.

The storage modulus (G') reflects the elastic fraction of the material, whereas the loss modulus (G'') is associated with the viscous dissipation of energy. Therefore, the predominance of G' over G'' indicates a cohesive three-dimensional network, with a greater elastic than viscous contribution, as described for fresh cheeses in the literature (Soltani; Boran; Hayaloglu, 2016; Wang et al., 2023).

The G' curves showed a positive slope throughout the evaluated frequency range, indicating an increase in the dynamic rigidity of the cheeses under higher deformation frequencies. This behavior demonstrates the sensitivity of viscoelastic moduli to the applied frequency, a characteristic commonly observed in structured systems whose mechanical response varies according to the timescale of the imposed stress. The maintenance of this pattern across all treatments indicates that the partial replacement of chymosin by aspartic protease did not promote substantial changes in the overall viscoelastic behavior of the samples. According to Dussault-Chouinard, Britten, and Pouliot (2019), frequency dependence is related to the time required for the recovery of structural interactions after deformation.

G'' values also increased with frequency; however, they remained lower than G' across all treatments, demonstrating that energy dissipation was lower than the energy stored elastically. This behavior reinforces the stability of the protein network formed, even under partial replacement of chymosin. Furthermore, the differences among treatments did not indicate disruption of the expected pattern for Minas Frescal cheese, but rather gradual adjustments in firmness and structural resistance, without compromising the product's identity.

The relationship between G'' and G' , expressed as $\tan \delta$, also reinforced the predominance of the elastic response in the samples. Across all treatments and evaluated frequencies, values remained below 1, indicating a greater contribution of the elastic component relative to the viscous one (Yang et al., 2019; Biswas; Salunke; Metzger, 2025). This behavior confirms the preservation of the structural integrity of the protein matrix, even with the incorporation of aspartic protease, maintaining the viscoelastic profile expected for fresh cheeses.

3.5 Mid-infrared spectroscopy (MIR)

In goat milk cheese, the casein matrix is stabilized by different intermolecular interactions, mainly electrostatic interactions, as well as hydrophobic interactions and hydrogen bonds. The absorption peaks associated with the functional groups of lipids and proteins in FTIR spectra may indicate possible alterations in these structural interactions within the cheese matrix (Wang et al., 2023). As shown in Figure 3, the FTIR spectra of goat milk Minas Frescal cheeses, produced with different concentrations of aspartic protease and proportional reduction of commercial chymosin, exhibited similar spectral profiles, with strong overlap of bands throughout the entire analyzed spectral range. This behavior suggests that the incorporation of aspartic protease did not promote detectable global chemical modifications nor significant alterations in the functional groups associated with the proteins and lipids of the cheese matrix.

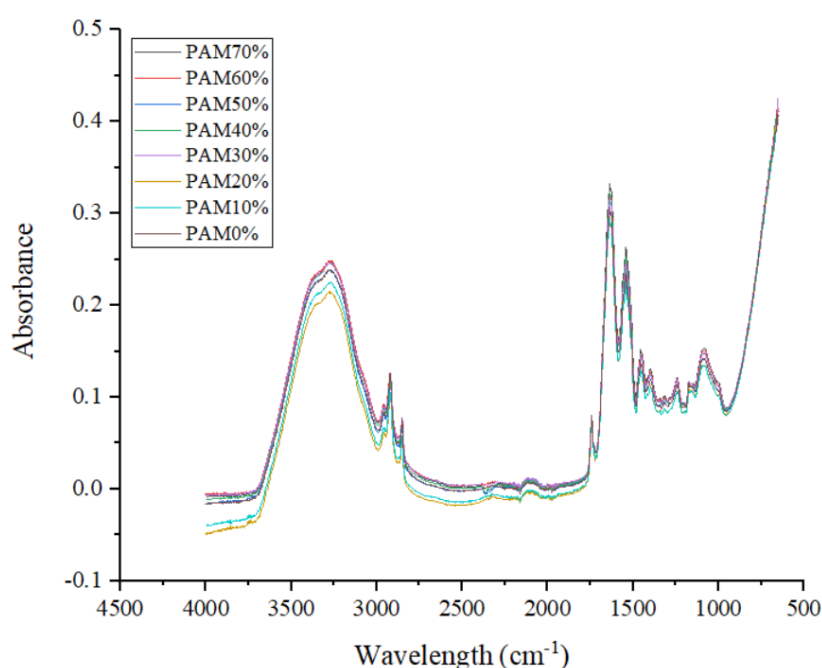


Figure 3. Infrared spectra of Minas Frescal cheeses produced from goat milk, formulated with different concentrations of microbial aspartic protease (0–70%) and proportional reduction of commercial chymosin, obtained by Fourier-transform infrared spectroscopy (FTIR) within the spectral range of 4,000 cm^{-1} to 600 cm^{-1} .

Overall, the FTIR spectra showed similar patterns among the samples, corresponding to the main constituents of cheese, such as water, proteins, and lipids. This was expected, since all samples corresponded to Minas Frescal cheese produced from the same goat milk, differing only in the concentrations of aspartic protease and the proportional reduction of commercial chymosin. In the high-frequency region, the broad band between 3600 and 3100 cm^{-1} is

attributed to the $\nu(\text{O-H})$ stretching modes of water molecules. Only slight variations in the intensity of this band were observed among the samples, possibly associated with minor differences in water-protein interactions, with no evidence of relevant structural alterations. The triple bands in the $3000\text{--}2800\text{ cm}^{-1}$ range correspond to $\nu(\text{C-H})$ stretching vibrations of methyl ($-\text{CH}_3$) and methylene ($-\text{CH}_2$) groups present in proteins and lipids (Ong et al., 2020). These bands maintained similar intensities across treatments, indicating preservation of the lipid fraction of the cheese.

In the low-frequency region, the absorption observed at approximately 1751 cm^{-1} was attributed to the stretching vibrations of the C=O bond of fatty acid esters (Ozturk et al., 2022), with no relevant differences in position or intensity among the samples. The spectral range between 1680 and 1239 cm^{-1} is associated with protein vibrations. Specifically, the regions of $1680\text{--}1631\text{ cm}^{-1}$, $1560\text{--}1533\text{ cm}^{-1}$, and $1472\text{--}1239\text{ cm}^{-1}$ correspond to the amide I, amide II, and amide III bands, respectively. These bands are widely used to evaluate the structural organization of proteins in food matrices. Amide I is predominantly associated with the C=O stretching of peptide bonds, whereas amide II mainly results from the combination of N-H and C-N stretching vibrations. Amide III, in turn, is related to the combination of N-H bending and C-N stretching (Yaman et al., 2022).

Although these bands showed slight variations in intensity among the spectra, no peak shifts or changes in band shape were observed, suggesting that the replacement of chymosin with aspartic protease did not promote detectable modifications in the global conformation of proteins, being limited to subtle differences in spectral intensity.

In addition, the bands observed in the range of 1120 to 1040 cm^{-1} are associated with C-O stretching modes and O-H angular deformation of organic acids, as well as the presence of residual lactose. The consistency of these spectral signals among the different treatments suggests that the chemical organization of the protein matrix and soluble constituents was preserved, even with variation in the concentrations of aspartic protease employed during coagulation.

3.6 Near-infrared spectroscopy (NIR)

The average raw NIR spectrum of the cheese samples, obtained using the NIR spectrometer, is presented in Figure 4. The overall spectral pattern of the cheese samples produced with different concentrations of aspartic protease and proportional reduction of commercial chymosin was highly similar, with only slight variations in the intensities of the absorption bands.

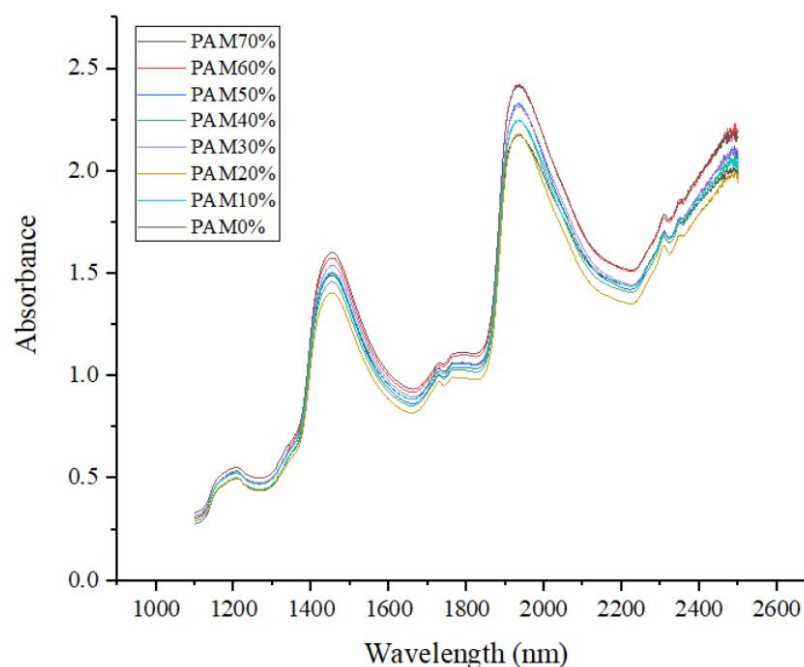


Figure 4. Near-infrared (NIR) absorption spectra of Minas Frescal cheeses produced from goat milk, formulated with different concentrations of microbial aspartic protease (0–70%) and proportional reduction of commercial chymosin, obtained within the spectral range of 1100 to 2500 nm.

The NIR absorbance spectra of the cheeses exhibited bands predominantly associated with the presence of water in the regions of 1400–1455 nm and 1900–1950 nm, corresponding, respectively, to the first and second overtones of the stretching and bending vibrations of the O–H group. Spectral regions between 1210–1220 nm, 1700–1730 nm, and 2300–2350 nm can be mainly related to combination vibrations and overtones of C–H bonds, associated with the lipid fraction. Proteins and peptides contributed to the broad bands observed between 1500–2056 nm and near 2174 nm, primarily attributed to vibrations involving N–H bonds (Manuelian et al., 2017; Karaziack et al., 2024).

Overall, the results obtained by NIR spectroscopy indicate that variation in the concentration of aspartic protease employed during coagulation did not promote relevant changes in the global chemical composition of the cheeses, since the spectra exhibited similar profiles, differing mainly in the relative intensity of bands associated with the main constituents of the matrix. This behavior reinforces the interpretation that the modifications resulting from enzyme use occurred at a functional or microstructural level, without detectable repercussions on the overall chemical composition assessed by NIR. It should be emphasized that the analysis was conducted based on raw spectra, allowing a qualitative evaluation of the components.

CONCLUSIONS

The partial replacement of commercial chymosin with aspartic protease produced by *Penicillium roqueforti* proved to be technically feasible in the production of goat milk Minas Frescal cheese. The results indicated that substitution can be carried out at partial levels without compromising yield, physicochemical stability, or product identity, with intermediate treatments showing the best balance between technological performance and maintenance of the structural characteristics expected for Minas Frescal cheese.

The use of the protease promoted subtle modifications in the organization of the protein matrix, reflected mainly in the textural, mechanical, and rheological properties of the cheese. These changes, evidenced by viscoelastic behavior and instrumental texture parameters, occurred without altering the typical structure of the product, which maintained characteristics compatible with a high-moisture fresh cheese. FTIR and NIR spectroscopic analyses reinforced that these modifications were predominantly structural and functional, associated with adjustments in the intermolecular interactions of the casein network, rather than with chemical or global conformational transformations of the proteins.

Thus, the protease shows potential as an alternative or complementary coagulant to chymosin, expanding the technological possibilities for the production of fresh goat cheeses and contributing to the diversification of coagulating agents in the dairy sector. In addition, the findings open perspectives for future investigations involving sensory evaluation, storage behavior, and more precise definition of substitution limits at industrial scale, aiming to consolidate its technological application.

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CHAPTER V

FINAL CONSIDERATIONS

FINAL CONSIDERATIONS

This thesis demonstrated that the valorization of agro-industrial residues through solid-state fermentation represents a promising, sustainable, and technically efficient biotechnological strategy for obtaining microbial proteases with high potential for application in the food industry. The use of alternative plant-based substrates, especially combinations of ora-pro-nóbis and soybean meal, proved to be viable for the production of aspartic protease by *Penicillium roqueforti*, highlighting that regional and low-cost raw materials can be converted into biocatalysts of industrial interest.

The results confirmed that the integration of statistical design and solid-state fermentation was essential to maximize enzyme production, enabling the definition of suitable operational conditions and contributing to the advancement of biotechnological processes applied to residue valorization. In addition, the produced protease exhibited biochemical and technological properties compatible with food applications, including high milk-clotting activity, stability under processing-relevant conditions, and favorable catalytic specificity.

In the functional field, the enzyme demonstrated high efficiency in the hydrolysis of caseins from different origins, promoting the release of bioactive peptides with significant antioxidant and α -amylase inhibitory activities. These findings broaden the potential use of dairy proteins as a source of natural ingredients for the development of functional foods and nutraceuticals, highlighting the versatility of the protease as a biotechnological tool for generating high value-added compounds.

From a technological perspective, its application in goat milk coagulation confirmed the feasibility of partially replacing commercial chymosin in the production of Minas Frescal cheese, without compromising yield, physicochemical stability, or product identity. The observed modifications were predominantly structural, reflected in adjustments in the protein matrix and in textural and rheological properties, without detectable global chemical alterations. This result reinforces the possibility of incorporating the enzyme as an alternative or complementary coagulant in dairy systems.

Overall, this research demonstrates that the aspartic protease from *Penicillium roqueforti* combines functional and technological attributes that position it as a strategic input for different segments of the food industry. By associating sustainability, innovation, and value addition to agro-industrial residues, this thesis contributes to strengthening the bioeconomy and to the development of solutions aligned with contemporary demands for more circular and efficient production processes.

Finally, the findings open perspectives for future studies focused on large-scale purification, structural identification of the generated peptides, sensory and stability evaluation of the developed products, as well as the consolidation of the enzyme's industrial application in different food matrices. In this way, the work not only achieves its scientific objectives but also establishes solid foundations for technological and market advances in the sustainable use of agro-industrial resources.