



UNIVERSIDADE ESTADUAL DO SUDOESTE DA BAHIA – UESB
PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA E CIÊNCIA DE
ALIMENTOS

Área de Concentração: Engenharia de Alimentos

FUNCIONALIZAÇÃO DE NANOPARTÍCULAS MAGNÉTICAS COM
GENIPINA PARA A IMOBILIZAÇÃO DE PROTEASES E VALORIZAÇÃO
BIOTECNOLÓGICA DO SORO DE LEITE

JONATHAN BARBOSA SANTOS

ITAPETINGA
BAHIA – BRASIL
Fevereiro de 2026

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Tese apresentada como parte das exigências para a obtenção do título de Doutor em Engenharia e Ciência de Alimentos, no Programa de Pós-Graduação em Engenharia e Ciência de Alimentos da Universidade Estadual do Sudoeste da Bahia.

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Itapetinga-BA, 24 de fevereiro de 2026.

Avançar nem sempre é confortável, mas permanecer imóvel diante do medo tem um custo ainda maior

A Deus.
Aos meus pais.
Dedico!

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SUMÁRIO

LISTA DE TABELAS	xiii
LISTA DE FIGURAS	xiv
RESUMO	xvi
ABSTRACT	xvii
CAPÍTULO 1	18
1. INTRODUÇÃO GERAL	19
2. OBJETIVO GERAL	22
2.1 Objetivos específicos	22
REFERÊNCIAS	23
CAPÍTULO 2 – ARTIGO 1	25
REVIEW ARTICLE: IMMOBILIZATION OF PROTEASES ON MAGNETIC NANOPARTICLES: A CRITICAL REVIEW OF FUNCTIONALIZATION STRATEGIES, CHARACTERIZATION, AND CATALYTIC PERFORMANCE	25
1. INTRODUCTION	26
2. PROTEASES: PROPERTIES AND REQUIREMENTS FOR IMMOBILIZATION	29
2.1 General protocols for enzyme immobilization	29
2.1.1 Chemical methods	29
2.1.2 Physics methods	30
2.2 Proteases: Properties and specific requirements for immobilization	30
2.3 Influence of composition and structure on coupling strategy	34
2.4 Regulatory Aspects and Safety (GRAS Status)	34
3. MAGNETIC NANOPARTICLES	35
3.1 Magnetic nanoparticles	35
3.2 Methods for Nanoparticle Production	36
3.2.1 Chemical methods	39
3.2.2 Physical Methods	40
3.3 Advantages and disadvantages of synthesis methods	40

3.4 MNPs characteristics	43
4. STRATEGIES FOR FUNCTIONALIZING MNPs	44
4.1 Surface functionalization.....	45
4.2 Coating techniques	47
4.2.1 Organic coating.....	48
4.2.2 Inorganic coatings.....	49
5. IMPACTS ON THE MICROENVIRONMENT DUE TO FUNCTIONALIZATION AND IMMOBILIZATION	50
5.1 Effect of the microenvironment on specificity and hydrolysis profile	50
5.2 Modulation of kinetic parameters (k _{cat} /K _m)	51
6. PROTEOLYTIC BIOREACTORS AND THEIR APPLICATIONS.....	51
6.1 Trends in protease immobilization in MNPs	51
6.2 Trends in functionalization and support	56
6.3 Operational stability and reuse	56
6.4 Classes of proteases and applications	56
7. FUTURE PERSPECTIVES AND CHALLENGES IN THE IMMOBILIZATION OF PROTEASES IN MNPs	57
7.1 Rational Design via Molecular Modeling and Dynamics (MD)	58
7.2 Intelligent and stimulus-responsive systems	59
7.3 Sustainability and technological integration.....	59
8. CONCLUSIONS	59
REFERENCES	61
CAPÍTULO 3 – ARTIGO 2.....	72
GENIPIN-FUNCTIONALIZED MAGNETIC MATERIALS: A SUSTAINABLE AND INNOVATIVE METHOD FOR IMMOBILIZING PROTEASES TO PRODUCE ANTIOXIDANT PEPTIDES	72
1. INTRODUCTION	74
2. MATERIALS AND METHODS	75

2.1 Materials	75
2.2 Synthesis of Fe ₃ O ₄ magnetic nanoparticles (MNPs)	76
2.3 Functionalization of MNPs.....	76
2.4 Characterization of MNPs	77
2.5 Immobilization of trypsin and bromelain	77
2.6 Enzyme activity determination.....	78
2.7 Effect of pH and temperature on enzyme activity	79
2.8 Evaluation of bioreactor reuse cycles.....	79
2.10 Chromatographic profile of peptides obtained from casein hydrolysis.....	79
2.11 Antioxidant Capacity of the Obtained Peptides (DPPH, ABTS and BCAL)	80
3. RESULTS AND DISCUSSION	80
3.1 Obtaining MNPs	80
3.2 Characterization of the MNPs produced	81
3.2.1 Evaluation of MNP functionalization by FTIR spectroscopy	81
3.2.2 X-ray Diffraction Analysis (XRD)	82
3.2.3 Thermogravimetric analysis	83
3.2.4 Surface morphology (SEM) and elemental composition (EDS)	85
3.2.5 Particle Size and Zeta Potential of MNPs	87
3.3 Immobilization and catalytic activity of free enzymes and enzyme-loaded bioreactors	88
3.4 Effect of pH and temperature on the activity of free and immobilized enzymes	90
3.5 Operational stability and reuse of the bioreactors	92
3.6 Chromatographic profiles of casein and the peptides obtained from its hydrolysis..	93
3.7 Assessment of the antioxidant activity of peptides obtained from enzymatic hydrolysis	94
4. CONCLUSIONS	96
REFERENCES	97
SUPPLEMENTARY DATA FILE	107

CAPÍTULO 4 – ARTIGO 3.....	111
AGREGAÇÃO DE VALOR AO SORO DE LEITE POR HIDRÓLISE ENZIMÁTICA COM TRIPSINA IMOBILIZADA EM NANOPARTÍCULAS MAGNÉTICAS: DESEMPENHO EM UMA MATRIZ COMPLEXA	111
1. INTRODUÇÃO.....	113
2. MATERIAS E MÉTODOS	115
2.1 Reagentes.....	115
2.2 Síntese, Funcionalização e Imobilização de tripsina em MNPs.....	115
2.3 Determinação da atividade enzimática	116
2.3 Produção dos peptídeos via hidrólise enzimática do soro de leite	116
2.4 Perfil cromatográfico dos peptídeos obtidos da hidrólise do soro de leite.....	117
2.5 Determinação do Grau de Hidrólise (DH).....	117
2.6 Capacidade antioxidante dos extratos peptídicos obtidos da hidrólise do soro de leite	118
2.6.1 Atividade de sequestro de radicais livres DPPH	118
2.6.2 Atividade de sequestro de radicais livres ABTS.....	118
2.6.3 Ensaio do Poder antioxidante redutor férrico (FRAP)	119
2.6.4 Inibição da oxidação do sistema β -caroteno-ácido linoléico.....	119
3. RESULTADOS E DISCUSSÕES	120
3.1 Imobilização e Atividade enzimática da tripsina livre e imobilizada	120
3.2 Reutilização dos biorreatores.....	121
3.3 Perfil cromatográfico do soro de leite e peptídeos obtidos da hidrólise.....	123
3.4 Avaliação do Grau de Hidrólise (DH) pelo ensaio o-Ftaldialdeído (OPA).....	124
3.5 Determinação do potencial antioxidante dos peptídeos produzidos.....	125
4. CONCLUSÃO.....	131
CAPÍTULO 5	139
CONSIDERAÇÕES FINAIS	140

LISTA DE TABELAS

CAPÍTULO 2

Table 1. Origin, industrial applications, and immobilization requirements of the main proteases	33
Table 2. Chemical and physical approaches for magnetic nanoparticle production and their recent applications.....	37
Table 3. Evaluation of the main magnetic nanoparticle synthesis techniques based on their benefits and challenges.....	41
Table 4. Performance of proteases immobilized in magnetic nanoparticles and their applications in bioprocesses	54

CAPÍTULO 3

Table 1. Estimation of crystallite size and crystallization state of MNPs by XRD.	83
Table 2. Effect of functionalization on the particle size and zeta potential of MNPs....	87
Table 3. Immobilization parameters and catalytic performance of the bioreactors	88
Table 4. Antioxidant activity measured by DPPH, ABTS, and BCAL assays for hydrolysates produced from casein using free Trypsin (FT), immobilized Trypsin (IT), free Bromelain (FB), and immobilized Bromelain (IB).	94

CAPÍTULO 4

Tabela 1. Eficiência de imobilização e atividade enzimática	120
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LISTA DE FIGURAS

CAPÍTULO 2

Figure 1. Representation of the catalytic mechanisms of proteases and their interactions with the substrate	31
Figure 2. Schematic representation of the synthesis of magnetic nanoparticles by coprecipitation method	39
Figure 3. Functionalization strategies and coatings of magnetic nanoparticles	44
Figure 4. Possible mechanisms for protease immobilization in MNPs using different functionalization agents: APTES (A); Glutaraldehyde (B); Genipin (C); EDC/NHS (D); Chitosan (E).....	45
Figure 5. Future outlook for the protease immobilization on Magnetic Nanoparticles.	58

CAPÍTULO 3

Figure 1. Schematic representation of the proteolytic bioreactor production process (functionalization and immobilization steps).	78
Figure 2. Images of the MNPs exposed to a magnetic field.	80
Figure 3. FTIR spectra of Fe ₃ O ₄ control (CT), Fe ₃ O ₄ @APTES (AP), and Fe ₃ O ₄ @APTES–Genipin (GP), in the range of 600–4000 cm ⁻¹	81
Figure 4. Diffractograms of MNPs: Fe ₃ O ₄ control (CT), Fe ₃ O ₄ @APTES (AP), and Fe ₃ O ₄ @APTES–Genipin (GP).	82
Figure 5. TG and DTA curves of MNPs: (A) Fe ₃ O ₄ control (CT); (B) Fe ₃ O ₄ @APTES (AP); and (C) Fe ₃ O ₄ @APTES–Genipin (GP).	84
Figure 6. Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS) of the MNPs: (A) Fe ₃ O ₄ control (CT); (B) Fe ₃ O ₄ @APTES (AP); and (C) Fe ₃ O ₄ @APTES–Genipin (GP).	86
Figure 7. Effect of pH on the relative enzymatic activity of Bromelain (A) and Trypsin (B).....	90
Figure 8. Effect of temperature on the relative enzymatic activity of Bromelain (A) and Trypsin (B).....	91
Figure 9. Relative activity of free and immobilized enzymes over multiple reuse cycles of the bioreactors: Bromelain (A) and Trypsin (B).	92
Figure 10. Chromatograms of the casein solution and peptides produced by the hydrolysis of Bromelain (A) and Trypsin (B).	93

CAPÍTULO 4

Figura 1. Atividade relativa da enzima livre e imobilizada sobre múltiplos ciclos de reuso	122
Figura 2. Perfil cromatográfico do WP e padrões de β -LG e α -LA (A); e peptídeos obtidos da hidrólise com tripsina livre e imobilizada livre e imobilizada (B) em diferentes tempos (0,5 a 4 h) (B)	123
Figura 3. Grau de hidrólise dos peptídeos pelo ensaio espectrofotométrico o-ftaldialdeído	124
Figura 4. Potencial antioxidante <i>in vitro</i> dos peptídeos obtidos da hidrólise do soro de leite medidos pela capacidade de inibir o radical DPPH.....	126
Figura 5. Potencial antioxidante <i>in vitro</i> dos peptídeos obtidos da hidrólise do soro de leite medidos pela capacidade de inibir o radical ABTS.....	126
Figura 6. Potencial antioxidante <i>in vitro</i> dos peptídeos obtidos da hidrólise do soro de leite medidos ensaio de redução de ferro (FRAP).....	127
Figure 7. Potencial antioxidante <i>in vitro</i> dos peptídeos obtidos da hidrólise do soro de leite avaliados pela inibição da oxidação do sistema BCAL.....	129

RESUMO

SANTOS, J. B. **Funcionalização de nanopartículas magnéticas com genipina para a imobilização de proteases e valorização biotecnológica do soro de leite.** Itapetinga-BA: UESB, 2026. 140 p (Tese – Doutorado em Engenharia e Ciência de Alimentos).

A utilização de enzimas na indústria de alimentos tem se expandido significativamente, embora desafios relacionados à estabilidade operacional, recuperação e reutilização catalítica ainda limitem sua aplicação em larga escala. Nesse contexto, esta tese investigou o desenvolvimento e a aplicação de nanopartículas magnéticas (MNPs) como suportes para a imobilização de proteases, visando a otimização de processos biotecnológicos e a valorização de subprodutos agroindustriais sob a ótica da economia circular. Inicialmente, realizou-se uma revisão sistemática sobre os avanços recentes na síntese e funcionalização de MNPs para a imobilização de proteases. O estudo destacou que, embora as MNPs ofereçam benefícios como maior vida útil operacional e facilidade de recuperação magnética, a padronização de métodos e a redução de custos permanecem como obstáculos críticos para a viabilidade industrial. Com base nessas lacunas, o primeiro estudo experimental focou na síntese de nanopartículas de magnetita funcionalizadas com (3-aminopropil)triethoxysilano (APTES) e genipina, um agente de funcionalização verde e biocompatível. Esta plataforma foi utilizada para a imobilização de tripsina e bromelina, alcançando eficiência de imobilização superior a 65% para ambas e mantendo alta atividade específica das mesmas. As enzimas imobilizadas apresentaram estabilidade estrutural e operacional, retraindo sua atividade após seis ciclos de reutilização, além de serem efetivos na produção de peptídeos com expressivo potencial antioxidante a partir da hidrólise da caseína. Visando sua aplicação tecnológica, um segundo estudo experimental aplicou o sistema de tripsina imobilizada para um novo direcionamento ao soro de leite, direcionado à produção de suplemento com potencial antioxidante. O processo de hidrólise enzimática mediado pelas MNPs mostrou-se superior ao uso da enzima livre, alcançando um grau de hidrólise máximo de 42,55% em 4 horas. A caracterização por Cromatografia Líquida de Alta Eficiência (HPLC) revelou um perfil peptídico diversificado, cujos peptídeos bioativos apresentaram elevada capacidade antioxidante, com índices de inibição de radicais DPPH e ABTS superiores a 90%. Em conjunto, os resultados desta tese demonstram que a imobilização de proteases em suportes magnéticos funcionalizados com agentes verdes não apenas supera as limitações catalíticas tradicionais, mas também constitui uma estratégia eficiente e sustentável para a produção de peptídeos bioativos. A abordagem proposta reforça o cumprimento dos Objetivos de Desenvolvimento Sustentável (ODS), em especial os ODS 3, 9 e 12, que visam, respectivamente, a promoção da saúde e do bem-estar por meio do desenvolvimento de ingredientes com potencial funcional, o fomento à inovação tecnológica e a processos industriais mais eficientes, e a adoção de padrões sustentáveis de consumo e produção, com ênfase na redução de resíduos e na valorização de coprodutos agroindustriais.

Palavras-Chave: Tripsina; Bromelina; Magnetita; Caseína; Hidrólise; Reaproveitamento; Reutilização.

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ABSTRACT

SANTOS, J. B. **Functionalization of magnetic nanoparticles with genipin for the immobilization of proteases and biotechnological valorization of whey.** Itapetinga-BA: UESB, 2026. 140 p (Thesis – Doctorate in Food Engineering and Science).

The use of enzymes in the food industry has expanded significantly, although challenges related to operational stability, recovery, and catalytic reuse still limit their large-scale application. In this context, this thesis investigated the development and application of magnetic nanoparticles (MNPs) as supports for protease immobilization, aiming at the optimization of biotechnological processes and the valorization of agro-industrial by-products from the perspective of the circular economy. Initially, a systematic review was conducted on recent advances in the synthesis and functionalization of MNPs for protease immobilization. The study highlighted that, although MNPs offer benefits such as longer operational lifespan and ease of magnetic recovery, method standardization and cost reduction remain critical obstacles to industrial viability. Based on these gaps, the first experimental study focused on the synthesis of magnetite nanoparticles functionalized with (3-aminopropyl)triethoxysilane (APTES) and genipin, a green and biocompatible functionalizing agent. This platform was used for the immobilization of trypsin and bromelain, achieving immobilization efficiency greater than 65% for both and maintaining high specific activity. The immobilized enzymes showed structural and operational stability, retaining their activity after six reuse cycles, and were effective in producing peptides with significant antioxidant potential from casein hydrolysis. Aiming at its technological application, a second experimental study applied the immobilized trypsin system to a new application in whey, targeting the production of a supplement with antioxidant potential. The enzymatic hydrolysis process mediated by MNPs proved superior to the use of the free enzyme, reaching a maximum hydrolysis degree of 42.55% in 4 hours. Characterization by High-Performance Liquid Chromatography (HPLC) revealed a diverse peptide profile, whose bioactive peptides showed high antioxidant capacity, with DPPH and ABTS radical inhibition indices greater than 90%. Taken together, the results of this thesis demonstrate that the immobilization of proteases on magnetic supports functionalized with green agents not only overcomes traditional catalytic limitations, but also constitutes an efficient and sustainable strategy for the production of bioactive peptides. The proposed approach reinforces the fulfillment of the Sustainable Development Goals (SDGs), especially SDGs 3, 9 and 12, which aim, respectively, at promoting health and well-being through the development of ingredients with functional potential, fostering technological innovation and more efficient industrial processes, and adopting sustainable consumption and production patterns, with an emphasis on reducing waste and valorizing agro-industrial co-products.

Keywords: Trypsin; Bromelain; Magnetite; Casein; Hydrolysis; Recycling; Reuse.

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CAPÍTULO 1

INTRODUÇÃO GERAL

OBJETIVOS

1. INTRODUÇÃO GERAL

As enzimas constituem ferramentas centrais da biotecnologia industrial por sua capacidade de catalisar reações específicas, permitindo a conversão de matérias-primas simples em produtos de maior valor agregado (Fasim et al., 2021). Dentre os diferentes grupos enzimáticos, as proteases ocupam posição de destaque tanto do ponto de vista científico quanto econômico, sendo responsáveis por hidrolisar ligações peptídicas e liberar aminoácidos e peptídeos com propriedades específicas (Boon et al., 2020). O mercado global de proteases reflete essa importância, tendo sido avaliado em aproximadamente USD 2,64 bilhões em 2025, com projeções de alcançar USD 4,22 bilhões até 2032 (Global Proteases Market, 2025). No setor de laticínios, essas enzimas desempenham papel central em etapas que vão desde a coagulação da caseína para a produção de queijos até a modulação de proteínas para aplicações nutricionais específicas (Mir Khan & Selamoglu, 2020).

No entanto, a aplicação de enzimas na forma livre ainda apresenta limitações importantes, como instabilidade térmica e operacional, autólise, dificuldade de recuperação e elevados custos associados à impossibilidade de reutilização do biocatalisador, fatores que restringem sua viabilidade em processos industriais de larga escala (Prabhakar et al., 2025). Diante desses desafios, a imobilização enzimática em suportes sólidos surge como uma alternativa tecnológica promissora, capaz de aumentar a estabilidade, facilitar a recuperação e permitir a reutilização das enzimas, além de contribuir para a melhoria do desempenho catalítico (Prabhakar et al., 2025).

Ao considerar o desenvolvimento de novos sistemas de imobilização, torna-se fundamental a busca por alternativas mais sustentáveis e fundamentadas nos princípios da química verde, visando reduzir o uso de fontes não renováveis e agentes tóxicos. Nesse contexto, a genipina destaca-se como um agente de ligação de origem natural, reconhecido por sua elevada biocompatibilidade e baixa toxicidade, surgindo como uma alternativa promissora ao glutaraldeído (Hong et al., 2021; Zimmermann et al., 2024). Como suporte, as nanopartículas magnéticas (NPMs) têm recebido notável atenção por permitirem a intensificação de processos e a recuperação eficiente do catalisador via campo magnético externo (Farinha et al., 2021; Vallinayagam et al., 2021). Entre os diversos materiais, as nanopartículas de óxido de ferro (Fe_3O_4) são amplamente exploradas por sua alta área superficial e facilidade de funcionalização (Materón et al., 2021; Matveeva & Bronstein, 2021).

Uma aplicação de elevado interesse para esses sistemas imobilizados é o uso de proteases para a produção de peptídeos bioativos, os quais têm despertado interesse devido às suas propriedades biológicas, como atividades antioxidante, anti-hipertensiva e antimicrobiana (Guha et al., 2021; Murtaza et al., 2022). A atividade antioxidante, em particular, é relevante por mitigar o estresse oxidativo associado a doenças crônicas e aumentar a estabilidade de alimentos (Yu et al., 2021). O setor lácteo é um dos principais beneficiados por essa tecnologia, uma vez que o leite é uma matéria-prima rica em proteínas, como a caseína, que representa 80% da fração proteica total, e as proteínas do soro, ambas excelentes precursoras de peptídeos funcionais (Auestad & Layman, 2021; Liu et al., 2024).

Alinhado aos conceitos de economia circular e redução de resíduos, o uso do soro de leite apresenta-se como uma alternativa estratégica. Embora contenha proteínas de alto valor biológico, esse subproduto possui elevada carga orgânica e pode causar impactos ambientais significativos se descartado inadequadamente (Peydayesh et al., 2023). Assim, a utilização do soro de leite por meio da hidrólise enzimática permite transformar um subproduto ainda é descartado inadequadamente em regiões subdesenvolvidas, em uma fonte de compostos de alto valor agregado, atendendo aos Objetivos de Desenvolvimento Sustentável (ODS), especialmente no que tange ao consumo e produção responsáveis (Peydayesh et al., 2023).

Diante desse contexto, o objetivo geral desta tese consiste em propor uma rota integrada e ambientalmente sustentável para a utilização do soro de leite, direcionada à produção de peptídeos bioativos com potencial antioxidante. Para tal, foi desenvolvido um novo sistema de imobilização de proteases, especificamente tripsina e bromelina, baseado em nanopartículas magnéticas funcionalizadas com genipina. O estudo avalia de forma sistemática os efeitos da imobilização sobre o desempenho catalítico e a estabilidade das enzimas, aplicando o biocatalisador obtido na conversão proteica do soro de leite. Ao combinar inovação em materiais, biotecnologia enzimática e reaproveitamento de subprodutos agroindustriais, a tese contribui para a redução de impactos ambientais associados à indústria de laticínios e se insere no escopo dos princípios da economia circular. Adicionalmente, a proposta dialoga de maneira transversal com os Objetivos de Desenvolvimento Sustentável, ao promover estratégias associadas à produção e ao consumo responsáveis (ODS 12), ao desenvolvimento de soluções tecnológicas mais eficientes e sustentáveis para processos industriais (ODS 9) e

à obtenção de compostos com potencial aplicação na promoção da saúde e do bem-estar humano (ODS 3).

2. OBJETIVO GERAL

O objetivo desta tese foi desenvolver e caracterizar biocatalisadores baseados em proteases imobilizadas em nanopartículas magnéticas funcionalizadas com genipina, visando a obtenção de sistemas catalíticos estáveis e reutilizáveis para a produção de peptídeos bioativos por meio da utilização sustentável de subprodutos da indústria de laticínios.

2.1 Objetivos específicos

Para alcançar o objetivo geral, foram estabelecidos os seguintes objetivos específicos:

- **Desenvolvimento e Caracterização do Suporte:** Sintetizar nanopartículas magnéticas (NPMs) estáveis pelo método de coprecipitação química e realizar a funcionalização superficial utilizando APTES e genipina, caracterizando o material quanto às suas propriedades morfológicas, térmicas, estruturais e magnéticas.
- **Imobilização enzimática e Desempenho Catalítico:** Imobilizar as proteases Tripsina e Bromelina no suporte desenvolvido, investigando a influência da natureza da enzima nas características de desempenho dos biorreatores e avaliando o efeito dos parâmetros operacionais pH e temperatura na atividade das enzimas livres e imobilizadas.
- **Estabilidade e Reutilização:** Estimar a eficiência e a estabilidade operacional dos biorreatores magnéticos por meio de sucessivos ciclos de uso, visando a redução de custos e o aumento da vida útil do biocatalisador.
- **Produção de Peptídeos a partir de Matriz Modelo:** Avaliar a eficiência dos biorreatores na hidrólise da caseína, analisando o perfil peptídico gerado e o potencial antioxidante dos peptídeos bioativos produzidos.
- **Aplicação Tecnológica e Valorização de Subprodutos:** Aplicar o sistema de imobilização otimizado na hidrólise enzimática do soro de leite, investigando o grau de hidrólise, o perfil peptídico via HPLC e a bioatividade (antioxidante) dos produtos gerados.
- **Sustentabilidade e Inovação:** Alinhar os processos desenvolvidos aos princípios da economia circular e aos Objetivos de Desenvolvimento Sustentável (ODS), promovendo a geração de inovação tecnológica e a difusão do conhecimento científico através de publicações de alto impacto.

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CAPÍTULO 2 – ARTIGO 1

REVIEW ARTICLE: IMMOBILIZATION OF PROTEASES ON MAGNETIC NANOPARTICLES: A CRITICAL REVIEW OF FUNCTIONALIZATION STRATEGIES, CHARACTERIZATION, AND CATALYTIC PERFORMANCE

Immobilization of proteases on magnetic nanoparticles: a critical review of functionalization strategies, characterization, and catalytic performance.

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Abstract

Magnetic nanoparticles (MNPs) have attracted increasing attention due to their potential in biotechnological applications, particularly as supports for enzyme immobilization, such as proteases. These enzymes play a key role in several industrial sectors, but their use is often limited by challenges related to stability, recovery, and autolysis. Immobilization on MNPs emerges as a promising strategy to overcome these limitations, enabling higher reusability, extended operational lifetime, and improved performance under adverse conditions. Despite recent advances in the synthesis, functionalization, and characterization of MNPs, which have shown encouraging results in food and health-related applications, important obstacles remain, including the standardization of production methods, cost reduction at industrial scale, and the assurance of reproducibility. This review examines the latest progress in the development of MNPs for protease immobilization, highlighting their benefits and limitations, as well as the challenges that must be addressed to enable large-scale application and expand their prospects in the biotechnological field.

Keywords: Reusability; Oxides; Stability; Bioprocess engineering; Surface functionalization; Nanobiotechnology.

1. INTRODUCTION

Rapid industrialization and the continuous growth of human demands have driven the development of innovative technologies, with biotechnology playing a central role in this advancement. Within this field, enzymes serve as highly efficient biocatalysts that accelerate biochemical reactions by lowering the activation energy, thereby influencing reaction rates and regulating complex metabolic pathways [1]. Among these biomolecules, proteases stand out as one of the largest and most versatile classes of enzymes, responsible for the cleavage of peptide bonds in protein structures, leading to

the formation of peptides of various sizes and functionalities [2]. Owing to their broad applicability, proteases hold significant industrial and economic importance, generating billions of dollars annually. According to Market Research Reports [3], this sector generated USD 2.64 billion in 2024, with projections reaching USD 4.22 billion by 2032, corresponding to a compound annual growth rate (CAGR) of 6.05%.

These enzymes have wide applicability in various industries, including pharmaceutical, chemical, textile, and medical. In the food industry, proteases are used in dairy products, baking, protein hydrolysates, infant formulas, dietary products, and processed foods. However, the efficiency of these processes depends on the stringent control of various intrinsic and extrinsic parameters, which reinforces the need for optimization and innovation in these applications [4].

Enzymatic efficiency is influenced by parameters such as pH, temperature, pressure, substrate concentration, and inhibitors. Free enzymes pose challenges, including product contamination, difficult recovery, high costs, and the maintenance of residual enzymatic activity after processing. In dairy applications, for example, proteases that are not properly inactivated may continue acting on caseins, generating bitter peptides and causing defects in flavor and texture, especially when highly proteolytic plant enzymes are used, depending on the type of cheese produced. Immobilization of enzymes emerges as a promising strategy to overcome these limitations and improve process efficiency [5]. Although several immobilization methods have been proposed, there is still a demand for simpler and more economical alternative techniques to produce these bioreactors. Among the promising methods, physical and chemical adsorption stand out because they are quick and easy-to-apply strategies, besides preserving the enzymatic structure. Therefore, the choice of the immobilization method should consider not only the enzyme's performance but also factors such as economic feasibility and compatibility with industrial requirements [6].

In addition to defining the immobilization method, the selection of the support is a crucial factor in ensuring the efficiency of the process. Essential requirements for determining the most suitable support include low cost, high surface area and porosity, chemical and mechanical stability, high presence of reactive groups, and resistance to microbiological attacks [7].

Among the supports, magnetic nanoparticles (MNPs) of iron oxide stand out, widely used for their unique property, superparamagnetism, which facilitates their separation from the reaction medium. Other advantages of MNPs include high surface

area, low toxicity, biocompatibility, and stability. In the field of biotechnology, enzymatic immobilization has gained notoriety due to its compatibility with MNPs, allowing applications in processes such as bioseparation, immunoassays, drug delivery, biosensors, and others. However, MNPs present some limitations, such as instability in aqueous media, aggregation, and susceptibility to oxidation in the presence of oxygen. These challenges can be minimized through different functionalization strategies, improving stability and performance. Thus, the development of more efficient and stable immobilized systems is not only a technological challenge but also an important step toward more sustainable industrial processes, since enzyme reuse, reduction of residues, and optimization of resources are directly related to current demands for environmentally responsible production [8,9].

In this context, the search for technologies that increase process efficiency while reducing environmental impact is aligned with global sustainability initiatives. Intending to promote sustainable development, the United Nations (UN) established the Sustainable Development Goals (SDGs), which aim to eradicate poverty, protect the environment, and ensure prosperity for all. These goals include actions against climate change, reduction of social inequalities, and encouragement of responsible industrial development [10]. In line with this plan, this manuscript addresses studies and presents strategies related to SDG 9: Industry, Innovation and Infrastructure, and SDG 12: Responsible Consumption and Production. The review focuses on the application of magnetic materials in the transport of proteases, to enable their reuse, facilitating their recovery, and promoting innovations in the processes of immobilization and enzymatic catalysis, thus contributing to the efficient use of resources in different industrial segments.

Furthermore, this study differs from previously published works by offering a specific analysis of the use of magnetic materials for protease immobilization, whereas most existing reviews address enzymes in general terms, without considering the structural and functional specificities of this enzyme group. Here, we address fundamental aspects such as protease classification, their catalytic mechanisms, and different synthesis routes for MNPs, comparing their advantages and limitations. Surface functionalization strategies, characterization techniques, and immobilization methodologies are also discussed, highlighting how these choices affect enzymatic activity, stability, and reusability. Studies employing MNPs with proteases are presented, with particular emphasis on applications in food processing and healthcare. Finally, the

review discusses current challenges and future prospects for expanding the use of these platforms on a biotechnological scale.

2. PROTEASES: PROPERTIES AND REQUIREMENTS FOR IMMOBILIZATION

The efficiency of immobilizing proteases on magnetic nanoparticles (MNPs) is intrinsically dependent on the structural and biochemical properties of the enzyme [11]. Different classes of proteases exhibit variations in terms of isoelectric point (pI), molecular mass, surface amino acid composition, and active site location factors that determine the binding strategy and orientation of the enzyme on the substrate [12].

2.1 General protocols for enzyme immobilization

Immobilization methods can be broadly categorized into physical and chemical methods, each with its advantages and disadvantages for the stability and activity of the biocatalyst [12].

2.1.1 Chemical methods

Chemical methods involve the formation of covalent bonds or cross-linking, providing a stable anchor between the enzyme and the support. This results in a significant reduction in enzyme leaching and often in greater thermal and operational stability of the immobilized proteases [13].

Covalent Immobilization: This method forms stable chemical bonds between complementary functional groups of the enzyme (such as aldehyde and amine groups, or carboxyl groups activated by EDC/NHS) and the support [14]. Although covalent immobilization offers high stability and minimal leaching, it can, in some cases, restrict the conformational flexibility of the protease, essential for the catalysis of large protein substrates, or even block the active site if binding occurs in critical regions. For proteases, covalent immobilization can be advantageous for suppressing autolysis (degradation of the enzyme itself) but requires strict control of orientation to maintain accessibility of the active site [15].

Cross-linking: In this strategy, enzymes are interconnected by means of cross-linking agents, without the need for external material support. Agents such as glutaraldehyde and genipin are widely used due to their efficiency in forming stable enzyme networks. [16]. This approach maximizes biocatalyst density and significantly reduces enzymatic leaching. However, excessive cross-linking can lead to aggregate

formation and decreased enzymatic activity due to restricted enzyme mobility and impaired substrate access [17].

2.1.2 Physics methods

In physical methods, enzymes are immobilized without the use of strong chemical bonds, generally better preserving the conformational structure of the protease.

- **Adsorption:** This is a reversible process based on hydrophobic and electrostatic interactions, hydrogen bonds, and van der Waals forces. It generally preserves the conformational structure of the protease, and enzyme orientation on the surface occurs naturally. However, because it is a reversible and spontaneous method, enzyme leaching can occur under variations in pH or ionic strength [18].
- **Encapsulation:** This represents a promising approach where the enzyme is confined within a semipermeable matrix or membrane, such as alginate, metal-organic frameworks (MOFs), or chitosan [19]. This technique acts as a protective barrier against adverse conditions (extreme temperature, pH, organic solvents), preserving biological activity since the protease does not bind directly to the material's wall, minimizing conformational changes. However, the diffusion of substrates and products can be limited by the matrix, affecting catalytic efficiency [11].

2.2 Proteases: Properties and specific requirements for immobilization

Proteases are classified according to their catalytic mechanism and the essential amino acid residues in their active site. For immobilization purposes, this classification is relevant because it defines the enzyme's sensitivity to different functionalization methods and the need to protect the active group during the binding process [20]. The catalytic mechanisms of proteases, as depicted in Figure 1, are crucial for understanding their function and role in various biological processes.

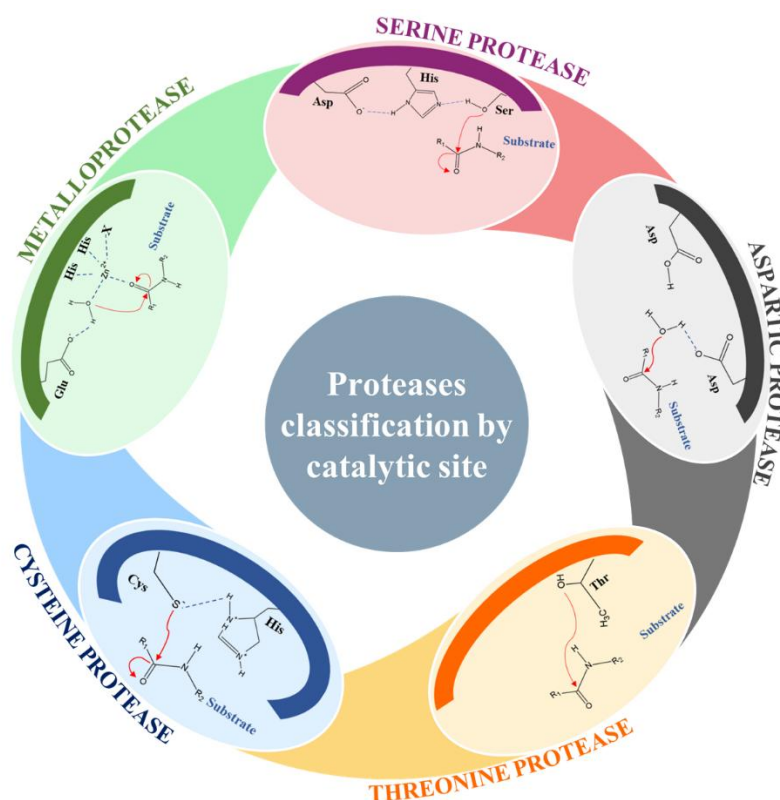


Figure 1. Representation of the catalytic mechanisms of proteases and their interactions with the substrate

- Serine proteases (e.g., trypsin, chymotrypsin): Possess a catalytic triad (His, Asp, Ser). Immobilization must avoid steric blocking of these residues. Coupling strategies via amino groups (lysines) are common, but orientation must be controlled to keep the active site accessible to the substrate [21].
- Cysteine proteases (e.g., papain, bromelain): Characterized by a nucleophilic cysteine residue. Because the sulfhydryl group (-SH) is highly reactive, immobilization methods using thiol-reactive reagents may inactivate the enzyme if the active site is not protected or if binding occurs directly to the catalytic residue [17].
- Aspartic proteases (e.g., Pepsin): These enzymes function in acidic pH and have a very low pI. This requires functionalized supports that maintain enzyme stability under acidic conditions and possess a compatible surface charge to promote efficient adsorption or covalent binding [11].
- Metalloproteases: These depend on metal ions (usually Zn^{2+}) for catalysis. Immobilization on MNPs must ensure that the support or functionalizing agents do not act as chelating agents, removing the metal cofactor and inactivating the biocatalyst [12].

- Threonine protease: These have an N-terminal threonine residue in the active site, whose hydroxyl group (-OH) acts as a nucleophile in attacking the peptide bond. Unlike serine proteases, the catalytic mechanism involves the intramolecular activation of threonine, often aided by the α -terminal amino group of the catalytic residue itself [22].

Table 1 summarizes the main sources of proteases, their industrial applications, and the critical considerations for their immobilization on solid supports.

Table 1. Origin, industrial applications, and immobilization requirements of the main proteases

Origin	Protease	Industrial Application	Immobilization Requirements	References
Animal	Trypsin	Protein hydrolysates, pharmaceutical	Orientation control to prevent autolysis; coupling of amino groups.	[21]
	Pepsin	Dairy industry (coagulation)	Stability at low pH; supports resistant to acid corrosion.	[11]
Plant	Papain	Meat tenderization, leather clarifying.	Protection of the active (-SH) group; thermal stability	[17,23]
	Bromelain	Dietary supplements, leather processing	Maintaining activity across a wide pH range; biocompatible functionalization.	[23]
Microbial	<i>Bacillus</i> protease	Detergents, waste processing	Surfactant resistance; immobilization that withstands high temperatures.	[15]
	Fungal Protease	Baking, cheeses	Controlling substrate porosity for access to complex substrates.	[11]

Table 1 summarizes the main proteases used industrially, highlighting that the enzyme's origin is directly related to its applications and the requirements for its immobilization. Proteases of animal origin, such as trypsin and pepsin, are mainly used in the production of protein hydrolysates, in the pharmaceutical industry, and in coagulation processes in the dairy sector; however, they have limitations related to structural stability, requiring immobilization strategies that control enzyme orientation and use supports resistant to acidic conditions or that minimize autolysis.

Proteases of plant and microbial origin, in turn, demonstrate greater industrial versatility, being used in processes such as meat tenderizing, baking, detergents, and waste treatment. In these cases, immobilization requirements are mainly associated with preserving the active site, maintaining activity over a wide pH and temperature range, and resistance to severe operating conditions, such as the presence of surfactants or complex substrates. In general, it is observed that the choice of immobilization strategy should be defined according to the structural characteristics of the enzyme and the process conditions, in order to guarantee catalytic stability, substrate accessibility, and feasibility of application on an industrial scale.

2.3 Influence of composition and structure on coupling strategy

The amino acid composition on the protease surface dictates the choice of cross-linking agent. For example, an abundance of lysine residues favors the use of glutaraldehyde for covalent linking in MNPs functionalized with amino groups [15]. On the other hand, the presence of carbohydrates in glycosylated proteases (common in plant and fungal sources) allows for immobilization via oxidation of sugar groups, which can offer a more favorable orientation and preserve the active site [24].

The orientation of the enzyme on the magnetic support is crucial to minimize steric hindrance, especially for proteases that act on bulky protein substrates [25]. Functionalizing MNPs with spacers of different lengths can increase the flexibility of the immobilized enzyme, improving substrate access and, consequently, apparent catalytic activity [24].

2.4 Regulatory Aspects and Safety (GRAS Status)

Plant-derived proteases, such as bromelain, papain, and ficin, have a long history of safe use in food. For this reason, they are classified as Generally Recognized as Safe (GRAS) by the United States Food and Drug Administration (FDA). This status is fundamental for applications in the food industry, such as meat tenderizing and the

production of hydrolysates, as it facilitates the approval of processes that use these immobilized enzymes, provided that the carrier (MNPs) and functionalizing agents also meet the safety and toxicity requirements [26].

The use of magnetic nanoparticles (MNPs) as immobilization supports introduces additional regulatory considerations, particularly in food applications. Although iron oxide nanoparticles are generally regarded as having low toxicity and are used in biomedical fields, their use in food processing requires specific assessment regarding migration, potential ingestion, and long-term safety [27]. Therefore, the application of MNP-immobilized enzymes is more readily accepted in processes where the nanoparticles can be completely removed from the final product, such as in magnetic recovery systems, ensuring compliance with food safety regulations.

3. MAGNETIC NANOPARTICLES

3.1 Magnetic nanoparticles

To meet the demands of various fields of knowledge, nanotechnology research has sought to promote innovation and improve existing technologies. In this context, magnetic nanoparticles (MNPs) stand out for their versatility and broad applicability, demonstrating affinity with various scientific and technological fields [28]. To date, numerous magnetic nanoparticles have been synthesized, including pure metallic particles (Fe, Co, Ni), metal oxides (such as magnetite, maghemite, and hematite), ferrites (MFe_2O_4), ferromagnetic materials, and certain metal alloys. The synthesis of MNPs requires careful consideration of several critical factors, including intrinsic magnetic properties, surface coating, particle morphology, surface charge, stability in aqueous environments, and, most importantly, biocompatibility and non-toxicity. Challenges such as dipolar interactions, surface effects, and size control are crucial in MNP synthesis. However, several synthetic chemical approaches have been developed to facilitate the control of nanoparticle formation and growth processes [29].

Iron oxides generally play a fundamental role in selecting magnetic materials. Among them, maghemite ($\gamma\text{-Fe}_2\text{O}_3$), magnetite (Fe_3O_4), and hematite ($\alpha\text{-Fe}_2\text{O}_3$) are the most extensively studied due to their high biocompatibility and stability against degradation [30]. Oxides can exhibit different structural forms, with their crystalline configuration determined by the arrangement of iron cations in octahedral or tetrahedral interstitial sites associated with oxygen anions. For example, in the structures of maghemite and magnetite, oxygen anions are arranged in a cubic close-packed

configuration, whereas in hematite, oxygen ions form a compact hexagonal structure. Since magnetite and maghemite have very similar crystalline structures, differentiating them solely by X-ray diffraction (XRD), based on the analysis of scattering angles, becomes limited, especially in nanoparticulate systems. In these cases, complementary techniques are necessary for a more precise characterization, including thermal analysis (TGA) and electron microscopy (SEM), as well as spectroscopic techniques sensitive to the oxidation state of iron, such as X-ray photoelectron spectroscopy (XPS) or Mössbauer [31].

Among spinel-type iron oxides, such as magnetite and ferrites, tetrahedral sites are predominantly occupied by Fe^{3+} ions, while octahedral sites can contain both Fe^{2+} and Fe^{3+} ions. Stoichiometrically, magnetite exhibits a 1:2 molar ratio of Fe^{2+} and Fe^{3+} ions, respectively, characterizing an inverted spinel structure. In contrast, hematite ($\alpha\text{-Fe}_2\text{O}_3$) exhibits a corundum-type structure, in which Fe^{3+} ions exclusively occupy octahedral sites. Its oxidation can result in the complete conversion of Fe^{2+} to Fe^{3+} , forming maghemite through weathering or other mechanisms [32]. The synthesis method strongly influences all these obtaining processes, which can be physical, chemical, or biological.

3.2 Methods for Nanoparticle Production

Various approaches have been employed for the synthesis of MNPs, and the key point lies in the development of simple and economically viable techniques that allow the production of high-quality materials and suitable physicochemical and morphological properties. The strategies for particle production are classified into physical, and chemical methods [33]. Table 2 briefly summarizes the different methods applied in the production of MNPs.

Table 2. Chemical and physical approaches for magnetic nanoparticle production and their recent applications

Technique	Source	Objective	References
Chemicals			
Co-Precipitation	FeSO ₄ , FeCl ₃ and Agarose	Desalination by forward osmosis for freshwater production	[34]
Pyrolysis	Fe(NO ₃) ₃ and corn cob	Development of Fenton catalyst to degrade methyl orange	[35]
Hydrothermal/Solvothermal	FeCl ₃ , Polyethylene glycol 8000, Hydrazine	Development of particles with antibacterial properties	[36]
Sol-Gel	(Co(NO ₃) ₂ ·6H ₂ O), (Fe(NO ₃) ₃ ·9H ₂ O) and (Cu(NO ₃) ₂ ·6H ₂ O)	Preparation of copper-substituted cobalt ferrite nanoparticles for evaluation of antibacterial property	[37]
Microemulsion	Fe(CO) ₅ , oleic acid, and octyl ether	Development of magnetic microemulsion for drug delivery	[38]
Sonochemical	FeSO ₄ · 7H ₂ O and NaOH	Production and characterization of magnetic nanotubes	[39]
Electrodeposition	Fe ₃ O ₄	Development of magnetic carbon fibers	[40]
Polyol	FeCl ₂ · 4H ₂ O, heptanoic acid (C ₇ H ₁₄ O ₂) and NaOH	Production of magnetite by the polyol method and evaluation of the material	[41]
Physical			
Ball milling	α-Fe ₂ O ₃	Production of nanostructured magnetite-maghemite particles from microstructured hematite	[42]

Laser evaporation	α -Fe ₂ O ₃	Development of hematite nanopowders by laser vaporization	[43]
Explosion of electrical wires	Fe ₃ O ₄ and α -Fe ₂ O ₃	Synthesize nanoparticles and examine their potential as adsorbents for arsenic removal from water.	[44]

3.2.1 Chemical methods

The first methods used to synthesize MNPs were chemical methods, which rely on solvents, controlled temperatures, and chemical reactions to obtain these materials. Among the colloidal systems, the most commonly used include microwave-assisted synthesis, sonochemical reactions, sol-gel synthesis, microemulsions, hydrothermal/solvothermal reactions, thermolysis, and co-precipitation reactions. Among the methods mentioned, co-precipitation is the most common and one of the simplest techniques, notable for its ability to synthesize large quantities of MNPs. The co-precipitation process involves mixing iron salts, which are precipitated by a basic agent under high temperatures and an inert atmosphere, forming the oxides [45]. For example, Fe^{2+} and Fe^{3+} ions can be used to produce magnetite, as shown in the following chemical Equation 1 and Figure 2:

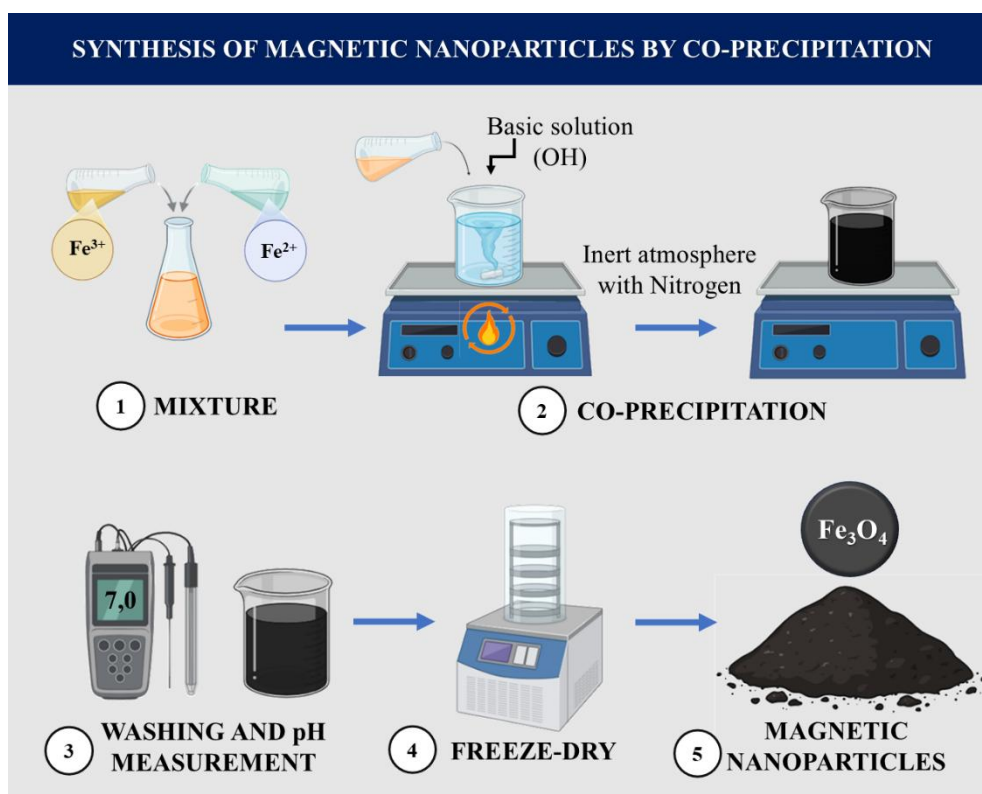
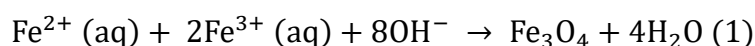


Figure 2. Schematic representation of the synthesis of magnetic nanoparticles by coprecipitation method

Co-precipitation is particularly advantageous when aiming to obtain MNPs in large quantities, in addition to allowing the use of different metal salts for their production. However, this method requires strict control of parameters to ensure higher

efficiency. For example, the temperature of the process directly influences the formation of the oxide phase: temperatures below 60°C favor the formation of hematite, while temperatures above 80 °C promote the formation of magnetite. One of the challenges is controlling the particle size, as MNPs exhibit high surface energy due to their small size. To minimize this effect and reduce the tendency for aggregation, intense stirring and slow dripping between the process phases are recommended [46].

3.2.2 Physical Methods

The production of MNPs through physical methods is based on approaches defined as "top-down" and "bottom-up." In the top-down strategy, macroscopic materials are reduced to nanometric particles through mechanical processes, such as high-energy ball milling. In the bottom-up approach, MNPs are synthesized from the controlled organization and growth of atoms or molecules, allowing for the production of fine, homogeneous, and well-dispersed particles. One of the most common examples of the bottom-up approach is laser ablation, where a high-energy laser is applied to vaporize a metallic material, leading to condensation and the formation of MNPs. Despite the advantages of physical methods, such as the absence of chemical agents and the ability to obtain purer nanoparticles, these processes present challenges, such as high energy costs and difficulty in precisely controlling particle size [47].

3.3 Advantages and disadvantages of synthesis methods

The choice of MNP synthesis method directly influences the physicochemical properties of the final material, such as size, shape, crystallinity, stability, and surface functionality. Each synthetic route presents specific characteristics that must be carefully evaluated according to the desired application, whether on a laboratory, industrial, or biotechnological scale [46]. Table 3 below presents a detailed comparison between the main MNP synthesis methods, highlighting their advantages, limitations and the bibliographic references supporting these observations.

Table 3. Evaluation of the main magnetic nanoparticle synthesis techniques based on their benefits and challenges

Method	Advantages	Disadvantages	References
1. Co-Precipitation	• Simple and inexpensive • High reproducibility	• Less control over shape • May generate crowding	[28]
2. Pyrolysis	• Rapid heating followed by rapid cooling	• High cost	[28]
3. Hydrothermal/Solvothermal	• High crystallinity • Better control over shape and size	• Requires autoclave and high temperature • Time-consuming process	[48]
4. Sol-Gel	• High purity • High crystallinity	• Long steps • May need calcination	[46]
5. Microemulsion	• Excellent dispersion • Great size control	• Low income • Use of organic solvents	[49]
6. Sonochemical	• High crystallinity • Saturation magnetization	• The mechanism is not yet fully known	[48]
7. Ball milling	• Simple process • Produces large quantities	• Irregular size and shape • Possibility of contamination	[28]
8. Laser evaporation	• No use of Chemicals	• Needs high amounts of energy	[28]

9. Explosion of electrical wires	• Ecofriendly • Highly productive	• Possibility of contamination	[46]
10. Biological Synthesis	• Sustainable and ecological • Low toxicity	• Difficult control of properties	[46]

3.4 MNPs characteristics

The characterization of MNPs involves different analytical techniques to examine their physicochemical properties, including particle size, which plays a fundamental role in their applications.

As mentioned earlier, the size and morphology of MNPs directly influence their physicochemical characteristics. Thus, techniques such as Brunauer-Emmett-Teller (BET) and Dynamic Light Scattering (DLS) are widely used to measure surface area, particle size, and size distribution. Additionally, microscopy techniques provide images that assist in determining shape and size through calculated diameters. Scanning Electron Microscopy (SEM) is an essential technique for characterizing MNPs, as it allows for detailed information on surface topography and sample composition. However, the crystalline structure of magnetic nanoparticles (MNPs) is frequently evaluated by X-ray diffraction (XRD), a technique that allows the identification of crystalline phases and the estimation of the average crystallite size. The presence of amorphous material is inferred from the absence of well-defined diffraction peaks and the observation of diffuse halos in the diffractogram, reflecting the lack of long-range crystalline order [50].

The elemental composition and chemical mapping of MNPs can be assessed using various techniques, including Energy Dispersive Spectroscopy (EDS/EDXD), X-ray Fluorescence (XRF), X-ray Photoelectron Spectroscopy (XPS), Atomic Absorption Spectrophotometry (AAS), Inductively Coupled Plasma Mass Spectrometry (ICP-MS), Transmission Electron Microscopy (TEM), and Scanning Electron Microscopy (SEM). However, some of these techniques have limitations. For instance, AAS cannot be directly applied to solid MNPs, requiring prior dissolution of the samples in acids or bases. Meanwhile, XPS and EDS provide detailed information on the elemental composition and chemical structure of the sample [51].

In addition to structural analysis, spectroscopic techniques are employed to investigate the chemical bonds and structural characteristics of MNPs. Methods such as Fourier Transform Infrared Spectroscopy (FT-IR), X-ray Absorption Spectroscopy (XAS), Thermogravimetric Analysis (TGA), and Raman Spectroscopy (RS) are widely used. The combination of XPS and FT-IR, for example, aids in identifying bonds between organic and inorganic compounds, determining the binding energy of MNPs, and assessing their oxidation state. Meanwhile, TGA is used to estimate binding efficiency

and provide insights into structural modifications, especially when polymers and surfactants are employed as coatings for MNPs [52].

Finally, the magnetic properties of MNPs can be analyzed under the influence of external magnetic fields. Magnetic behavior can be measured using a Vibrating Sample Magnetometer (VSM) and SQUID Magnetometry (Superconducting Quantum Interference Device), each with distinct applications. SQUID is particularly useful in the magnetic characterization of fine powders, crystals, liquids, and films due to its high sensitivity. These techniques also allow for the determination of saturation magnetization and remanent magnetization when a constant external magnetic field is applied [53].

4. STRATEGIES FOR FUNCTIONALIZING MNPs

The interactions between MNPs and the external environment are strongly influenced by the chemical groups present on their surface. Therefore, the development of functionalization strategies aims to control MNPs' solubility, improve their stability, enhance their biocompatibility, and optimize their application in various fields. Figure 3 illustrates the main functionalization approaches used in MNPs, highlighting the different types of coatings and chemical modifications employed to enhance their properties.

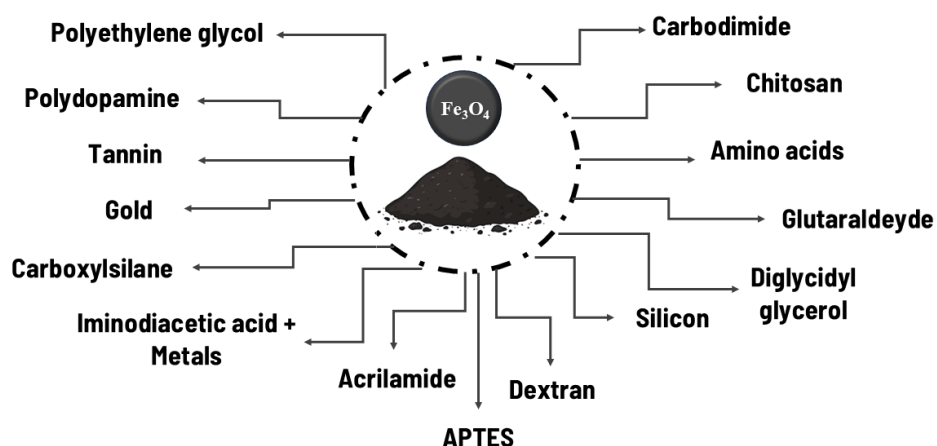


Figure 3. Functionalization strategies and coatings of magnetic nanoparticles

Similarly, Figure 4 presents some possible functionalization mechanisms and coatings for MNPs using different agents.

Figure 4. Possible mechanisms for protease immobilization in MNPs using different functionalization agents: APTES (A); Glutaraldehyde (B); Genipin (C); EDC/NHS (D); Chitosan (E).

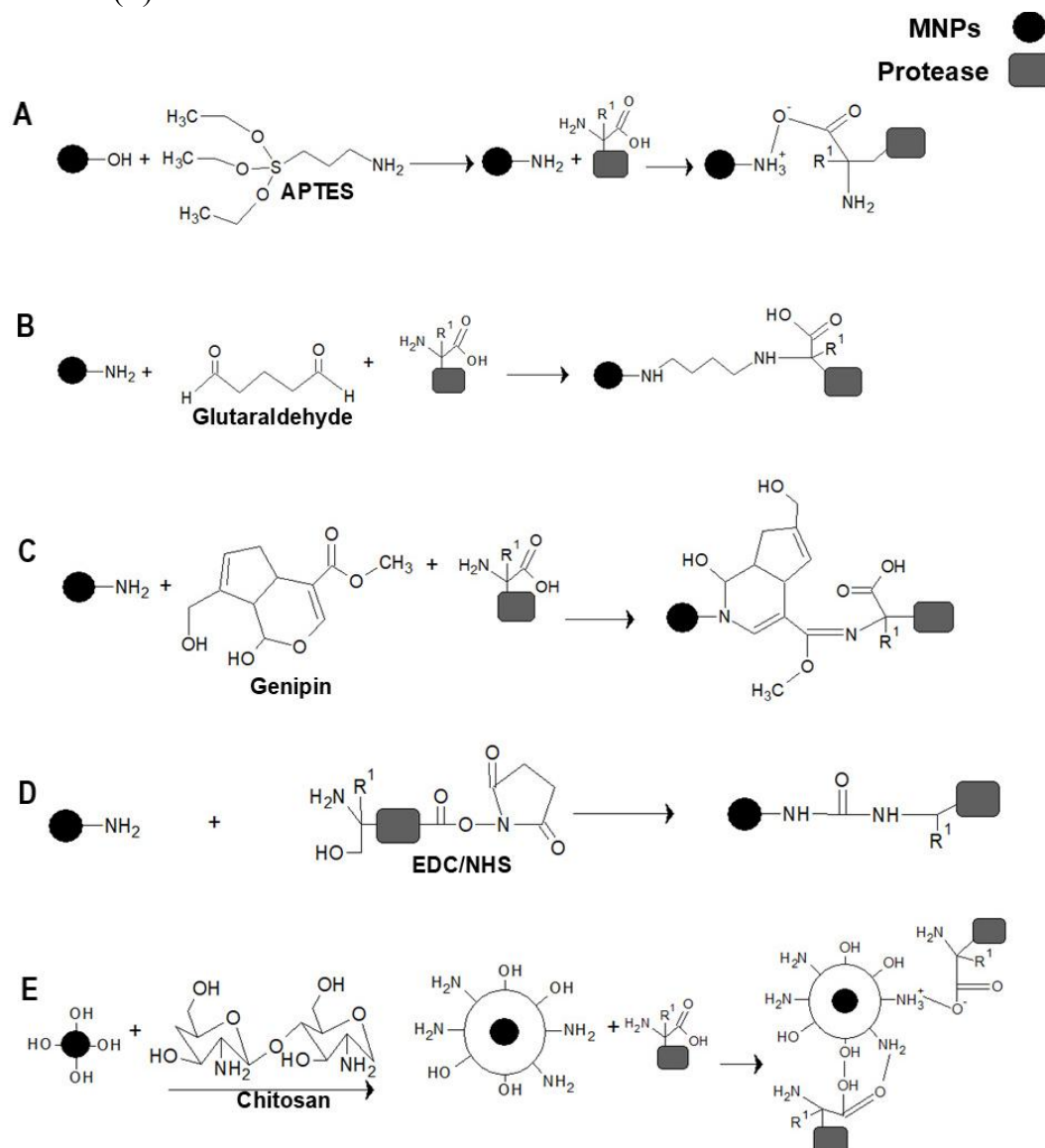


Figure 5 shows different functionalization strategies that allow the immobilization of proteases on magnetic nanoparticles through covalent or noncovalent interactions. Agents such as APTES, chitosan, glutaraldehyde, genipin, and the EDC/NHS system modify the particle surface, introducing functional groups capable of promoting amide bonds, hydrogen bonds, or electrostatic interactions with the enzyme. These approaches adjust the surface chemistry of the support, influencing the stability, orientation, and catalytic activity of the immobilized protease.

4.1 Surface functionalization

The modification or functionalization of supports occurs by inserting organic and/or reactive groups onto the surface of MNPs, enabling greater efficiency in enzyme

immobilization. The increase in these functional groups on the surface of MNPs can lead to bioreactors with higher catalytic efficiency, reducing the diffusion of substrates and reaction products, in addition to improving operational stability in both static and dynamic processes [54] .

Many of the produced MNPs exhibit a hydrophobic character; however, hydroxyl groups on their surface enable more efficient functionalization. Several studies have employed amination techniques to introduce new chemical groups, facilitating the immobilization of enzymes [55]. Among the most commonly used amination agents are (3-aminopropyl) triethoxysilane (APTES), [3-(2-aminoethyl)propyl]trimethoxysilane (AEAPTMS), and 3-[2-(2-aminoethylamino)ethylamino]propyltrimethoxysilane (AEAEAPTMS). These agents have a structure composed of a terminal amine group, a silica core, and hydrophobic tails.

Studies have demonstrated the effectiveness of these agents in the immobilization of proteases. Aslani et al. [56] evaluated the immobilization of porcine trypsin on Fe₃O₄ using APTES as a spacer, while Kumar et al. [57] applied APTES in the immobilization of Serratiapeptidase. In general, functionalizing MNPs with APTES increases the charge density on the surface, resulting in a greater number of active sites available for protease immobilization, primarily through electrostatic interactions with acidic amino acids such as aspartic acid and glutamic acid. It also enables covalent bonds with the protease's carboxyl or amine groups via glutaraldehyde or genipin. An improvement in the enzyme's thermal and chemical stability is also observed, as covalent bonds would reduce its mobility. However, it is important to control the number of amino acid groups on the surface, as excess can generate electrostatic repulsion, impairing proteolytic activity [58].

Among the most widely used covalent strategies, glutaraldehyde stands out. This bifunctional compound contains aldehyde groups at its ends and is widely used in the immobilization of biomolecules. It provides enzymes with greater thermal and operational stability and offers resistance to conformational changes caused by denaturing agents and organic solvents. The interaction occurs primarily between amine (-NH₂) groups, as well as hydroxyl (OH) and thiol (-SH) groups. Another little-discussed point about this agent is that its use can introduce charged groups to the surface of the material, depending on the reagents used. To increase this functionalization, APTES is often combined with glutaraldehyde. However, glutaraldehyde has disadvantages, such as its tendency to self-crosslink in aqueous media, which can alter the enzyme's conformation, reducing its efficiency. Furthermore, the agent's toxicity requires the study of minimum

safe concentrations [18,59]. New strategies are being developed as an alternative, such as the use of Genipin as a covalent binding agent.

Genipin, a natural compound derived from *Gardenia jasminoides*, stands out for its low toxicity and its ability to react spontaneously with primary amines present in proteins, forming stable covalent bonds. Like glutaraldehyde, genipin can modify the surface charge of the material; however, the crosslinking density must be carefully controlled to ensure that the binding sites remain accessible. The use of this agent in protease immobilization helps reduce autolysis, maintain the enzyme's structural integrity, and minimize leaching during reuse cycles [18,60]. Studies conducted by Sousa et al. [61] investigated the effect of genipin concentration on the immobilization of pepsin, demonstrating that using 50 % of the genipin extract concentration led to better biocatalyst performance.

Carbodiimide chemistry is widely used in enzyme immobilization. This strategy is based on the activation of carboxylic groups ($-\text{COOH}$) present on the surface of materials using EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide), which forms an unstable O-acylsurea intermediate. In the presence of NHS (N-hydroxysuccinimide), this intermediate is converted to active esters, which react with free amine groups of proteases, forming amide covalent bonds. Studies report that the interaction obtained through this pathway reduces leaching and increases the thermal and operational stability of immobilized proteases, as it promotes smoother and more controlled conjugation compared to rigid cross-links, such as those formed by glutaraldehyde. However, when activation occurs in excess of carboxylic groups, multipoint immobilization can occur, resulting in conformational rigidity and loss of catalytic activity. Thus, optimization of reaction conditions such as activation time, EDC/NHS concentration and pH is essential to avoid indiscriminate modification of essential enzyme groups [62,63].

The reports indicate the use of various agents for immobilization, providing great versatility for MNPs in the immobilization of different proteases.

4.2 Coating techniques

Coating strategies are applied to enhance the stability and interaction capacity of MNPs. This functionalization preserves the primary structure of the nanoparticles and prevents aggregation, which occurs due to the strong attraction between particles [46]. The following topics present the main organic and inorganic coating approaches, highlighting their advantages and applications.

4.2.1 Organic coating

Organic coatings on MNPs can be applied during or after the synthesis of the particles. Materials such as polymers, resins, waxes, or even oils are typically used. The application of these coatings aims to protect against moisture, enhance mechanical and chemical resistance, and modify the surface of the MNPs, enabling their use in various applications [64].

Chitosan is one of the most widely used polymers for coating magnetic nanoparticles (MNPs). It is a cationic polysaccharide structurally similar to cellulose, whose amine groups ($-NH_2$) allow both the formation of covalent bonds through agents such as glutaraldehyde, genipin, or EDC/NHS, and the electrostatic interactions between its protonated amines and carboxylic (acidic) groups present in proteases. This coating significantly increases the number of active sites available for immobilization, resulting in greater thermal and operational stability, and enzymatic reusability. However, a surface exposed to amine groups can cause electrostatic repulsion or induce conformational changes in the enzyme, compromising its activity. Despite this, chitosan has advantageous characteristics, such as biodegradability, biocompatibility, and the absence of immunogenic or allergenic properties, making it an ideal material for coating MNPs for biomedical and environmental applications [65].

Another widely studied polymer is dextran, a hydrophilic, nontoxic, and biodegradable polysaccharide composed of glucose units linked by α -1,6-glycosidic bridges. When used as a coating on MNPs, dextran confers colloidal stability and provides numerous hydroxyl groups ($-OH$) available for subsequent functionalization steps. Dextran coating contributes to a more flexible support–enzyme interface, increasing the density of accessible functional groups and, consequently, the number of binding sites available for immobilization. Furthermore, the dextran layer can improve biocompatibility and reduce the aggregation or denaturation of proteases during the immobilization process. However, as with most coating agents, an excessively thick layer can restrict substrate diffusion, compromising the enzyme's catalytic activity [46].

Among synthetic polymers, polyethylene glycol (PEG) stands out as one of the most effective coatings for magnetic nanoparticles (MNPs) due to its amphiphilic nature, which combines hydrophilic and hydrophobic characteristics. PEG significantly improves the dispersibility of MNPs in aqueous media and reduces nonspecific interactions between the support surface and biomolecules. Although it does not have

intrinsically reactive groups, the polymer can be functionalized with different chemical groups, enabling the covalent or noncovalent coupling of enzymes. Recent studies have explored the combination of PEG with other polymers, such as dextran, seeking synergistic effects that increase stability and retention of protein activity. Furthermore, PEG is biocompatible, flexible, nontoxic, and nonimmunogenic properties that make it especially attractive for medical applications. Its coating acts as a steric barrier that prevents the recognition and rapid elimination of MNPs by the immune system, prolonging their circulation time in the body and enhancing their therapeutic efficacy [66].

Another promising approach involves using polydopamine as a coating for MNPs, since its catechol and amine groups allow for multiple types of interactions, both covalent and non-covalent. As a versatile coating agent, polydopamine provides high protease loading due to its broad functional surface area, while maintaining the enzymes' biological activity, since the interactions formed are less restrictive than rigid cross-links. Like other modifying agents, polydopamine can impart negative or positive charges to the surface, depending on the microenvironmental conditions, enabling different types of interactions with biomolecules [67]. Recent studies have demonstrated the development of a recyclable material functionalized with halogenated amines on Fe_3O_4 and coated with polydopamine, exhibiting antimicrobial activity [68].

4.2.2 Inorganic coatings

Inorganic coatings play a crucial role in enhancing the stability and functionality and expanding the applications of MNPs. Materials such as silica and biocompatible metals are widely used to reduce aggregation and oxidation, improving the performance of MNPs in biomedical, industrial, and environmental applications [69]. The following outlines the main strategies for inorganic coatings, their effects, and their applications.

Silica (SiO_2) coating is one of the most established approaches for the inorganic encapsulation of MNPs. Silica is a nontoxic, optically transparent, chemically inert, and thermally stable material with high porosity and a well-established surface for additional functionalization. These properties make silica an ideal coating for magnetic applications [70].

Mortazavi-Derazkola et al. [71] synthesized magnetite and coated it with silicon dioxide (SiO_2), observing a significant increase in the biocompatibility of the particles. In studies conducted by Xia et al. [72] magnetic iron was produced via a hydrothermal

method, followed by coating with silicone to obtain $\text{Fe}_3\text{O}_4@\text{SiO}_2$. The MNPs were then functionalized with APTES for lipase immobilization, resulting in an increase in immobilization efficiency from 52.8 % to 86.5 % and enhanced stability and reusability of the bioreactor.

The modification of MNPs with precious metals, such as gold (Au), has gained attention due to its application in biosorption, catalysis, and biomolecule immobilization. This coating can be achieved by different methods, such as microemulsion, redox transmetalation, and interactive seeding with hydroxylamine, which is an effective approach to prevent the oxidation of MNPs [46]. Gold-coated magnetic nanoparticles ($\text{Au}@\text{MNPs}$) have been widely used in analytical chemistry and the development of electrochemical biosensors. Liu et al. [73] developed an innovative method for synthesizing gold-coated MNPs by combining wet chemistry and laser irradiation. In this process, MNPs and gold powder particles were irradiated with a laser in a reactive liquid medium, resulting in a highly efficient and stable coating.

5. IMPACTS ON THE MICROENVIRONMENT DUE TO FUNCTIONALIZATION AND IMMOBILIZATION

Traditionally, the immobilization of proteases on magnetic nanoparticles (MNPs) has been approached as a means of increasing stability and enabling enzymatic reuse. However, recent advances indicate that the microenvironment generated at the support-enzyme interface plays a much more active role, acting as a modulator of catalytic specificity and hydrolysis profile [74]. This section discusses how the physicochemical properties of MNPs and their coatings can be exploited to target the production of specific bioactive peptides.

5.1 Effect of the microenvironment on specificity and hydrolysis profile

The microenvironment of MNPs, defined by factors such as surface charge density, hydrophobicity, and the presence of coating polymers, can alter the accessibility of the protease's active site to different regions of the protein substrate [64].

- **Cleavage Direction:** The orientation of the enzyme on the support, induced by specific functionalization (e.g., use of affinity tags or spacers), can expose or conceal catalytic domains. Studies demonstrate that the oriented immobilization of trypsin on MNPs functionalized with metal chelates can alter the cleavage

pattern of complex proteins, such as bovine serum albumin (BSA), resulting in peptide profiles distinct from those obtained with the free enzyme [21].

- Thick or highly charged coatings (e.g., chitosan or branched polymers) can create diffusion barriers that select which protein fractions reach the active site. This is particularly relevant in whey protein valorization, where microenvironment modulation can favor the hydrolysis of beta-lactoglobulin over other proteins, directing production towards peptides with greater antioxidant or antihypertensive potential [75].

5.2 Modulation of kinetic parameters (kcat/Km)

Immobilization alters the apparent kinetic constants of protease due to conformational changes and diffusional limitations imposed by the MNP microenvironment.

- Catalytic Efficiency (kcat/Km): Functionalization with flexible polymers, such as polyethylene glycol (PEG) or dextran, establishes a more permissive environment that minimizes conformational stress. In contrast, supports with a high density of reactive groups can induce multiple covalent bonds, restricting the structural mobility required for the transition state, which often results in a reduction of kcat[76].
- Substrate Affinity (Km): The apparent Km can be reduced (indicating higher affinity) if the microenvironment of the support concentrates the substrate close to the enzyme through favorable electrostatic interactions. For example, MNPs with a surface charge opposite to that of the protein substrate can facilitate the formation of the enzyme-substrate complex, compensating for losses in intrinsic activity[77].

6. PROTEOLYTIC BIOREACTORS AND THEIR APPLICATIONS

6.1 Trends in protease immobilization in MNPs

Several strategies have been developed for producing and modifying MNPs for protease immobilization. These approaches enhance enzyme stability, facilitate enzyme separation and reuse, and optimize their catalytic performance for applications in various fields [78]. Table 4 presents a series of studies on the conjugation of proteases with MNPs for different applications.

Proteolytic hydrolysis mediated by immobilized proteases has been widely explored, particularly in the production of bioactive peptides with antimicrobial, antioxidant, antitumor, chelating, antihypertensive, and anti-inflammatory activities, making it applicable in healthcare and the food industry [61]. Siddiqui and Husain [79] evaluated the biocatalysis of conjugated proteases in the hydrolysis of bovine serum albumin, casein, and ovalbumin. It was observed that immobilized trypsin exhibited higher hydrolysis efficiency than the free enzyme. Approximately 50 % of casein was hydrolyzed in 40 minutes by the free enzyme, while 80 % was hydrolyzed by the immobilized enzyme, demonstrating the superiority of the magnetic bioreactor. The lower efficiency of free trypsin may be related to reduced steric hindrance in the immobilized system, allowing for an optimal interaction between the enzyme and the substrate. Additionally, the limited autolysis of immobilized trypsin ensured its prolonged stability during digestion.

Proteases also play a crucial role in sustainable leather production, particularly in the depilation and fiber opening process. These enzymes facilitate the hydrolysis of non-collagenous substances, such as elastin, casein, and proteoglycans, while preserving the integrity of keratin and collagen. The use of proteases emerges as an eco-friendly alternative to the traditional sodium sulfide, offering a more sustainable approach to leather processing [80]. The immobilization of proteases on MNPs has been evaluated for the removal of interfibrillar material from the skin, showing enhanced enzymatic activity after immobilization [81]. Additionally, bioreactors based on MNPs immobilized with alkaline proteases derived from hair waste have been applied to hydrolyze oat bran, egg whites, soy protein isolates, and casein. This approach yielded a 24 % higher hydrolysis performance than the free enzyme. The use of MNPs for immobilizing proteases not only enhances the hydrolysis process but also offers the advantage of enzyme reuse and better operational stability, making it a promising strategy for improving the efficiency of protein hydrolysis in various industrial applications [80].

Proteases are commonly used in enzymatic detergents; however, their stability can be compromised in alkaline environments and high temperatures. Immobilizing these enzymes on MNPs enhances their resistance and efficiency in protein stain removal [82]. The recombinant Subtilisin protease from *Bacillus subtilis* PTTC 1023 retained its full enzymatic activity in the presence of various detergents [83]. These results indicate that the immobilization of proteases can make them more effective under adverse conditions, expanding their potential in cleaning product formulations.

In the food industry, proteases can be used in animal hide treatment, gelatin extraction, and meat and fish processing. Furthermore, they have been studied for the degradation of organic matter in wastewater, reducing costs and environmental impacts [84]. Ariaeenejad et al. [85] investigated the degradation of chicken feathers using keratinase immobilized on MNPs. The bioreactor exhibited 88 % hydrolysis efficiency after 72 hours of reaction, demonstrating its viability as an alternative to conventional physicochemical treatment. The immobilization of proteases in magnetic nanoparticles has proven efficient for applications in the food industry and waste treatment, allowing the degradation of complex organic materials with high conversion, as evidenced by the hydrolysis of chicken feathers with immobilized keratinase, and representing a viable and sustainable alternative to conventional physicochemical methods.

In the biomedical sector, proteases are used due to their wound-healing and antimicrobial properties, and they are being investigated for the degradation of microbial biofilms [80]. Kim et al. [86] developed magnetic nanocarriers based on halloysite functionalized with APTES for the immobilization of type I protease, assessing its antibiofilm performance. The bioreactor exhibited high activity against biofilms of *Escherichia coli*, *Staphylococcus aureus*, and methicillin-resistant *Staphylococcus*, demonstrating great potential for therapeutic applications.

In summary, the immobilization of proteases on magnetic nanoparticles represents a convergence of stability engineering, catalytic efficiency, and sustainable process design. Beyond simply improving thermal and operational stability, these systems allow fine control over enzyme orientation, microenvironment, and substrate accessibility parameters that directly influence the selectivity and generation of bioactive peptides. Such versatility enables their application across diverse sectors, from biomedicine to environmental remediation and food processing, where enzymatic reuse, magnetic recovery, and reduced waste generation are critical advantages. Therefore, the integration of magnetic nanomaterials as enzyme carriers not only enhances catalytic performance but also aligns with the current demand for greener, more efficient, and recyclable biocatalytic technologies

Table 4. Performance of proteases immobilized in magnetic nanoparticles and their applications in bioprocesses

MNPs	Protease	Results	Application	References
Graphene oxide@Fe ₃ O ₄	L-Asparaginase	80 % activity retained after eight cycles	Ammonia production	[87]
Fe ₃ O ₄	Urokinase	MNPs showed 50 % more activity than the free enzyme	Thrombus removal	[88]
Fe ₃ O ₄	Sortease A	5 activity cycles	Protein modification	[89]
Fe ₃ O ₄	2S1 Keratinase	43 % activity retained after ten cycles	Hydrolysis of keratin residues	[90]
Fe ₃ O ₄	Protease from <i>Bacillus</i> KHB3	80 % activity retained after ten cycles	Hydrolysis of blood clots and protein residues	[91]
Fe ₃ O ₄ @Silicon dioxide	Alkalase	80 % activity retained after ten cycles	Production of hydrolysates with emulsifying, emulsifying, and antioxidant properties	[92]
Magnetic Chitosan	Fish trypsin	16 % activity lost after ninety days of storage	Detection of antinutritional compounds	[93]
Fe ₃ O ₄	Enterokinase	90 % activity retained after ten cycles	Removal of histidine from recombinant proteins	[94]
Fe ₃ O ₄	Trypsin	Catalysis efficiency after fifteen minutes observed in electrophoresis	Assisted digestion of BSA and human serum	[95]

CuFe ₂ O ₄	Papain	50,5 % activity retained after ten cycles	Antibacterial studies against <i>S. aureus</i> and <i>E coli</i>	[96]
Graphene oxide@ Magnetic nickel	Urokinase	Immobilized Urokinase showed a 77.6 % increase in specific activity compared to free Urokinase.	Hydrolysis of human serum for thrombolysis treatment	[97]
Fe ₃ O ₄ @CLEA	Thermophilic-tolerant protease	100 % activity retained after eight cycles	Whey protein hydrolysis, silver recovery, hair removal, and application as an additive in detergents	[98]
Fe ₃ O ₄ @Chitosan	Fibrinolytic Serine Metalloprotease	70 % activity retained after ten cycles	Applied to clot lysis	[99]
Fe ₃ O ₄ @HEMA-GMA	L-Asparaginase	80 % activity retained after five cycles	<i>In vivo</i> cancer treatment	[100]

6.2 Trends in functionalization and support

A predominance of pure or minimally modified Fe_3O_4 MNPs is observed as a base support, indicating their versatility and ease of synthesis. However, surface functionalization is crucial to optimize performance. Coatings such as graphene oxide (Graphene oxide@ Fe_3O_4 , Graphene oxide@Magnetic nickel) and chitosan (Magnetic Chitosan, Fe_3O_4 @Chitosan) are common. Graphene oxide, with its high surface area and functional groups, is used for L-Asparaginase and Urokinase, suggesting a role in improving activity and stability for biomedical and industrial applications. Chitosan, due to its biocompatibility and ability to form bonds, is used for fish trypsin and fibrinase, highlighting its relevance in biological and food contexts. Other modifications, such as silicon dioxide (Fe_3O_4 @Silicon dioxide) and CLEA (Cross-linked Enzyme Aggregates), are employed for proteases like Alkalase and thermotolerant proteases, aiming at the production of hydrolysates with specific properties and robustness under industrial conditions.

6.3 Operational stability and reuse

Most studies demonstrate excellent operational stability, with enzymatic activity retention typically ranging from 70% to 90% after 8 to 10 reuse cycles. Notable cases include L-Asparaginase (80% after 8 cycles), Bacillus proteases (80% after 10 cycles), and Enterokinase (90% after 10 cycles). Thermotolerant protease immobilized via CLEA on Fe_3O_4 achieves an impressive 100% activity after 8 cycles, underscoring the potential of certain immobilization strategies to confer extreme robustness. Fish trypsin on magnetic chitosan, although evaluated by storage time, shows a loss of only 16% of activity after ninety days, indicating good long-term stability. These results reinforce the effectiveness of immobilization in MNPs to increase the longevity and economic viability of biocatalysts.

6.4 Classes of proteases and applications

Immobilized proteases encompass several classes, notably serine proteases (Trypsin, Urokinase, Fibrinolytic Serine Metalloprotease) and metalloproteases (Fibrinolytic Serine Metalloprotease). Applications are broad and can be categorized as follows:

- Biomedical: Thrombus removal (Urokinase), cancer treatment (L-Asparaginase), hydrolysis of human serum for thrombolytic treatment (Urokinase), clot lysis (Fibrinolytic Serine Metalloprotease). In these cases, the biocompatibility of the support (such as chitosan) and the targeting capabilities of MNPs are crucial.
- Industrial and Food: Production of hydrolysates with emulsifying and antioxidant properties (Alkalase), hydrolysis of keratin residues (2S1 Keratinase), hydrolysis of whey proteins (thermotolerant protease), detection of antinutritional compounds (fish trypsin), and applications in detergents. For these, robustness, reusability, and large-scale catalytic efficiency are the main drivers.
- Biotechnological/Research: Protein modification (Sortase A), histidine removal from recombinant proteins (Enterokinase), assisted digestion of BSA and human serum (Trypsin). In these, the specificity and purity of the process are of paramount importance.

7. FUTURE PERSPECTIVES AND CHALLENGES IN THE IMMOBILIZATION OF PROTEASES IN MNPs

The prospects for immobilizing proteases on magnetic nanoparticles (MNPs) point to innovations that transcend simple stabilization, focusing on rational design and intelligent functionality. Although the field has advanced significantly, the transition from laboratory systems to high-precision industrial and biomedical applications requires overcoming specific challenges related to molecular dynamics and microenvironment responsiveness[101]. Figure 5 summarizes the main innovation fronts that will shape the future of this field.

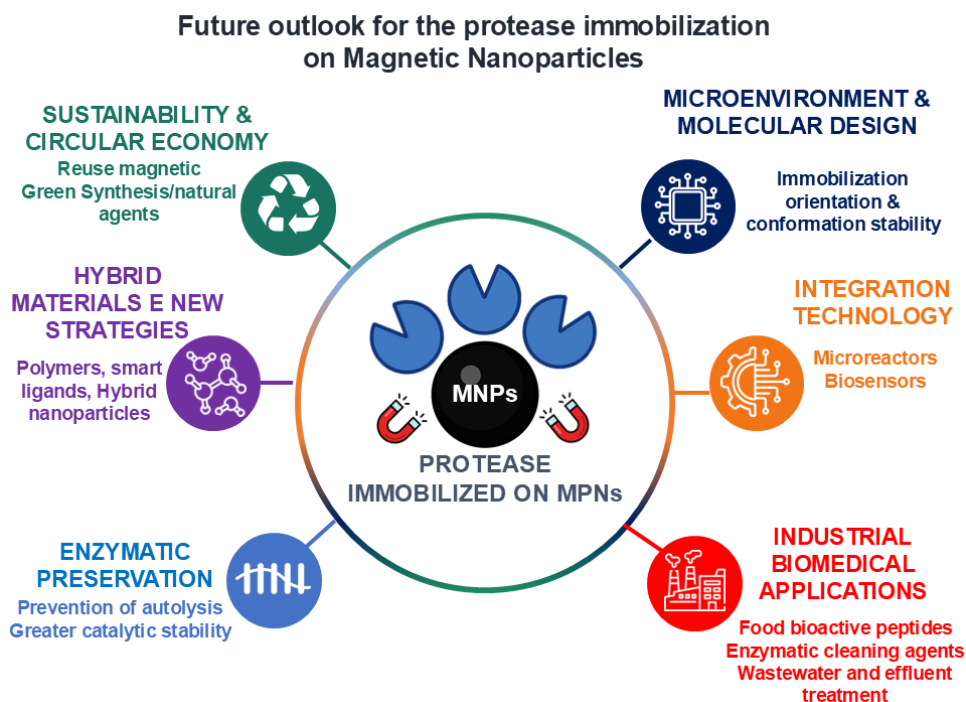


Figure 5. Future outlook for the protease immobilization on Magnetic Nanoparticles

7.1 Rational Design via Molecular Modeling and Dynamics (MD)

One of the greatest current challenges is predicting how the functionalized surface of MNPs interacts with the protease's active site. The use of Molecular Dynamics (MD) and Molecular Docking simulations emerges as an indispensable tool for the rational design of supports [102].

- **Enzyme-Support Interface Simulation:** Future studies should focus on how nanoparticle curvature and the charge density of the coating layer (e.g., chitosan or PEG) affect the conformational flexibility of the protease. MD simulations can reveal whether adsorption or covalent bonding induces deformations in the active site, allowing in silico selection of the ideal length of "spacers" that ensure correct orientation and avoid steric hindrance for bulky substrates[103].
- **Thermal and Chemical Stability Prediction:** Computational modeling will allow predicting the resistance of immobilized protease to organic solvents and extreme temperatures even before experimental synthesis, accelerating the development of biocatalysts for drug synthesis and industrial wastewater treatment[104].

7.2 Intelligent and stimulus-responsive systems

The next generation of enzymatic MNPs will be characterized by "intelligent" behaviors, where protease activity is modulated by external stimuli or changes in the microenvironment[105].

- **Site-Specific Release and Activation:** In the biomedical field, a central objective is the development of MNPs that release proteases (such as urokinase or asparaginase) only under specific conditions, such as the acidic pH of the tumor microenvironment or in the presence of matrix metalloproteinases (MMPs) overexpressed in diseased tissues[106]. This would minimize systemic side effects and increase therapeutic efficacy.
- **Magnetic Field-Controlled On/Off Systems:** The application of low-frequency oscillating magnetic fields can be explored to induce local microheating or conformational changes in the support, allowing remote control of the hydrolysis rate in continuous-flow bioreactors[107].

7.3 Sustainability and technological integration

In line with green chemistry, the replacement of toxic crosslinking agents with natural alternatives, such as genipin and plant polyphenols, will cease to be an alternative and become the industrial standard[17]. Furthermore, the integration of enzymatic MNPs into continuous flow microreactors and biosensors will allow the automated production of bioactive peptides with strictly controlled hydrolysis profiles, facilitating the valorization of by-products of the food industry, such as whey, on a global scale[75].

In short, the future of proteases immobilized in MNPs lies in the ability to manipulate matter at the atomic scale to create biocatalytic systems that not only survive industrial conditions but also intelligently "respond" to the biotechnological challenges of the 21st century[108].

8. CONCLUSIONS

The immobilization of proteases on magnetic nanoparticles (MNPs) is consolidating itself as a cutting-edge biotechnological strategy, offering effective solutions to critical challenges such as autolysis, operational instability, and difficulty in enzyme recovery. This review demonstrated that the careful choice of support and functionalization strategy is crucial for the success of the biocatalyst, allowing not only

its reuse for multiple cycles but also the maintenance of high activity rates under adverse industrial conditions.

One of the main differentiating factors presented in this work is the understanding that immobilization transcends simple physical stabilization. The microenvironment generated at the interface between the enzyme and the nanoparticle acts as an active modulator of catalytic selectivity. By manipulating properties such as surface charge and the use of polymeric coatings, it is possible to direct the hydrolysis profile towards the generation of specific bioactive peptides, adding value to industrial byproducts and opening new avenues in the production of functional foods and nutraceuticals.

In line with the principles of green chemistry and the circular economy, the use of MNPs promotes cleaner and more efficient processes. However, the future of the field lies in overcoming technological gaps, especially regarding rational design via molecular modeling and the development of intelligent systems responsive to stimuli. In short, this manuscript provides a solid and critical basis for the development of new biocatalytic platforms that are simultaneously highly efficient, sustainable, and capable of responding to complex contemporary biotechnological challenges.

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CAPÍTULO 3 – ARTIGO 2

GENIPIN-FUNCTIONALIZED MAGNETIC MATERIALS: A SUSTAINABLE AND INNOVATIVE METHOD FOR IMMOBILIZING PROTEASES TO PRODUCE ANTIOXIDANT PEPTIDES

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Genipin-functionalized magnetic materials: a sustainable and innovative method for immobilizing proteases to produce antioxidant peptides.

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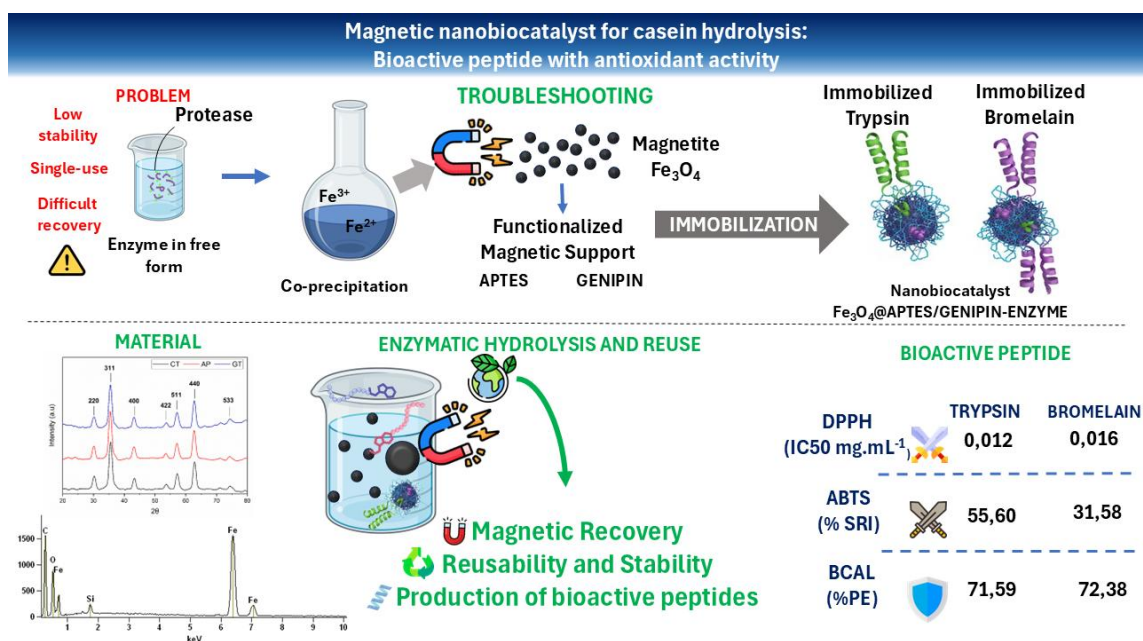
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Graphic Abstract



Abstract: The use of enzymes in the food industry continues to expand, although challenges that still limit their large-scale applications like stability, reuse, and contamination. Enzyme immobilization on magnetic nanoparticles (MNPs) is an efficient strategy to overcome these limitations, enabling greater structural stability, enhanced catalytic activity, and easy magnetic recovery. In this study, magnetite nanoparticles were synthesized and functionalized with (3-aminopropyl)triethoxysilane (APTES) and genipin, a green alternative, resulting in a biocompatible and reactive support for the immobilization of trypsin and bromelain. Structural analyses (XRD, FTIR, zeta potential, SEM/EDS, and TGA) confirmed the magnetite crystalline phase and the successful surface modification, indicating linkage between the enzymes and the support. Immobilization efficiencies of 67.68% for bromelain and 65.19% for trypsin were achieved, maintaining high specific activities (8.14 and $60.85 \text{ U} \cdot \text{g}^{-1}$, respectively). The immobilized enzymes presented operational stability and retained their activity after six reuse cycles. Casein hydrolysis with the immobilized enzymes yielded peptides with significant antioxidant potential (DPPH = $0.016 \text{ mg} \cdot \text{mL}^{-1}$; ABTS = 55.60%; β -carotene/linoleic acid = 72.38%). Thus, the developed platform demonstrates high catalytic performance, combined with sustainability and reuse, and represents a promising alternative for biotechnological and industrial processes in the food sector.

Keywords: Magnetic recovery; Surface functionalization; Trypsin; Bromelain; Casein hydrolysis

1. INTRODUCTION

The study of nanotechnology has grown significantly in recent decades, with promising applications in different scientific areas, including industry and biotechnology. This field includes development, characterization, and application of materials with dimensions ranging from 1 to 100 nm, which exhibit unique physical and chemical properties. Among these materials, magnetic nanoparticles (MNPs) stand out, having been widely investigated for their biocompatibility, low toxicity, easy functionalization, besides separation and recovery via external magnetic fields application [1,2].

MNPs can be obtained through different synthetic routes, with chemical methods, coprecipitation in special, being the most widely used once its allow greater control over particle size, crystallinity, and morphology [3–5]. Surface functionalization is essential to expand the applicability of these nanostructures, as in biomedical and industrial contexts. Genipin, a natural crosslinker agent obtained from the *Genipa americana* fruit, has been studied as a less toxic alternative to glutaraldehyde and its use in magnetic nanoparticles is still few explored [6,7]. In addition, MNPs have broad functional versatility being able for diverse applications, including bioactive properties such as antimicrobial, antioxidant [8], and anticancer activities [9] controlled drug delivery systems [9], therapeutic treatments such as magnetic hyperthermia applied to tuberculosis [10], as well as environmental processes, for example, the removal of dyes from wastewater [11].

To enzyme immobilization, MNPs offer advantages due to their wide availability of reactive functional groups, allowing efficient interactions with enzymes through amino, carboxyl, hydroxyl, or thiol groups [12,13]. The use of MNPs for immobilizing different enzymes, such as glucoamylase [14], α -amylase [15], pectinase [16], laccase [17], lipase [18] has been reported in the literature. This process is especially relevant for proteases, which present an autolysis risk when used in their free form. Proteases are one of the most widely used groups of enzymes in industry, catalyzing the hydrolysis of peptide bonds and generating peptides capable of exhibiting antioxidant, antimicrobial, and antitumor activities, among others [19,20]. The global market for this biocatalyst is growing steadily, with forecasts reaching US\$4.74 billion by 2029, driven by applications in the pharmaceutical, diagnostic, and food industries [21].

Among the most technologically relevant proteases are bromelain (EC 3.4.22.44), a cysteine protease derived from pineapple and widely used in food, pharmaceutical, and industrial formulations [22–24], and trypsin (EC 3.4.32.4), a serine enzyme used in the production of protein hydrolysates and highly digestible dairy products [25,26]. The possibility of reusing these enzymes by immobilizing them on magnetic nanomaterials represents an economically attractive strategy, increasing operational stability and facilitating recovery after use.

Considering this scenario, this study proposes the development of genipin-functionalized magnetic nanoarchitectures for the immobilization of trypsin and bromelain, with the aim of producing peptides with antioxidant activity. To our knowledge, this is a pioneering study that combines APTES and genipin as binding agents for immobilizing these proteases on magnetic nanoparticles, and compares the catalytic performance and antioxidant potential of peptides produced by free and immobilized enzymes. In addition, the study advances sustainable technologies aligned with Sustainable Development Goal 9 (Industry, Innovation, and Infrastructure), reinforcing the potential of magnetic materials as efficient, reusable platforms for biocatalysis on an industrial scale.

2. MATERIALS AND METHODS

2.1 Materials

The reagents used in this study were of at least analytical grade (PA-ACS) and purchased from Sigma-Aldrich Brazil. These included: ferrous sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; CAS: 7782-63-0), ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$; CAS: 10025-

77-1), sodium hydroxide (NaOH; CAS: 1310-73-2), 3-aminopropyltriethoxysilane (APTES; CAS: 919-30-2), stem bromelain from pineapple (≥ 3 U/mg protein; CAS: 37189-34-7), bovine pancreatic trypsin ($\geq 10,000$ BAEE units/mg protein; CAS: 9002-07-7), casein (CAS: 9005-46-3), trichloroacetic acid (TCA; CAS: 73-03-9), Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3 ; CAS: 497-19-8), 2,2-diphenyl-1-picrylhydrazyl (DPPH; CAS: 1898-66-4), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; CAS: 30931-67-0), β -carotene (CAS: 7235-40-7), and linoleic acid (CAS: 60-33-3). Other reagents used are described throughout the methodology, and all solutions were prepared using deionized water. For surface functionalization, a genipin extract previously characterized by Santos et al. [27] was used at a concentration of 6.46 mg.mL⁻¹.

2.2 Synthesis of Fe₃O₄ magnetic nanoparticles (MNPs)

Magnetic nanoparticles (MNPs) were prepared via co-precipitation of Fe²⁺ and Fe³⁺ ions, following the methodology described by Aslani et al. [3]. The full description of the methodology and its modification is available in the supplementary data file.

2.3 Functionalization of MNPs

For the functionalization of MNPs, introducing amino groups into their structure, the methodology described by Aslani et al. [3] was followed with modification. Briefly, 500 mg of MNPs were dispersed in 100 mL of 70% (v/v) ethanol and placed in an ultrasonic bath for 30 min. Subsequently, 1.5 mL of APTES was added to the solution, which was maintained under orbital stirring (20 rpm) for 20 h at 50 °C in a BOD incubator. Afterward, the MNPs were washed with 100 mL of 70% ethanol, then with 100 mL of acetone. The MNPs were then separated using a magnet, lyophilized, and proceeded to the next functionalization step. This procedure rendered the MNPs amino-activated.

The amino-activated MNPs were then converted into carbonyl-activated MNPs. For this purpose, 500 mg of APTES-activated MNPs were mixed with 20 mL of acetone and 20 mL of genipin-containing *Genipa americana* extract. The mixture was maintained under orbital stirring for 2 h. Subsequently, the material was washed with distilled water, separated using a magnet, and lyophilized. The resulting carbonyl-activated MNPs were then stored in an oxygen-free container.

2.4 Characterization of MNPs

The functional groups present on the MNPs were analyzed by Fourier-transform infrared (FTIR) spectroscopy, following the methodology described by Silva et al. [28], using the attenuated total reflectance (ATR) technique in the infrared range of 4000–600 cm^{-1} on a spectrometer (Cary 630 FTIR, Agilent Technologies Inc., Santa Clara, CA, USA).

The synthesized materials were evaluated using a Shimadzu XRD-6000 X-ray diffractometer (Kyoto, Japan), where approximately 20 mg of previously ground sample was placed in the sample holder and analyzed under the following conditions: 30 kV voltage, 30 mA, $\text{CuK}\alpha$ radiation ($\lambda = 0.15406 \text{ nm}$), 2θ range of $5\text{--}80^\circ$, goniometer speed of 2° min^{-1} , and step size of 0.02° . Mass loss was determined by thermogravimetric analysis (TGA) using a calorimeter (Linseis STA PT1000, Selb, Germany) with approximately 10 mg of MNPs, heated from 20 to 900°C at $10^\circ\text{C}\cdot\text{min}^{-1}$ under a nitrogen atmosphere. From the X-ray diffractograms, the crystalline fraction of the materials was estimated by integrating the peak areas after subtracting a local baseline [29]. Furthermore, the average crystallite size was calculated using the Debye–Scherrer equation, considering the full width at half height (FWHM) of the peaks, determined by direct interpolation on the experimental diffractogram [30,31].

The morphology and chemical composition of the MNPs were assessed by scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS). Samples were mounted on double-sided conductive carbon tape and coated with a conductive layer using the Desk V Gold Film Deposition System (Denton Vacuum LLC, Moorestown, NJ, USA) equipped with a carbon accessory. The samples were then analyzed using a JSM-6610 scanning electron microscope (JEOL, Tokyo, Japan) equipped with a Thermo Scientific EDS system (NSS Spectral Imaging, USA).

Measurements of zeta potential and particle size of the produced materials were carried out using a Malvern Zetasizer NanoSZ (Malvern Instruments, UK). The samples were dispersed in aqueous solution at a concentration of $0.1 \text{ mg}\cdot\text{mL}^{-1}$ and sonicated for 10 minutes before analysis to ensure proper dispersion and minimize particle aggregation.

2.5 Immobilization of trypsin and bromelain

For the immobilization of trypsin and bromelain, the methodology described by Santos et al. [20] was followed with modifications (which can be observed in the supplementary data file). For this, 30 mg of carbonyl-activated MNPs were mixed with 3

mL of a solution containing proteases (trypsin or bromelain 1 mg.mL⁻¹) overnight (14 h). The immobilized protein was quantified using the Bradford method [32]. The immobilization capacity of the materials was calculated according to Equation 1:

$$C_{im} = \frac{(C_o - C) \times V}{M} \quad (1)$$

Where C_{im} is the immobilization capacity (mg.g⁻¹); C_o is the initial protein concentration in the solution (mg.mL⁻¹); C is the final protein concentration (mg.mL⁻¹); V is the solution volume (mL); and M is the mass of the support material (g).

A schematic representation of the bioreactor development is shown in Figure 1.

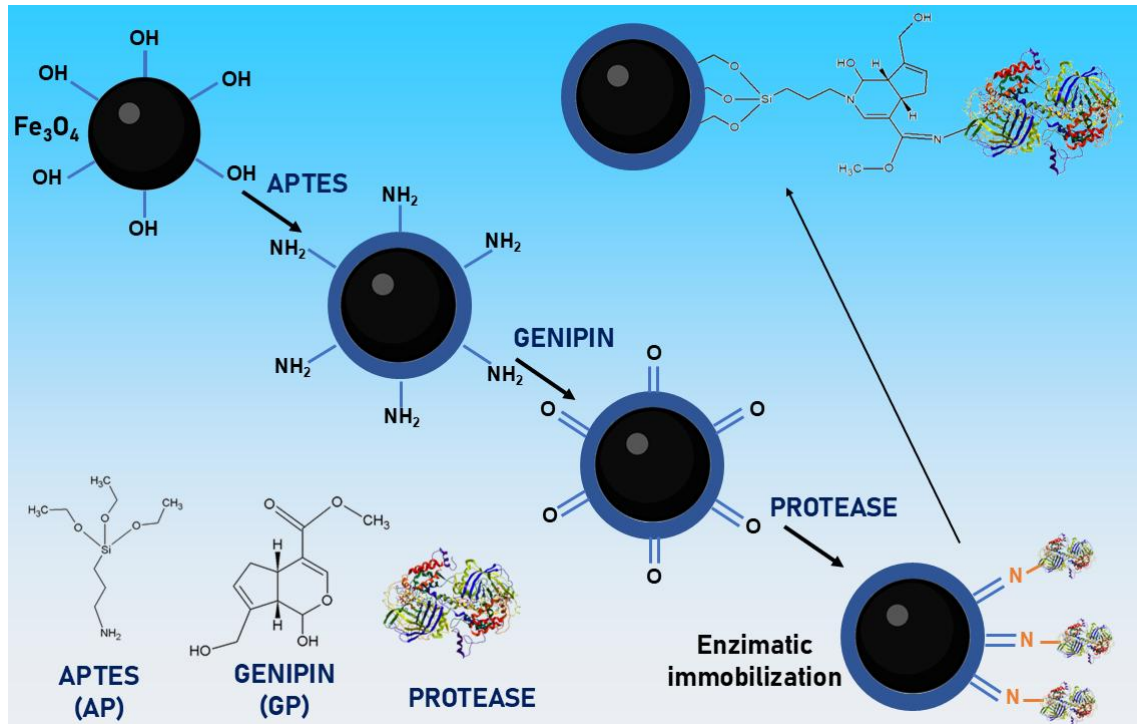


Figure 1. Schematic representation of the proteolytic bioreactor production process (functionalization and immobilization steps).

2.6 Enzyme activity determination

The enzymatic activity of free and immobilized enzymes in the bioreactors was determined following a methodology adapted from Santos et al. [20] and Souza et al. [33], with modifications. The full description of the methodology and modifications is available in the supplementary data file. For this purpose, a calibration curve was constructed using tyrosine at concentrations ranging from 0.1 to 1 mg.mL⁻¹, according to the Lowry method. One unit (U) of proteolytic activity was defined as the amount of enzyme required to release 100 µg of tyrosine equivalent under the assay conditions, as determined by Equation 2.

$$\text{Specific enzyme activity}(U/g) = \left(\frac{\frac{C_{\text{Tyrosine}}(\frac{\mu\text{mol}}{\text{mL}})}{\text{Time}(\text{min})}}{\text{Enzyme mass}(g)} \right) (2)$$

2.7 Effect of pH and temperature on enzyme activity

The enzymatic activity of trypsin and bromelain, in both free and immobilized forms, was evaluated across different pH and temperature ranges. For the pH assessment, buffer solutions with pH values ranging from 5 to 9 were prepared. A sodium acetate buffer at pH 5, a sodium phosphate buffer at pH 6–8, and a sodium carbonate buffer at pH 9 were used at 0.1 M, and temperature-dependent activity was assessed between 20 and 70 °C using a thermostatic water bath. Enzymatic activity was determined using the same protocol described in Section 2.6, with the respective pH and temperature conditions of interest.

2.8 Evaluation of bioreactor reuse cycles

The proteolytic bioreactors were evaluated over multiple use cycles, with 6 cycles performed to assess retention of enzymatic activity. The methodology for determining activity was described in Section 2.6. After each activity cycle, the bioreactors were washed with 3 mL of sodium phosphate buffer for 10 min under orbital stirring (20 rpm) to remove any residual casein from the medium.

2.10 Chromatographic profile of peptides obtained from casein hydrolysis

The chromatographic profile of the casein solution and the hydrolyzed solution containing peptides was analyzed by high-performance liquid chromatography (HPLC). A C18 column (250 × 4.6 mm, 5 μm particle size; ZORBAX Eclipse Plus C18) coupled to a pre-column (ZORBAX SB-C18, 4.6 mm ID × 12.5 mm, 5 μm) and connected to an HP Agilent 1260 Infinity II system was used. Two mobile phases were employed: phase A, composed of 5% acetonitrile and 0.1% trifluoroacetic acid (TFA), and phase B, consisting of 50% acetonitrile and 0.1% TFA. A 20 μL aliquot of the sample was automatically injected into the column at a flow rate of 0.8 mL·min⁻¹ of mobile phases A and B for 70 min. The run began with 100% phase A, which decreased linearly over 60 min until 100% of the flow was represented by phase B. From 60 to 65 minutes, a new linear gradient was applied to re-establish 100% phase A, which was maintained until the end of the analysis at 70 minutes. Column temperature was maintained at 30 °C, and peptides were detected at 280 nm using a UV–Vis detector. The chromatographic profile was evaluated by comparing peak retention times and their UV spectra.

2.11 Antioxidant Capacity of the Obtained Peptides (DPPH, ABTS and BCAL)

Antioxidant analyses of peptides generated by proteases were performed to evaluate potential differences in the antioxidant characteristics of peptides produced by free and immobilized enzymes action. All assays were conducted in triplicate. The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging activity was evaluated according to the protocol described by Li et al. [34], with modifications. The ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging activity was determined following the methodology described by Re et al. [35]. The antioxidant capacity was also determined using the β -carotene–linoleic acid system (BCAL), following the methodology described by Aminjafari et al. [36] with modifications. The full description of the methodologies and their modifications are available in the supplementary data file.

3. RESULTS AND DISCUSSION

3.1 Obtaining MNPs

The obtained MNPs and the co-precipitation reaction of Fe^{2+} and Fe^{3+} ions are shown in Figure 2.

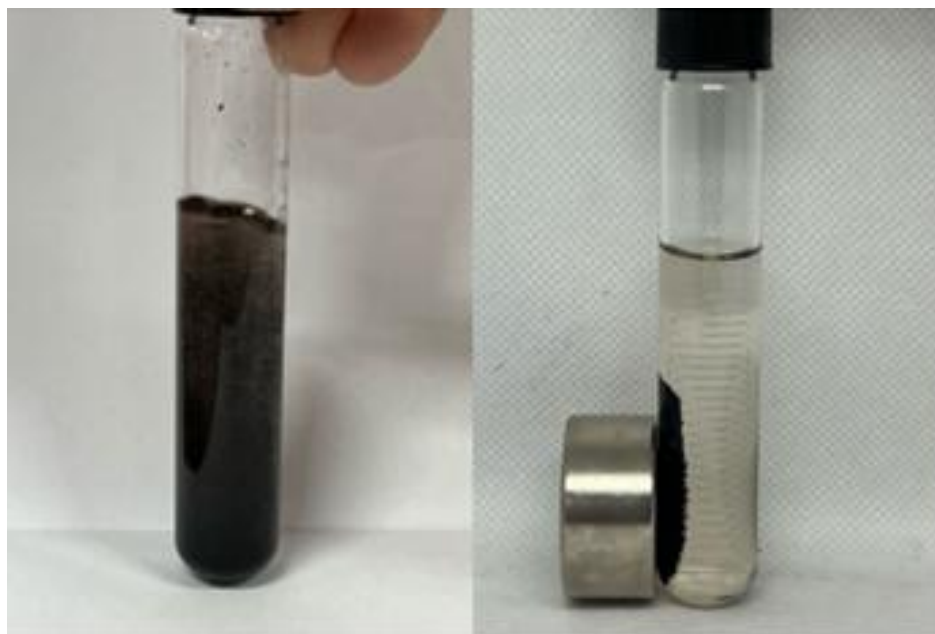


Figure 2. Images of the MNPs exposed to a magnetic field.

The produced particles exhibited magnetization, being attracted when exposed to external magnetic fields. A fine, granular, and firm powder was also observed after drying. According to Massart [37], a pioneer in the development of these materials, the efficiency in producing MNPs results from the co-precipitation of ferrous and ferric salts in the presence of strong oxidizing agents under an inert gas atmosphere, provided the

appropriate stoichiometric proportions are used. Therefore, the reaction efficiency depends on the strict control of these factors.

3.2 Characterization of the MNPs produced

3.2.1 Evaluation of MNP functionalization by FTIR spectroscopy

FTIR spectroscopy analysis was performed to identify the functional groups involved in the synthesis of MNPs and their subsequent functionalization with APTES and Genipin. The FTIR spectra are shown in Figure 3.

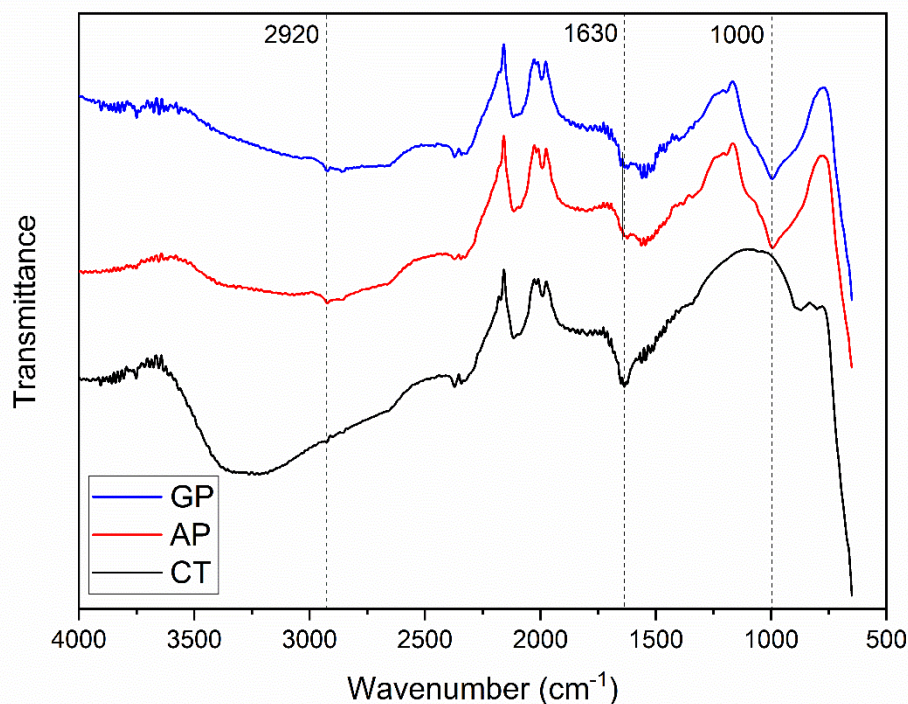


Figure 3. FTIR spectra of Fe_3O_4 control (CT), $\text{Fe}_3\text{O}_4@\text{APTES}$ (AP), and $\text{Fe}_3\text{O}_4@\text{APTES}$ -Genipin (GP), in the range of 600–4000 cm^{-1} .

The spectra show broadening and/or a reduction in vibrational modes when comparing the control MNPs to the functionalized ones. A pronounced band can be observed around $\sim 1000 \text{ cm}^{-1}$ for the AP sample. APTES is a compound that forms a siloxane network (Si–O–C), which exhibits asymmetric stretching vibrations, generating a characteristic vibrational mode in this region of the spectrum [38]. Numerous studies employing silicon-containing compounds have reported similar behavior [39,40].

Another characteristic vibrational mode of this modification was observed at $\sim 2920 \text{ cm}^{-1}$, corresponding to C–H stretching bands (CH_2 and CH_3) from the organic chain of APTES [38,39]. A reduction of the band around 1630 cm^{-1} was also noted from the control to the functionalized samples, which, according to Souza et al. [41], corresponds to the bending vibration of the water molecule (H–O–H), that is, water

adsorbed on the material surface. After the addition of the agent (APTES), this vibrational mode was almost completely reduced, as the coating decreased the amount of adsorbed water by rendering the surface more hydrophobic through the functional groups present in APTES and Genipin.

3.2.2 X-ray Diffraction Analysis (XRD)

The X-ray diffraction (XRD) analysis of the produced materials is shown in Figure 4.

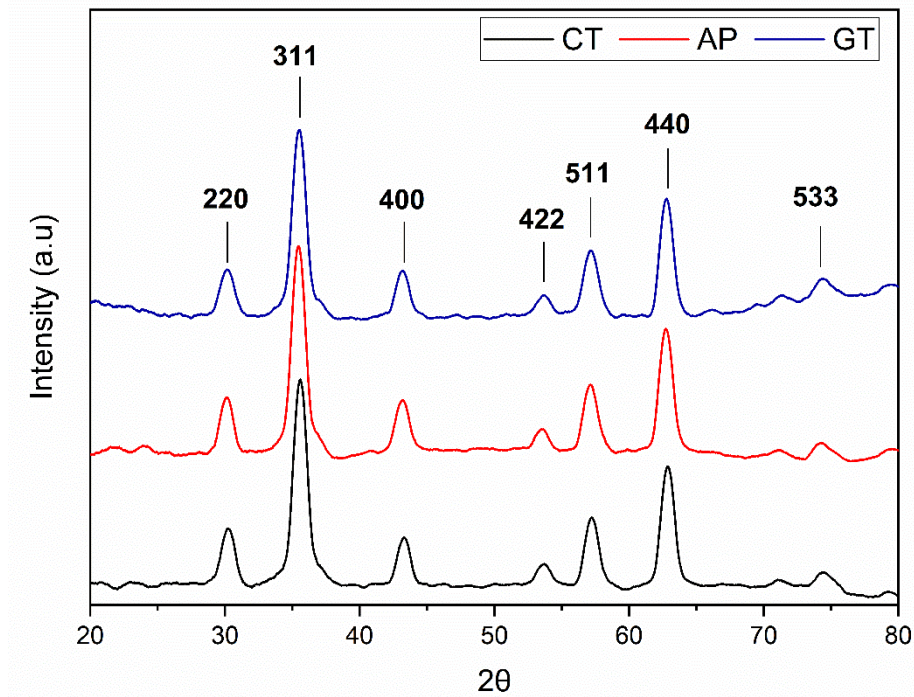


Figure 4. Diffractograms of MNPs: Fe_3O_4 control (CT), $\text{Fe}_3\text{O}_4@\text{APTES}$ (AP), and $\text{Fe}_3\text{O}_4@\text{APTES}$ -Genipin (GP).

The phase composition and crystal structure of the synthesized materials were analyzed by X-ray diffraction (XRD). The obtained diffractograms exhibited diffraction peaks at $2\theta \approx 30.2^\circ, 35.5^\circ, 43.2^\circ, 53.5^\circ, 57.2^\circ, 62.8^\circ,$ and 74.3° , corresponding to the crystallographic planes (220), (311), (400), (422), (511), (440), and (533), respectively. These reflections are consistent with the diffraction pattern of magnetite (Fe_3O_4), as reported by Mhammedsharif et al. [42]. Such peaks are characteristic of Bragg reflections associated with the face-centered cubic (FCC) structure of magnetite, confirming the formation of the expected phase Biswas et al. [43] and further validating the material's synthesis. Similar diffraction patterns have been reported for Fe_3O_4 nanoparticles, corroborating the high crystallinity and structural organization of the material [44,45].

Using the XRD data, the crystallinity and particle size were determined using the Debye-Scherrer equation, as presented in the table.

Table 1. Estimation of crystallite size and crystallization state of MNPs by XRD.

Sample	Particle Size by Debye-Scherer (nm)	Crystalline state (%)
CT	14.89	99.54
AP	14.80	90.47
GP	14.62	81.88

Legend: Fe₃O₄ control (CT), Fe₃O₄@APTES (AP), and Fe₃O₄@APTES-Genipin (GP)

The results showed fairly similar average crystallite sizes among the samples (14.6–14.9 nm), suggesting that the functionalization processes with APTES and genipin did not significantly affect the crystalline core size. This behavior is consistent with previous observations, which report that surface modifications of magnetite generally occur without altering the internal crystal structure [46].

Conversely, the crystalline fraction decreased progressively after the surface modification steps. Sample CT showed the highest crystallinity (99.5%), while AP and GP showed 90.5% and 81.9%, respectively. This decrease can be attributed to the introduction of organic groups and the formation of amorphous layers on the surface of the nanoparticles, as also reported [47].

The greatest reduction observed in the GP sample suggests that genipin retention promotes the formation of a thicker, less ordered organic layer, thereby increasing surface disorder. Despite this, such a modification favors enzyme immobilization, since the organic layer provides better stability, biocompatibility, and anchoring points for covalent binding of the biomolecule.

3.2.3 Thermogravimetric analysis

Figure 5 displays the thermal decomposition profiles, assessed by thermogravimetric analysis (TG) and differential thermal analysis (DTA).

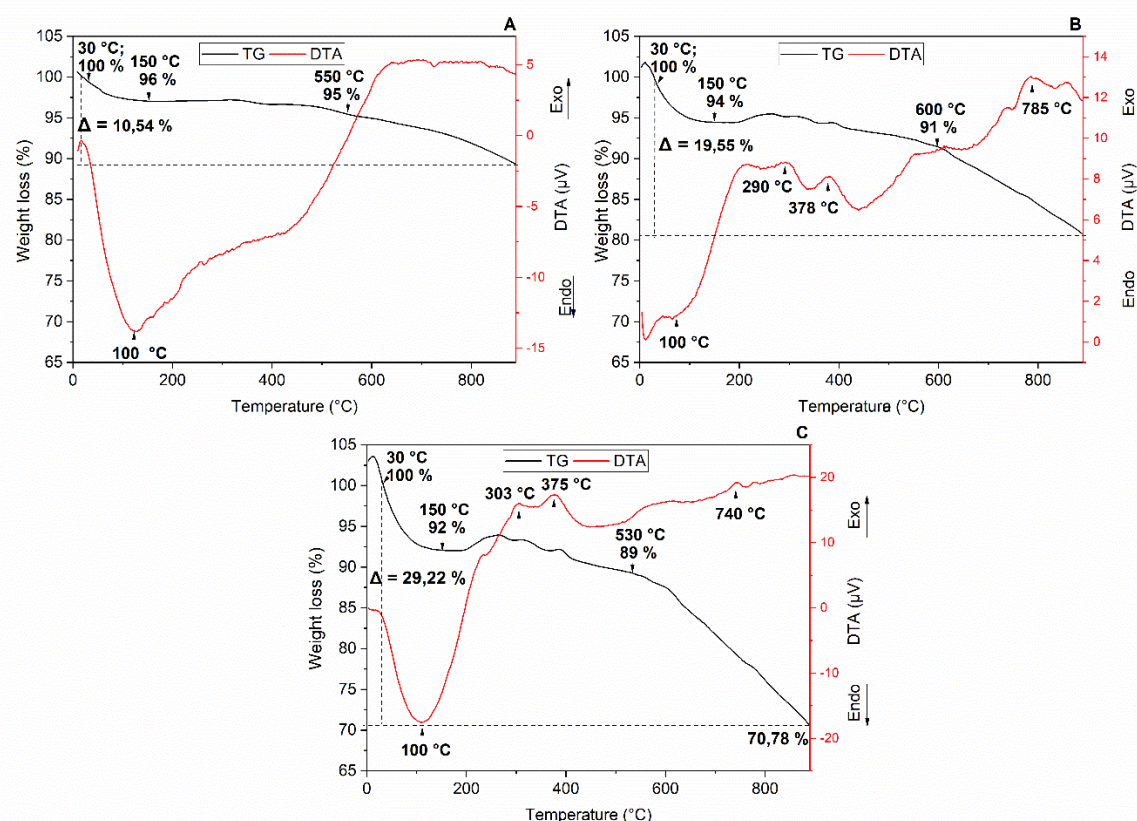


Figure 5. TG and DTA curves of MNPs: (A) Fe₃O₄ control (CT); (B) Fe₃O₄@APTES (AP); and (C) Fe₃O₄@APTES-Genipin (GP).

The mass-loss curves of the synthesized materials exhibit similar behavior due to their magnetic structure (Fe₃O₄). For all three samples, a thermal degradation event can be observed in the range of 30–150 °C, which is attributed to the vaporization of water within the structure [48]. In the GP sample, the mass loss reaches up to 8% compared to CT and AP during the first thermal event, a behavior that may be related to the agent's deposition on the material's surface.

For the CT sample, another thermal degradation event is observed, starting at approximately 550 °C. According to Kristl et al. [49] this degradation may be attributed to the presence of water bound to the magnetic structure. Finally, the CT sample shows a total mass loss of only 10.54%, indicating a tendency towards stabilization. This result is expected and is related to the inherent thermal stability of this type of material [49]. For comparison, Darwish et al. [50] reported a mass loss of 39.1% for Fe₃O₄. The improved stability observed in the present study is associated with better control of nanoparticle synthesis conditions, particularly through the use of nitrogen, which provided an inert atmosphere.

A second mass-loss event is observed between 150 °C and 600 °C for the AP sample. According to Ghutepatil et al. [51] the initial stages of this degradation are

associated with ethoxy groups and with the organic backbone of APTES. The mass loss occurring near 600 °C is related to the degradation of more stable organic residues and possible residual carbonization [52]. Finally, the APTES-functionalized particles (AP) exhibited a mass loss of 19.55%, which is higher than that of the control sample.

Regarding the Fe₃O₄@APTES-Genipin particles, a behavior similar to that of AP is observed, with a thermal event associated with APTES degradation. However, this degradation range, which extends up to 530 °C, is also related to the degradation of the olefinic ring of the genipin structure, a phenomenon similarly reported in a study evaluating the immobilization of xylanase and cellulase on MNPs using genipin [53]. Beyond 530 °C, the mass loss is attributed to the oxidation of carbonized residues or reaction by-products [48].

Thermogravimetric analysis showed that mass loss increased consistently at each step, due to the accumulation of organic compounds on the surface. This behavior confirms the efficiency of functionalizing the magnetic nanoparticles with APTES and Genipin.

Regarding the DTA, all three samples exhibit an endothermic peak at 100 °C, corresponding to the evaporation of water. However, for the APTES-functionalized MNPs (AP), the peak is less pronounced. According to Souza et al. [41], this behavior is attributed to the increased hydrophobicity of the sample, resulting from the incorporation of the APTES group, which reduces the amount of water on the material's surface. The control particles (CT) do not exhibit any further endo- or exothermic events, which corroborates the inorganic purity of the material [54].

Subsequent exothermic peaks are observed at 290 °C and 378 °C for the AP sample. According to Ulu et al. [55], this energy release is associated with the organic decomposition of APTES. For the Genipin-containing samples, similar peaks are observed at 303 °C and 375 °C, but with higher intensity. Danait-Nabar & Singhal. [56] attribute this increased energy to the degradation of the imine bond (Schiff base) formed between APTES and Genipin. Finally, the last exothermic peaks of the samples are observed at 785 °C for AP and 740 °C for GP, resulting from the formation of oxygen-rich intermediates in the early stages, particularly unstable species such as peroxides and hydroperoxides, which tend to decompose completely at higher temperatures [48].

3.2.4 Surface morphology (SEM) and elemental composition (EDS)

Figure 6 presents the SEM images and EDS spectra of the synthesized MNPs.

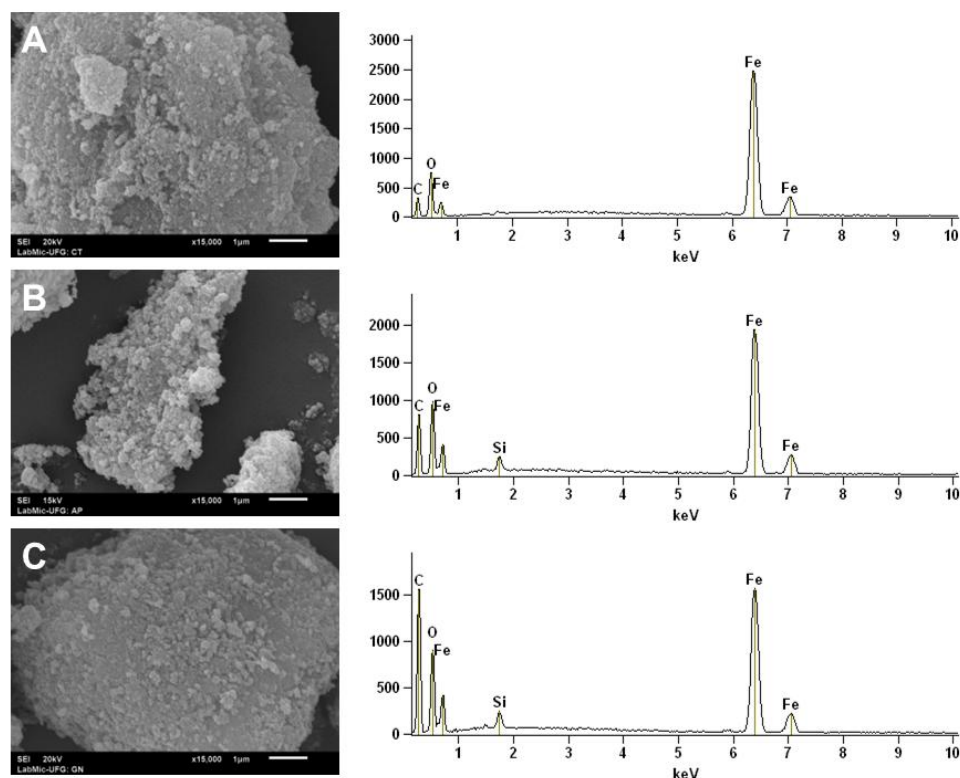


Figure 6. Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS) of the MNPs: (A) Fe_3O_4 control (CT); (B) Fe_3O_4 @APTES (AP); and (C) Fe_3O_4 @APTES-Genipin (GP).

The SEM images (Figure 6) reveal that the control MNPs (CT) exhibit a predominantly spherical morphology, with a relatively rough surface and a tendency to aggregate, a common feature in magnetic nanoparticles due to dipolar interactions between the magnetite cores. After the functionalization process, a slight surface smoothing is observed, attributed to the formation of a thin siloxane layer resulting from APTES immobilization, which promotes greater surface uniformity without altering the particles' spherical morphology.

The elemental composition of the materials was determined by EDS, which showed characteristic Fe and O peaks in all samples, consistent with the magnetite structure. In the control material, a C signal was also detected, mainly attributed to the adsorption of atmospheric contaminants on the surface of the APTES-functionalized MNPs (AP) and those subsequently treated with Genipin (GP). New Si signals were observed, along with a relative increase in C and O content. These results confirm the presence of silane and the incorporation of organic groups associated with Genipin, indicating the effectiveness of the functionalization process. Furthermore, the relative decrease in the Fe signal in the modified samples suggests that the magnetic core surface

has been coated, as the formed organic layer attenuates the signal intensity from the magnetite substrate.

3.2.5 Particle Size and Zeta Potential of MNPs

The values obtained for particle size and zeta potential are presented in Table 2.

Table 2. Effect of functionalization on the particle size and zeta potential of MNPs

	Particle Size (nm)	ζ -Zeta Potential (mV)
CT	430.6	- 15.8
AP	458.0	- 22.9
GP	466.7	- 20.4

Legend: Fe₃O₄ control (CT), Fe₃O₄@APTES (AP), and Fe₃O₄@APTES-Genipin (GP)

According to the data presented in Table 2, a gradual increase in the average particle size was observed throughout the functionalization steps, ranging from 430.6 to 466.7 nm. This growth is directly associated with the adsorption and formation of successive functional layers on the surface of the MNPs, such as APTES, which is responsible for the formation of a condensed siloxane network, and genipin, which acts as a natural crosslinking agent [57]. A similar behavior was reported by Natesan et al. [58] who observed an average size of approximately 445.9 nm for MNPs coated with chitosan, indicating that surface functionalization generally results in an apparent increase in particle diameter due to the formation of additional organic layers and the enhancement of surface thickness.

Significant variations in zeta potential were also observed throughout the functionalization process of the MNPs. The control material exhibited a value of –15.8 mV, characteristic of a moderately negative surface, which is associated with the presence of exposed hydroxyl (–OH) groups on the iron oxide surface [59]. Following the introduction of APTES, the zeta potential increased to –22.9 mV, indicating improved colloidal stabilization and reflecting the incorporation of amine and siloxane groups on the surface. These modifications alter the charge distribution and enhance electrostatic interactions within the dispersion medium [49,60].

With genipin functionalization, a slight reduction in zeta potential to –20.4 mV was observed, which may be due to crosslinking between the amine groups of APTES and the reactive groups of genipin, resulting in a less exposed surface. A similar behavior was reported by Arteche Pujana et al. [61], who incorporated genipin into a chitosan

polymer and observed a decrease in zeta potential due to material crosslinking, indicating a reduction in the fraction of free amine groups. According to Favela-Camacho et al. [62], materials with absolute zeta potential values greater than ± 20 mV are classified as moderately colloidally stable systems, owing to sufficient electrostatic repulsion to prevent particle aggregation.

3.3 Immobilization and catalytic activity of free enzymes and enzyme-loaded bioreactors

The results for the immobilization process and enzyme activity are summarized in Table 3.

Table 3. Immobilization parameters and catalytic performance of the bioreactors

Sample	Enzyme Immobilization Capacity (mg.g ⁻¹)	Enzyme Immobilization Efficiency (%)	Enzyme Specific Activity (U.g ⁻¹)
Bromelain			
Enzyme-Loaded Bioreactor	28.31 $\pm$ 0.54	67.68 $\pm$ 1.00	8.14 $\pm$ 0.68 ^a
Non-Immobilized Enzyme	-	-	4.75 $\pm$ 0.02 ^b
Trypsin			
Enzyme-Loaded Bioreactor	16.41 $\pm$ 0.62	65.19 $\pm$ 2.46	60.85 $\pm$ 2.50 ^a
Non-Immobilized Enzyme	-	-	28.41 $\pm$ 0.24 ^b

Means sharing the same letters within a column for a given enzyme are not significantly different according to the t-test ($p < 0.05$).

The protease immobilization assays indicated a higher retention efficiency for Bromelain than for Trypsin. In this regard, the pH of the reaction medium during enzyme immobilization may also influence the greater immobilization capacity of Bromelain. The Schiff base formation reaction, responsible for covalent binding with the aldehyde groups of Genipin, is favored when the free amines of the proteins are deprotonated, which occurs under slightly basic conditions [63]. Furthermore, considering that immobilization was carried out at pH 7, the surface of bromelain (pI $\sim$ 9) contains a large number of reactive amines [64], which are essential for this interaction. Trypsin (pI $\sim$ 10.5) Zamani et al. [65] although also positively charged at pH 7, it has a lower distribution of exposed amino groups near the surface, resulting in reduced immobilization efficiency.

Other factors may explain the differences in immobilization efficiency. Structural features specific to Bromelain, such as greater conformational flexibility and better exposure of reactive functional groups, even if in lower absolute amounts [66], as well as a more hydrophilic, less compact surface, may facilitate access to interactive regions of the functionalized nanoparticles. This occurs even in the presence of a lower lysine density, which is essential for support–enzyme interactions due to the requirement of free lateral amines [67].

The likely mechanism of enzyme immobilization involves the formation of Schiff bases between the reactive carbonyl (C=O) groups of Genipin on the support and the amino (NH₂) groups, particularly on the side chains of basic amino acids in the enzymes [68,69]. As reported in the literature, the pH used during the immobilization processes was within the range that favors Schiff base formation (pH 6.0–7.5), since the amine must not be protonated to act as a nucleophilic agent attacking the carbonyl group on the support [70–72].

The observed immobilization efficiencies in this study were 67.68% for Bromelain and 65.19% for Trypsin, higher than those reported by Costa et al. [73] with 55.30% for Bromelain, and Sahin & Ozmen [74] with 52% for Trypsin, using the same magnetic material. These results may be related to the different crosslinking agents employed in the immobilization processes. Genipin facilitated Schiff base formation to create the bioreactor, whereas the cited studies used glutaraldehyde. According to Pinheiro et al. [68] the use of Genipin for immobilization reacts specifically with amino (NH₂) groups, forming stable bonds through a condensation and intramolecular rearrangement mechanism, while better preserving the native structure of the enzyme, which promotes a more flexible binding network. Overall, using Genipin for enzyme retention may constitute a promising, environmentally friendly alternative to glutaraldehyde.

Immobilization of bromelain and trypsin on APTES/genipin-functionalized magnetic nanoparticles resulted in a significant increase in specific activity compared to the free enzymes, from 4.75 U·g⁻¹ to 8.14 U·g⁻¹ for bromelain and from 28.41 U·g⁻¹ to 60.85 U·g⁻¹ for trypsin. This approximately twofold increase in specific activity is consistent with the hyperactivation phenomenon widely documented in the literature for enzymes immobilized on magnetic nanoparticles[75–77]. Recent studies with trypsin immobilized on Fe₃O₄ specifically report increases in activity under similar conditions [78], while other immobilized enzymes demonstrate even more pronounced increases,

such as the 156% increase reported by Jeon et al. [79] for lipases and the 326-fold hyperactivation in thermal stability reported by Xing et al. [80] for β -xylosidase. This increase can be attributed to a synergistic combination of well-established molecular mechanisms.

Covalent anchoring reduces enzyme autolysis, a critical phenomenon for proteases, preserving a larger fraction of catalytically active molecules throughout the assay [79,80]. Furthermore, immobilization on non-porous nanomaterials minimizes steric hindrance and diffusional limitations, ensuring broad substrate access to the enzyme's active sites [81]. This type of immobilization can also stabilize structural conformations more favorable to catalysis, increasing the efficiency of the active site, as well as favoring a molecular orientation that expands substrate accessibility to the catalytic site [75]. Additionally, physical separation between enzyme molecules reduces intermolecular aggregation, which often compromises catalytic performance in solution [75,81].

It is also worth noting that the literature demonstrates that covalent immobilization on magnetic nanoparticles not only preserves enzymatic functionality but can also enhance catalytic performance, especially under specific operating conditions of temperature and pH [3,78,81]. Thus, the higher activity values observed in this study are in agreement with the state of the art in the field of enzymatic immobilization.

3.4 Effect of pH and temperature on the activity of free and immobilized enzymes

Figure 7 illustrates the effect of pH on protease enzymatic activity.

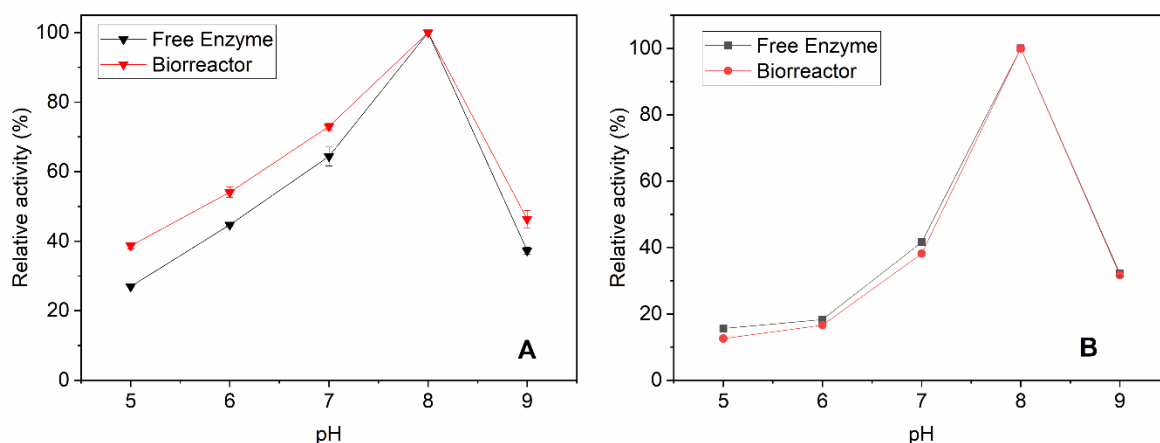


Figure 7. Effect of pH on the relative enzymatic activity of Bromelain (A) and Trypsin (B).

Bromelain exhibited higher activity at pH 8, which is consistent with data reported in the literature [7,64]. At pH values outside the optimum, activity decreased for both the free and immobilized enzyme, with the lowest values observed at pH 5. The reduction in enzymatic activity outside the optimal pH is likely related to the microenvironment created by the interaction between the enzyme and the support, which may alter the protonation states of amino acid residues involved in catalysis, thereby affecting the efficiency of the active site. Javaid et al. [82] additionally observed activity reduction outside the optimal pH, attributed to excess charges in the medium that caused conformational changes in the enzyme's active site, as well as nonspecific interactions in the bioreactor that reduced enzymatic efficiency.

In contrast to bromelain, which exhibited good stability over a wide pH range, Trypsin is characterized as a strictly basic enzyme [83,84], which is consistent with the results obtained. Similar findings were reported by Behshad et al. and Milek & Tatarchuk [85,86] when evaluating the immobilization of Trypsin on magnetic supports. It was observed that both free and immobilized Trypsin exhibited optimal activity at pH 8, suggesting that its catalytic triad (serine, histidine, and aspartate) likely remained unaltered during the immobilization process [65,87]. As observed for Bromelain, immobilization of Trypsin did not alter its optimal pH for activity, which remained at 8.

Figure 8 illustrates the effect of temperature on the relative enzymatic activity of the proteases.

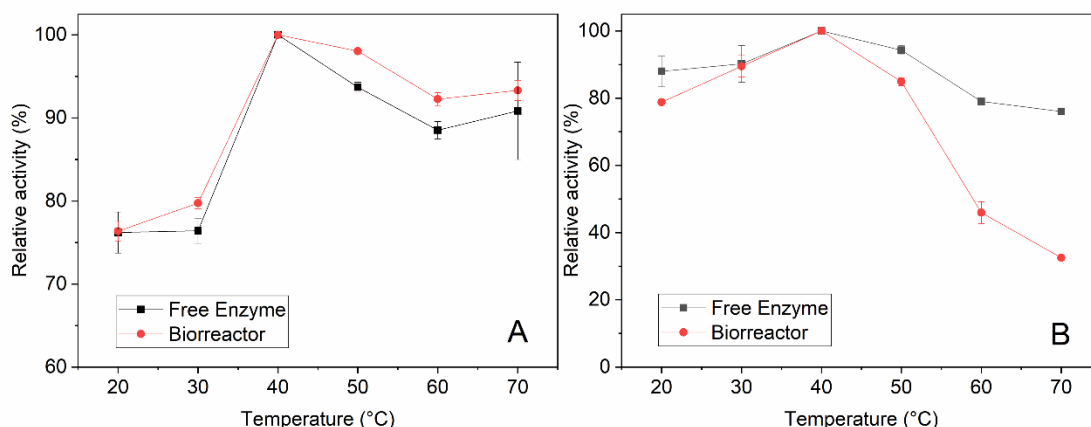


Figure 8. Effect of temperature on the relative enzymatic activity of Bromelain (A) and Trypsin (B).

It was observed that both proteases exhibited the highest activity at 40 °C, a behavior similar to that reported by Al-Sa'ady et al. and Piman et al. [83,88]. Bromelain showed high thermal stability over the studied range, maintaining an average relative

activity of ~90% for the free enzyme and ~94% for the immobilized enzyme at 70 °C, which is satisfactory for technological applications. These results are higher than those reported by Javaid et al. [82] who evaluated the immobilization of Bromelain on magnetic nanoparticles using carbodiimide and obtained a relative activity of ~80% at this temperature. The formation of the Schiff base using Genipin likely helped maintain the bioreactor's efficiency. Some studies attribute this improvement in activity to the increased rigidity of the secondary and tertiary structures, which in turn may preserve the relative positions of each amino acid residue around the support, leading to better substrate interaction [89].

Regarding immobilized Trypsin, relative activity values of 78% and 89% were observed at 20 and 30 °C, respectively, which are higher than those reported by Siddiqui et al. [90] in the same temperature range. These activity values indicate that no conformational changes occurred in the enzyme within this range that could lead to a loss of activity. However, a decrease in activity was observed at 50–70 °C for the immobilized form compared to the free enzyme. This may be related to the strengthening of undesired interactions (hydrogen bonding, hydrophobic interactions, and van der Waals forces) with the support, which could have reduced its activity [67].

3.5 Operational stability and reuse of the bioreactors

Figure 9 shows the results of bioreactor reuse, expressed as relative enzymatic activity.

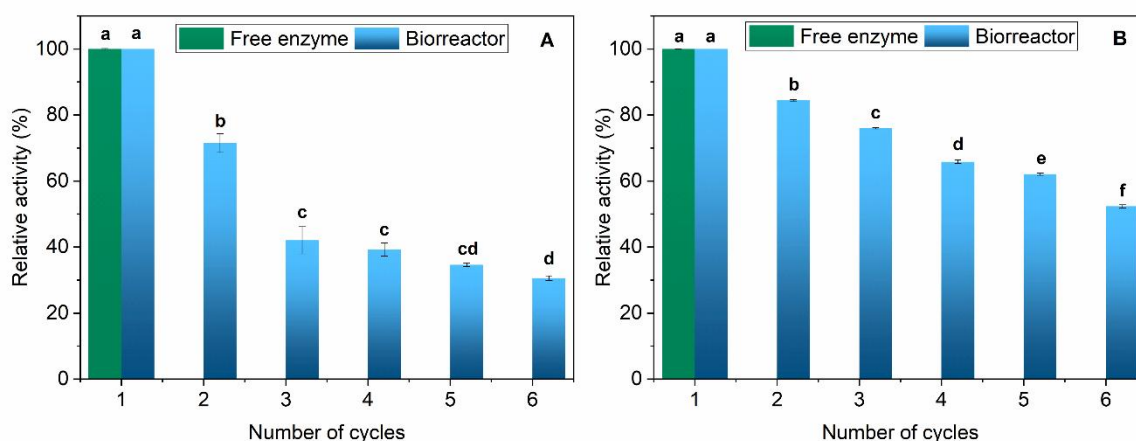


Figure 9. Relative activity of free and immobilized enzymes over multiple reuse cycles of the bioreactors: Bromelain (A) and Trypsin (B).

In each graph, means sharing the same letters are not significantly different according to Tukey's test ($p > 0.05$). Error bars represent the standard deviation for each sample

Both immobilized enzymes retained activity for up to six reuse cycles. However, the immobilized bromelain maintained only about 30% of its activity after the sixth cycle,

likely due to partial desorption from the support, which compromised its catalytic stability. In addition, the progressive decrease in activity may be associated with other factors commonly observed in reusable systems, such as possible obstruction of active sites by substrate or reaction products, conformational changes induced by repeated washing and reaction steps, and structural damage to the magnetic support over time. These phenomena indicate that, although the support exhibits promising performance, it is still necessary to optimize immobilization parameters, including pH, temperature, ionic strength, and initial enzyme concentration, to improve the operational stability of immobilized bromelain [75,91].

For bioreactors with immobilized Trypsin, greater retention of enzymatic activity was observed over six reuse cycles, with slightly more than 50% activity retained after the sixth cycle. Similar results were reported by Sahin & Ozmen [74] with a magnetite–Trypsin bioreactor, which retained 56% activity after the sixth cycle. Aggarwal & Ikram [84] also observed this behavior, reporting 60% activity after the same number of reuse cycles during magnetic support evaluations.

3.6 Chromatographic profiles of casein and the peptides obtained from its hydrolysis

Figure 10 shows the HPLC chromatographic profiles of the casein solution and the hydrolysates obtained with the different proteases.

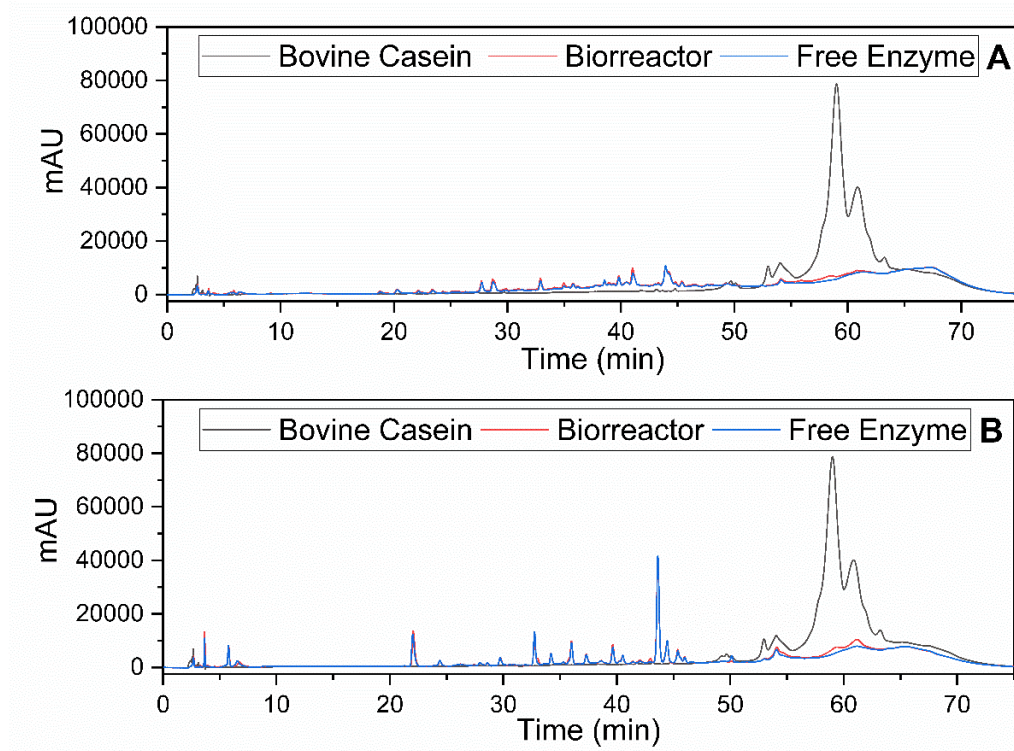


Figure 10. Chromatograms of the casein solution and peptides produced by the hydrolysis of Bromelain (A) and Trypsin (B).

The chromatographic profile of the bovine milk casein solution shows peaks with absorbance between 50 and 70 minutes, indicating the presence of more hydrophobic molecules.

After hydrolysis, the chromatographic profile at 280 nm revealed differences between the hydrolysates obtained with Trypsin and Bromelain, reflecting the cleavage specificity of each enzyme. Trypsin generated more peaks, indicating a more diverse peptide profile. This can be attributed to its specificity for basic residues, which are relatively abundant in the casein structure, leading to greater protein fragmentation. In contrast, Bromelain, which prefers hydrophobic and aromatic residues, promotes more limited and less frequent cleavages, reflected in fewer peaks. Additionally, absorbance at 280 nm is associated with aromatic residues, such as tyrosine, tryptophan, and phenylalanine. The peptides produced by Trypsin may contain these sequences more frequently or in greater abundance, explaining the more intense peaks.

It is worth noting that immobilizing both enzymes under optimal conditions resulted in minimal conformational changes, preserving their activity. This is evident in the chromatograms, where the profiles of free and immobilized enzymes are very similar, corroborating previous results.

3.7 Assessment of the antioxidant activity of peptides obtained from enzymatic hydrolysis

Table 4 presents the antioxidant activity, as determined by DPPH and ABTS radical scavenging assays, along with the percentage of protection in the BCAL system.

Table 4. Antioxidant activity measured by DPPH, ABTS, and BCAL assays for hydrolysates produced from casein using free Trypsin (FT), immobilized Trypsin (IT), free Bromelain (FB), and immobilized Bromelain (IB).

	FT	IT	FB	IB
DPPH				
(IC ₅₀ mg.mL ⁻¹)	0.020±0.01 ^a	0.012±0.01 ^b	0.014±0.01 ^b	0.016±0.01 ^{ab}
ABTS				
(% Radical Scavenging Inhibition)	45.32±0.62 ^b	55.60±0.33 ^a	35.08±1.20 ^c	31.58±0.28 ^d
BCAL (% Protective Effect)				
	71.77±0.79 ^a	71.59±0.97 ^a	73.80±1.06 ^a	72.38±0.53 ^a

In each row, means sharing the same letters are not significantly different according to Tukey's test ($p > 0.05$).

The generated peptides exhibited significant antioxidant activity against the DPPH radical, with IC₅₀ values ranging from 0.012 to 0.020 mg.mL⁻¹. These results indicate that the obtained fragments possess a high radical-scavenging capacity, surpassing the values reported for casein hydrolysates by Iwaniak et al. [92] which showed an IC₅₀ of approximately 1.246 mg.mL⁻¹. The superior performance may be associated with the presence of aromatic amino acids, such as tyrosine and tryptophan, which act as hydrogen donors, as well as hydrophobic residues that facilitate electron and proton transfer. This mechanism, known as Hydrogen Atom Transfer (HAT), plays a decisive role in radical stabilization and inhibition of oxidative reactions [93].

The antioxidant activity of the obtained peptides varied according to the enzyme used and its form (free or immobilized), directly reflecting the cleavage pattern on the casein structure. Trypsin, which showed the highest results, preferentially acts on lysine and arginine residues, generating shorter peptides rich in basic residue groups associated with hydrogen-donating capacity and redox activity [94,95]. Additionally, immobilization may protect the enzyme's structure and promote more controlled, selective cleavage, which explains the superior performance of immobilized Trypsin. Thus, the cleavage site directly influences the composition and antioxidant potential of the resulting peptides, with the target residues of the proteases being decisive for the final bioactivity.

In evaluating the antioxidant potential of the hydrolysates via ABTS radical scavenging, a profile similar to that observed for DPPH scavenging was observed, with the highest antioxidant potential in the Trypsin hydrolysates. The ABTS radical exhibits lower selectivity compared to DPPH, responding better to polar compounds [96]. In other words, the results for ABTS can be attributed to the lower amount of polar peptides produced during enzymatic hydrolysis, as evidenced by the higher polarity observed at the beginning of the chromatographic profile.

The results obtained for the two radicals are related to their distinct mechanisms of action. DPPH and ABTS operate via different pathways, which influence how peptides interact with each radical [93,96]. DPPH reacts predominantly via Hydrogen Atom Transfer (HAT), a mechanism favored by peptides containing hydrogen-donating amino acids, especially those with more nonpolar side chains. In contrast, ABTS can also react through Single Electron Transfer (SET), but it requires a higher overall redox capacity of the molecule, not just the presence of hydrogen-donating residues. This may explain the lower values observed for Bromelain, suggesting a lower redox efficiency of its hydrolyzed peptides in this context [93,96,97].

In the β -carotene/linoleic acid assay, the obtained samples, both in free and immobilized forms, showed values of approximately 70% protection against oxidation, with no statistically significant differences between the samples. These results indicate that immobilization did not compromise peptide bioactivity and that, regardless of enzyme form, the resulting peptides exhibited similar performance. The composition of the generated peptides can explain the high antioxidant capacity observed. In the case of Trypsin, cleavage at basic residues (lysine and arginine) favors the production of polar peptides, which interact more effectively in the aqueous phase, contributing to radical neutralization [94]. In contrast, Bromelain, due to its broader specificity, tends to release peptides containing nonpolar and aromatic residues, which insert into the lipid phase, delaying linoleic acid peroxidation [98–100]. Thus, although each hydrolysis produced distinct peptide profiles, the overall effect on antioxidant protection was equivalent, reinforcing the role of both polar and nonpolar residues in stabilizing the emulsified system.

Compounds with antioxidant activity act by inhibiting oxidative reactions, which can generate free radicals harmful to cells. Thus, the results obtained in this study reveal a promising potential and underscore the importance of further investigations focused on the use of enzymes in casein hydrolysis, aiming to develop antioxidant ingredients for application in the food industry

4. CONCLUSIONS

In this study, a new functionalized magnetic support ($\text{Fe}_3\text{O}_4@\text{APTES-Genipin}$) for the immobilization of proteases using Schiff base-type bonds was developed. The nanoparticles exhibited magnetic behavior which facilitate separation and reuse, as well as structural and thermal stability due to the organic layer formed. Enzymatic immobilization, especially of trypsin, resulted in high catalytic activity, greater stability during use, and the ability to be reused for multiple cycles, demonstrating the material's efficiency. In addition, peptides with significant antioxidant properties were produced, demonstrating the potential of the system to yield bioactive compounds of industrial and pharmaceutical interest. The use of genipin-functionalized nanoparticles represents an efficient, sustainable, and economically viable strategy for biocatalysis and the generation of higher-value-added peptides, expanding their application possibilities in the food, pharmaceutical, and biotechnology areas.

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SUPPLEMENTARY DATA FILE

to

Genipin-functionalized magnetic materials: a sustainable and innovative method for immobilizing proteases to produce antioxidant peptides

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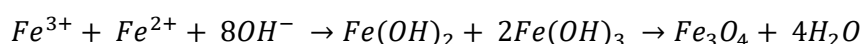
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SECTION S1 - Synthesis of Fe₃O₄ magnetic nanoparticles (MNPs)

For this purpose, 500 mL of 0.5 M NaOH solution was prepared and heated to 90 °C under nitrogen bubbling with continuous stirring. Subsequently, a 50 mL solution was prepared containing 1.0 M ferric chloride and 0.5 M ferrous sulfate. The iron ion solution was added dropwise to the NaOH solution, yielding a dark suspension, which was heated to 90 °C for 1 h. The MNPs were separated using an external magnetic field generated by a neodymium magnet (1.5 cm high × 3 cm diameter) and washed with deionized water until the pH was neutral. The resulting material was then lyophilized and stored in a sealed container at room temperature for further use. The equation below illustrates the co-precipitation reaction of the iron ions:



SECTION S2 - Immobilization of trypsin and bromelain

For trypsin immobilization, 30 mg of carbonyl-activated MNPs were mixed with 3 mL of a solution containing trypsin at 1 mg·mL⁻¹ and benzamidine hydrochloride at 0.1 mg·mL⁻¹ in 0.1 M sodium phosphate buffer (pH 7). For bromelain immobilization, a solution of the same concentration (1 mg·mL⁻¹) was prepared under the same conditions as described above. The 5 mL Eppendorf tubes were maintained under orbital stirring (20 rpm) at room temperature (25 ± 1 °C) overnight (14 h). After this period, the material was separated using an external magnetic field, and the amount of non-adsorbed protein was quantified using the Bradford method. Successive washes were performed with 3 mL of 0.1 M sodium phosphate buffer (pH 7) under orbital stirring (20 rpm) for 10 min, and

protein content was quantified until no protein was detected in the supernatant. The final protein concentration was defined as the sum of the supernatant concentration and the desorbed protein from the washing solutions. Experiments were conducted in triplicate, and each tube was subsequently used to determine enzymatic activity. The immobilization capacity of the materials was calculated according to the Equation below:

$$C_{im} = \frac{(C_o - C) \times V}{M} \quad (1)$$

Where C_{im} is the immobilization capacity ($\text{mg}\cdot\text{g}^{-1}$); C_o is the initial protein concentration in the solution ($\text{mg}\cdot\text{mL}^{-1}$); C is the final protein concentration ($\text{mg}\cdot\text{mL}^{-1}$); V is the solution volume (mL); and M is the mass of the support material (g).

SECTION S3 - Enzyme activity determination

For the determination of enzymatic activity, a 2% casein solution was prepared in 0.1 M sodium phosphate buffer (pH 8). For the bioreactor analysis (30 mg of support + immobilized enzyme), 3 mL of the casein solution was added, and the mixture was incubated in a water bath at 40 °C for 1 h. The activity of the free enzyme was assessed by adding 0.65 mL of enzyme solution ($1 \text{ mg}\cdot\text{mL}^{-1}$ for trypsin and $1 \text{ mg}\cdot\text{mL}^{-1}$ for bromelain) to 3 mL of casein solution and incubating the mixture at 40 °C for 1 hour. After incubation, the bioreactors were separated from the hydrolysate using an external magnetic field, and 1.2 mL of 6.5% trichloroacetic acid (TCA) was added to the hydrolyzed casein solution. The tubes were homogenized and then centrifuged at $3622 \times g$ for 10 min at 4 °C. Subsequently, 0.45 mL of the supernatant was transferred to amber test tubes and mixed with 3 mL of 4% sodium carbonate solution and 0.45 mL of 20% Folin–Ciocalteu reagent. The mixture was stirred and incubated in a water bath at 40°C for 20 minutes. Absorbance readings were then obtained using a UV-Vis spectrophotometer (UV-Vis Quimis, São Paulo, Brazil) at 660 nm. One unit (U) of protease activity was defined as the amount of enzyme required to release 100 μg of tyrosine equivalent under the assay conditions, as determined by the Equation below. The enzymatic activity assays for the free enzyme followed the same steps described for the bioreactors.

$$\text{Specific enzyme activity}(U/g) = \left(\frac{\frac{C_{Tyrosine}(\frac{\mu\text{mol}}{\text{mL}})}{\text{Time}(\text{min})}}{\text{Enzyme mass}(g)} \right)$$

SECTION S4 - Antioxidant Capacity of the Obtained Peptides

DPPH

First, a 70% ethanolic solution of DPPH (0.06 mM) was prepared and adjusted to an initial absorbance of 0.6–0.7 at 515 nm using a spectrophotometer. Then, 500 µL of the sample was placed in amber test tubes with 500 µL of the DPPH solution. The tubes were mixed and incubated at room temperature for 30 min, protected from light.

To determine the EC₅₀ value (the concentration required to scavenge 50% of the DPPH radical), a linear curve was constructed using five concentrations of the protein hydrolysates and their corresponding radical scavenging percentages.

ABTS

First, a 7 mM ABTS solution was prepared, followed by a 140 mM potassium persulfate solution. The ABTS radical was generated by mixing 5 mL of the ABTS solution with 88 µL of the potassium persulfate solution. The mixture was protected from light and kept at room temperature for 16 hours before use.

For the assay, the ABTS solution was diluted in 70% ethanol to achieve an absorbance of 0.7 at 740 nm. Then, 100 µL of the sample was added to an amber test tube containing 2 mL of the ABTS radical solution. The mixture was kept protected from light at room temperature for 6 min. Subsequently, absorbance readings were taken at 740 nm, using ethanol as the blank. Antioxidant activity was determined based on ABTS radical inhibition according to the Equation below:

$$\% \text{ ABTS radical inhibition} = \left(\frac{A_c - A_a}{A_c} \right) \times 100$$

Where A_c and A_a correspond to the absorbance readings of the ABTS radical and the sample, respectively

β-carotene–linoleic acid system (BCAL)

First, ultrapure water was saturated with oxygen (O₂) for 30 min. During the oxygenation, the reactive mixture was prepared by combining 50 µg of linoleic acid, 200 mg of Tween 40, and 1 mL of β-carotene solution (1 mg·mL⁻¹ in chloroform). The mixture was then placed in a drying oven (TECNAL, Model TE-393/1, Brazil) at 50 °C for 40 min to evaporate the chloroform.

After solvent evaporation, approximately 100 mL of saturated water was added to the mixture, which was then homogenized using magnetic stirring. The emulsion absorbance

was adjusted to 0.6–0.7 at 470 nm, using saturated water as the blank. For the analysis, 2.5 mL of the emulsion was transferred to tubes containing 250 µL of sample, then mixed immediately, and the absorbance was measured at 470 nm. After 2 h incubation at 50 °C in a thermostatic water bath (TECNAL, Model TE-2005, Brazil), absorbance was measured again to determine the percentage of protection. The control consisted of the emulsion and chloroform, and the results were expressed as the percentage of protection against oxidation, as shown in the Equation below.

$$\% \textit{Protection} = \left(1 - \left(\frac{(Abs_{sample \textit{ initial}} - Abs_{sample \textit{ final}})}{(Abs_{control \textit{ initial}} - Abs_{control \textit{ final}})} \right) \right) \times 100$$

CAPÍTULO 4 – ARTIGO 3

***AGREGAÇÃO DE VALOR AO SORO DE LEITE POR HIDRÓLISE ENZIMÁTICA
COM TRIPSINA IMOBILIZADA EM NANOPARTÍCULAS MAGNÉTICAS:
DESEMPENHO EM UMA MATRIZ COMPLEXA***

Agregação de valor ao soro de leite por hidrólise enzimática com tripsina imobilizada em nanopartículas magnéticas: desempenho em uma matriz complexa

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Resumo: Políticas sustentáveis, princípios da economia circular e os Objetivos de Desenvolvimento Sustentável (ODS) têm impulsionado em escala global estudos voltados ao reaproveitamento e valorização de resíduos e subprodutos agroindustriais. Nesse contexto, o soro de leite destaca-se como um dos subprodutos mais abundantes da indústria láctea e quando descartado inadequadamente apresenta elevado potencial poluidor. O seu reaproveitamento na geração de produtos de valor agregado, como peptídeos antioxidantes, oferece uma alternativa da sua utilização, indo de encontro a modernas iniciativas. Assim, este estudo teve como objetivo investigar o potencial de produção de peptídeos antioxidantes obtidos do soro de leite, a partir da hidrólise enzimática utilizando tripsina imobilizada em nanopartículas magnéticas, como estratégia para sua valorização. As nanopartículas foram sintetizadas, funcionalizadas com genipina, um agente de ligação renovável, e utilizadas no processo de imobilização enzimática, o qual se mostrou eficiente, apresentando atividade catalítica superior à enzima na forma livre. Os peptídeos gerados foram avaliados por Cromatografia Líquida de Alta Eficiência (HPLC), sendo observado um perfil peptídico diversificado, com grau de hidrólise máximo de 42,55 % após 4 h de reação para a tripsina imobilizada, de forma superior a enzima livre. A atividade antioxidante foi avaliada por diferentes ensaios in vitro, em que verificou-se 91,46 % de inibição do radical DPPH, 90,26 % para ABTS, 265 µM Fe(II) no ensaio FRAP e 60,79 % de proteção no sistema β-caroteno-ácido linoleico. De modo geral, os resultados demonstram que a estratégia proposta representa uma alternativa eficiente para a valorização do soro de leite, contribuindo para sua realocação como fonte de peptídeos bioativos e reforçando abordagens alinhadas à sustentabilidade e à economia circular.

Palavras-chave: Nanopartículas Magnéticas; Sustentabilidade; Reutilização; Saúde; APTES; Genipina.

1. INTRODUÇÃO

Nas últimas décadas, o avanço industrial impulsionou a economia global, mas também intensificou a geração de resíduos capazes de impactar diretamente solo, água e ar. Assim, a valorização de subprodutos surge como uma estratégia eficaz para reduzir impactos ambientais e transformar materiais antes descartados em produtos de alto valor agregado, alinhando-se aos princípios da economia circular [1–3]. Nesse cenário, a indústria de laticínios enfrenta um grande desafio: a produção de queijos gera volumes massivos de soro de leite, estimados mundialmente entre 180 e 190 milhões de toneladas por ano, dos quais cerca de 50 % ainda é descartada inadequadamente [4]. Esse rejeito possui elevada carga orgânica, com Demanda Química de Oxigênio (DQO) e Demanda Bioquímica de Oxigênio (DBO) que podem alcançar até 100 g/L, valores aproximadamente 150 vezes superiores aos encontrados em efluentes urbanos. Por isso, o reaproveitamento do soro torna-se essencial, integrando a necessidade de diminuição dos impactos ambientais com a criação de oportunidades econômicas mais sustentáveis[5].

Apesar dos impactos ambientais, o soro proveniente da produção de queijo (WP) possui uma composição rica e nutricionalmente valiosa. Ele contém água, lactose, gordura, minerais, vitaminas e diversas frações proteicas, como β -lactoglobulina (β -LG), α -lactoalbumina (α -LA), albumina sérica bovina (BSA), imunoglobulinas (IGs), Glicomacropéptideo (GMP) e lactoferrina (LF). Essas proteínas e peptídeos têm alto valor biológico e representam uma fonte rica em aminoácidos essenciais [6]. Assim, uma alternativa para aproveitar essas proteínas é a produção de peptídeos bioativos, que, segundo diversos estudos, podem apresentar atividade antioxidante, antimicrobiana, antitumoral, anti-inflamatório, imunomoduladora e anti-hipertensiva[7–10]. A eficácia desses peptídeos está diretamente relacionada à estrutura das proteínas do soro e, principalmente, às tecnologias aplicadas, que podem liberar peptídeos com estrutura e funções específicas diferentes [11,12].

Dentre as metodologias usadas para a obtenção de peptídeos, a hidrólise enzimática se destaca por oferecer condições mais controladas, permitindo a liberação de peptídeos com diferentes atividades biológicas. As enzimas mais aplicadas incluem proteases gastrointestinais (pepsina, pancreatina, tripsina, quimotripsina), enzimas de origem vegetal (papaína, bromelina, actinidina, ficina) e proteases microbianas [13]. Embora a hidrólise enzimática apresente bons resultados na produção de peptídeos

bioativos, um desafio importante é o uso de proteases na forma livre, que não permite o reaproveitamento da enzima, pode ter ação residual e resultar na formação de dipeptídeos e aminoácidos livres sem atividade para sequestrar radicais livres, além de aumentar os custos do processo. Assim, a imobilização enzimática se torna uma alternativa viável, possibilitando reutilização desses biocatalisadores [14,15].

A tecnologia de imobilização enzimática consiste em reter a enzima em um suporte sólido, permitindo seu reaproveitamento e oferecendo proteção contra degradação e desnaturação, o que aumenta sua estabilidade operacional[16]. Diversos materiais podem ser usados na imobilização de enzimas como criogeis de quitosana[17], nanotubos de halosita[18], carvão ativado[19], goma trancana[20], nanoflores orgânicas-inorgânicas[21], nanopartículas de ouro[22], mas as nanopartículas magnéticas (MNPs) têm ganhado destaque por permitirem uma separação rápida e eficiente quando submetidas a um campo magnético externo [23].

Em um estudo preliminar avaliou a coagulação contínua de leite usando extrato de flor de *Cynara cardunculus* e coalho de bezerro imobilizados em partículas magnéticas[24], os autores relataram que o sistema de coagulação contínua do leite, pode ser possível revolucionar a aplicação do coalho na indústria de laticínios em termos de produtividade e redução de custos. Com isso, a tecnologia de biorreatores formados pela combinação de nanomateriais e enzimas tem sido amplamente aplicada em biotransformações, principalmente por oferecer alta área superficial e excelente transferência de massa, resultando em maior eficiência catalítica[25].

Diante desse cenário, este estudo dá continuidade ao desenvolvimento de sistemas enzimáticos imobilizados aplicados à produção de peptídeos bioativos. Em trabalho anterior [26], o sistema contendo tripsina imobilizada foi avaliado utilizando caseína como proteína modelo, demonstrando eficiência na hidrólise controlada e na obtenção de peptídeos com potencial bioativo. A utilização de uma proteína modelo permitiu compreender o comportamento catalítico do biocatalisador e otimizar as condições reacionais, estabelecendo bases para sua aplicação em substratos mais complexos.

Com base nesses resultados, o presente estudo avança ao aplicar o mesmo sistema imobilizado na hidrólise do soro de leite proveniente da produção de queijo, uma matriz proteica complexa e um dos principais coprodutos da indústria láctea. Essa abordagem amplia a aplicabilidade do biocatalisador, ao mesmo tempo em que propõe o reaproveitamento de um resíduo de alto volume para a obtenção de peptídeos bioativos por uma rota sustentável. A estratégia contribui para a redução de impactos ambientais e

para o aumento da eficiência dos processos biotecnológicos, alinhando-se aos princípios da ODS 12, ao incentivar produção responsável; da ODS 9, ao empregar tecnologias limpas e processos otimizados; e da ODS 3, ao viabilizar a geração de compostos com potencial aplicação na promoção da saúde humana.

2. MATERIAS E MÉTODOS

2.1 Reagentes

Para o desenvolvimento desse estudo foram utilizados reagentes que têm no mínimo grau analítico PA-ACS foram adquiridos da Sigma Aldrich Brasil. Sulfato de ferro heptahidratado ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; CAS: 7782-63-0), Cloreto de ferro hexahidratado ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$; CAS: 10025-77-1); Hidróxido de sódio (NaOH ; CAS: 1310-73-2), 3-aminopropiltrietoxisilano (APTES; CAS: 919-30-2). Tripsina do pâncreas bovino (≥ 10.000 BAEE units/mg protein; CAS: 9002-07-7), ácido tricloroacético (TCA; CAS: 73-03-9), Folin-Ciocalteu (2 N; Sigma-Aldrich Brasil), Carbonato de Sódio (Na_2CO_3 ; CAS: 497-19-8), 2,2-difenil-1-picril-hidrazila (DPPH; CAS: 1898-66-4); Ácido 2,2'-azino-bis(3-etilbenzotiazolina-6-sulfônico) (ABTS; CAS: 30931-67-0); 2,4,6-Tris(2-piridil)-s-triazina (TPTZ; CAS: 3682-35-7); β -caroteno (CAS: 7235-40-7); ácido linoleico (CAS: 60-33-3). Os demais reagentes utilizados estão descritos ao longo da metodologia e todas as soluções foram preparados utilizando água ultrapura.

O soro proveniente da coagulação enzimática do queijo (soro doce) WP utilizado no trabalho foi doado pelo Laticínios Pytty, localizado no município de Itapetinga, Bahia (BR), nas coordenadas 15°14'56.0"S 40°14'52.0"W. O mesmo mantido sob congelamento até sua utilização.

2.2 Síntese, Funcionalização e Imobilização de tripsina em MNPs

A síntese de nanopartículas magnéticas, a funcionalização da superfície e a subsequente imobilização da tripsina foram realizadas de acordo com a metodologia previamente descrita em nosso trabalho anterior [26]. Resumidamente, as nanopartículas magnéticas foram preparadas por coprecipitação, seguida de modificação da superfície para permitir a ligação covalente da enzima. O procedimento de imobilização foi realizado sob as condições otimizadas estabelecidas no estudo anterior, no qual o sistema imobilizado foi aplicado com sucesso à hidrólise da caseína como proteína modelo.

A capacidade de imobilização foi calculada conforme a Equação 1.

$$C_{im} = \frac{(C_o - C) \times V}{M} \quad (1)$$

Na qual C_{im} é a capacidade de imobilização (mg.g-1); C_o é a concentração inicial de proteína em solução (mg.mL-1); C é a concentração final de proteína (mg.mL-1); M é a massa de enzima imobilizada (g); e V é volume de solução.

No presente trabalho, o mesmo biocatalisador imobilizado foi utilizado sem modificação estrutural, sendo aplicado à hidrólise de proteínas do soro do leite obtidas na produção de queijo, a fim de avaliar seu desempenho em uma matriz proteica complexa.

2.3 Determinação da atividade enzimática

A atividade enzimática da enzima livre e imobilizada nos biorreatores foi determinada seguindo metodologia de Santos et al. [26], com algumas modificações, utilizando o WP como substrato. Para isso, 30 mg do biorreator (30 mg de suporte contendo tripsina imobilizada) foram adicionados a tubos eppendorf contendo 3 mL de soro de leite ajustado para pH 8, incubando-se a mistura a 40 °C por 30 min. A atividade da enzima livre foi avaliada adicionando-se 0,65 mL de solução enzimática (1 mg.mL⁻¹) em 3 mL de WP nas mesmas condições de incubação.

Após esse período, os biorreatores foram separados por imã e 1,2 mL de ácido tricloroacético (6,5%) foi adicionado ao soro de leite hidrolisado, o mesmo procedimento foi realizado para as amostras contendo enzima livre. Os tubos foram centrifugados a 3.622 × g por 10 min a 4 °C. Em seguida, 0,45 mL do sobrenadante foi transferido para tubos âmbar e misturado com 3 mL de carbonato de sódio (4%) e 0,45 mL do reagente Folin–Ciocalteu (20%). A mistura foi incubada a 40 °C por 20 min e a absorbância medida em espectrofotômetro a 660 nm.

Uma unidade de atividade enzimática foi definida como a quantidade de enzima capaz de liberar 1 μmol de tirosina por minuto, sob as condições do ensaio, conforme determinado pela Equação 2.

$$Atividade\ Enzimática\ Específica\ (U/g) = \left(\frac{\left(\frac{Conc.Tirosina\ (Umol)}{Min(tempo)} \right)}{Massa\ de\ enzima\ (g)} \right) \quad (2)$$

2.3 Produção dos peptídeos via hidrólise enzimática do soro de leite

Para a produção dos peptídeos, o soro de leite foi centrifugado a 3622 xg por 15 min a 4 °C para remover possíveis impurezas. O sobrenadante coletado teve o pH ajustado para 8 com solução de NaOH (0,1 M). Em seguida, 3 mL de soro foram adicionados a

tubos contendo 30 mg de biorreator, e a hidrólise foi conduzida em cinco tempos distintos (0,5; 1; 2; 3 e 4 h) a 40 °C em banho termostático. Para hidrolise com enzima livre, foi utilizado 0,65 mL (tripsina 1 mg.mL⁻¹ em tampão fosfato de sódio) seguindo as mesmas etapas previamente descritas. A reação foi interrompida pela retirada do biorreator com auxílio de um ímã e posterior aquecimento da solução a 85 °C por 15 min, seguido de resfriamento rápido em banho ultratermostático a -12 °C. Após isso, os tubos contendo os hidrolisados foram centrifugados novamente a 3622 xg por 15 min a 4 °C. Para a enzima livre, o interrompimento da reação se deu pelo aquecimento seguido de resfriamento e centrifugação das amostras. Os sobrenadantes obtidos foram armazenados sob congelamento até as análises posteriores.

2.4 Perfil cromatográfico dos peptídeos obtidos da hidrólise do soro de leite

O perfil cromatográfico do WP e da solução hidrolisada contendo os peptídeos foram realizadas por cromatografia líquida de alta eficiência de fase reversa (HPLC-RP). Foi utilizada uma coluna C18 (250 x 4,6 mm de diâmetro, 5 mm de tamanho de partícula; ZORBAX Eclipse Plus C18) conectada a uma pré-coluna (ZORBAX SB-C18, 4,6 mm x 12,5 mm, 5 µm), acoplado ao um sistema Thermo Scientific Vanquish Flex UHPLC.

Para verificação dos perfis cromatográficos, foram utilizadas duas fases móveis: a primeira (A) composta por acetonitrila (5 %) e ácido trifluoracético (0,1 %), e a segunda (B) composta de acetonitrila (50 %) e ácido trifluoracético (0,1 %). Foi injetada uma alíquota de 20 µL de amostra automaticamente na coluna a um fluxo 1,0 mL.min⁻¹ das fases móveis A e B por 70 min. A corrida foi iniciada com 100 % de fase A e reduziu linearmente por 60 min até que a fase fosse representada por 100 % de fase B. Posteriormente, de 60 a 65 min, um novo gradiente linear foi realizado para estabelecer a vazão de 100 % de A, mantida até o final da análise, 70 min. A análise foi realizada em temperatura de 30 °C e a detecção dos peptídeos ocorreu no comprimento de onda de 215 nm em um detector UV-Vis. Os picos observados no perfil cromatográfico foram comparados pelo seu tempo de retenção e seus espectros UV.

2.5 Determinação do Grau de Hidrólise (DH)

A extensão da hidrólise foi avaliada pelo ensaio espectrofotométrico de o-ftaldialdeído (OPA), seguindo a metodologia descrita na literatura por Mirzaei et al. [27]. A solução de OPA foi preparada no dia da análise, contendo: 25 mL de tetrahidroborato de sódio (100 mM), 2,5 mL de dodecil sulfato de sódio (SDS) 20% (p/v), 40 mg de OPA dissolvidos em 1 mL de metanol e 100 µL de β-mercaptoetanol, ajustados para um volume

final de 50 mL com água deionizada. Para o ensaio, 50 µL de amostra foram adicionados a 1 mL do reagente OPA em tubos de ensaio. A mistura foi agitada em vórtex e incubada por 2 min a 25 °C. Em seguida, as amostras foram lidas a 340 nm em espectrofotômetro. A concentração de grupos amino livres foi determinada por meio de uma curva padrão de L-alanina (0,01 a 0,4 mg.mL⁻¹). O grau de hidrólise (DH) foi calculado pela Equação 3:

$$DH = \left(\frac{D_1 - D_0}{D_{max} - D_0} \right) \times 100 \quad (3)$$

Na qual, DH é a grau de hidrólise (%), D1 é a quantidade de grupos amino livres liberados após o processo de hidrólise, D0 é a quantidade de grupos amino livres do WP e Dmax é a quantidade total de grupos amino livres no WP obtido após hidrólise ácida (6 M HCl a 120 °C por 24 h).

2.6 Capacidade antioxidante dos extratos peptídicos obtidos da hidrólise do soro de leite

As soluções soro de leite hidrolisado (LWP) foram avaliados enquanto seu potencial antioxidante por diferentes métodos, listados na sequência.

2.6.1 Atividade de sequestro de radicais livres DPPH

A avaliação do sequestro do radical DPPH (2,2-difenil-1-picril, hidrazina) foi avaliado seguindo a metodologia descrita por Souza et al. [28] com modificações. Primeiramente, o radical DPPH (0,06 mM) foi preparado em solução etanólica até uma absorbância inicial na faixa de 0,6 a 0,7 unidades de absorbância em espectrofotômetro a 515 nm. Feito isso, 500 uL de amostra foi mantido em tubos com 500 uL de solução DPPH. Os tubos foram agitados e mantidos em temperatura ambiente por 30 min, protegidos da luz. Logo após, as soluções foram lidas em espectrofotômetro usando o etanol como branco. A atividade antioxidante foi determinada por meio da inibição do radical DPPH, de acordo a Equação 4:

$$\% \text{ Inibição do radical DPPH} = \left(\frac{A_c - A_a}{A_c} \right) \times 100 \quad (4)$$

Na qual, Ac e Aa são as leituras do radical DPPH e amostra, respectivamente.

2.6.2 Atividade de sequestro de radicais livres ABTS

A determinação do sequestro do radical ABTS (ácido 2,2-azino-bis(3-etilbenzo-tiazolino-6-sulfônico) foi avaliada seguindo a metodologia descrita por Re et al. [29] com modificações. Foi preparada uma solução de ABTS a 7 mM, seguida de uma solução de persulfato de potássio a 140 mM. O radical ABTS foi obtido pela mistura de 5 mL de

ABTS (7 mM) e 88 µL de persulfato de potássio (140 mM). A mistura foi incubada em frasco âmbar a temperatura ambiente por 16 h antes da análise.

Para o ensaio, a solução de ABTS previamente preparada foi diluída em etanol até que fosse obtida absorvância de 0,700 a 740 nm. Após isso, 100 µL de amostra foi adicionado em tubos âmbar contendo 2 mL de radical ABTS. Sequentemente, a mistura foi agitada em vórtex e armazenada ao abrigo da luz por 6 min. As leituras foram realizadas a 740 nm, usando etanol como branco. A atividade antioxidante foi determinada pela inibição do radical ABTS de acordo a Equação 5:

$$\% \text{ ABTS radical inhibition} = \left(\frac{A_C - A_A}{A_C} \right) \times 100 \quad (5)$$

Na qual, A_C e A_A correspondem as leituras de absorvância do radical ABTS e das amostras, respectivamente.

2.6.3 Ensaio do Poder antioxidante redutor férrico (FRAP)

O ensaio de FRAP foi realizado seguindo a metodologia descrita por Liu et al [30], com modificações. Foi preparado uma solução de FRAP utilizando tampão acetato (0,3 M pH 3,6), cloreto férrico (20 mM) e TPTZ (10 mM) em uma razão de 10:1:1 (v/v/v) respectivamente. Posteriormente, 90 µL de amostra foi misturado com 270 µL de água deionizada e 2,7 mL de solução FRAP, a solução foi misturada e mantida em banho termostático por 37 °C por 30 min. As leituras foram realizadas em espectrofotômetro a 595 nm, utilizando água como branco. Foi construída uma curva de calibração utilizando sulfato de ferro heptahidratado, com concentrações variando de 50 a 2000 µM. Os valores de FRAP foram determinados usando a curva padrão e os resultados foram expressos como µM de Fe (II).

2.6.4 Inibição da oxidação do sistema β-caroteno-ácido linoléico

Para determinação da atividade antioxidante usando o sistema β-caroteno-ácido linoleico foi utilizada a metodologia descrita por Aminjafari et al. [31] com modificações. Primeiramente, água deionizada foi saturada com oxigênio por 30 min. Foi preparado a mistura reativa, contendo 50 µg de ácido linoléico, 200 mg de Tween 40 e 1 mL da solução de β-caroteno (1 mg.mL⁻¹ em clorofórmio). A mistura foi acondicionada em estufa de secagem a 50 °C por 40 min para evaporação do clorofórmio.

Após a evaporação do solvente, 100 mL de água saturada foi adicionada a mistura e agitado. A emulsão foi ajustada para absorvância entre 0,6 a 0,7 a 470 nm usando água

como branco. Para a análise 2,5 mL de emulsão foi transferida para tubos âmbar contendo 250 uL de LWP, que foi misturado imediatamente e lido a 470 nm. Após, 2h de incubação a 50 °C em banho termostático, a absorbância foi medida novamente. O controle consistiu na emulsão com clorofórmio e os resultados foram expressos em porcentagem de proteção contra oxidação, de acordo a Equação 6:

$$\% \text{ Proteção} = \left(1 - \left(\frac{(Abs_{amostra\ inicial} - Abs_{amostra\ final})}{(Abs_{controle\ inicial} - Abs_{controle\ final})} \right) \right) \times 100 \quad (6)$$

3. RESULTADOS E DISCUSSÕES

3.1 Imobilização e Atividade enzimática da tripsina livre e imobilizada

A Tabela 1, apresenta os dados relativos ao processo de imobilização da tripsina e o desempenho catalítico da enzima na forma livre e imobilizada.

Tabela 1. Eficiência de imobilização e atividade enzimática

Amostra	Capacidade de imobilização enzimática (mg.g ⁻¹)	Eficiência imobilização enzimática (%)	Atividade enzimática específica (U.g ⁻¹)	Atividade Recuperada (%)
Enzima imobilizada	16,17±0,46	64,01±1,62	126,22±3,68 ^a	146,62
Enzima Livre	-	-	90,83±0,83 ^b	

Médias seguidas da mesma letra não diferem significativamente pelo teste-t (p>0,05)

A imobilização da tripsina em MNPs funcionalizadas com APTES/Genipina apresentou uma capacidade de imobilização de 16,17 mg.g⁻¹ e uma eficiência de 64,01%, indicando boa disponibilidade de grupos funcionais na superfície do material, já que aproximadamente dois terços da enzima foram retidos. Alguns estudos descrevem mecanismos que ajudam a explicar esse comportamento. De acordo com autores como Sahin et al. [32] e Prabhakar et al. [33], suportes ativados por agentes formadores de base de Schiff (como glutaraldeído e genipina) favorecem a imobilização via grupos amino terminais da enzima, que permanecem reativos na faixa de pH utilizada neste trabalho (pH 8). Nessa condição, resíduos de lisina apresentam maior nucleofilicidade e contribuem para o acoplamento covalente entre a tripsina e o suporte[33].

Outro fator relevante é a concentração de enzima utilizada no processo de imobilização. Neste estudo, empregou-se 1 mg·mL⁻¹, valor que, segundo os mesmos autores, está dentro da faixa ideal (0,75–1,5 mg·mL⁻¹) para alcançar boa eficiência de imobilização sem comprometer o desempenho catalítico. Concentrações inferiores

tendem a resultar em baixa densidade enzimática na superfície e menor atividade enzimática, enquanto concentrações mais altas podem causar limitações difusionais, formação de multicamadas e alterações conformacionais indesejadas [32,34].

No que diz respeito à atividade enzimática, o estudo utilizou o próprio soro de leite como substrato, com o objetivo de avaliar o desempenho do biorreator em uma matriz alimentícia complexa. O soro é rico em proteínas como β -LG, α -LA, lactoferrina e peptídeos derivados da caseína [35], oferecendo múltiplos alvos naturais para a ação da tripsina. Mesmo que parte da enzima não tenha sido aderida ao suporte, a fração imobilizada manteve sua funcionalidade, resultando em uma atividade específica de 126,22 U.g⁻¹, superior à da enzima livre (90,83 U.g⁻¹), com recuperação de atividade de 146,62%. Vale ressaltar que o soro de leite é rico em minerais e gordura, e mesmo assim esses compostos não influenciaram na ação da enzima.

A melhoria observada no desempenho enzimático pode estar associada a diferentes fatores. A presença do APTES como braço espaçador, por exemplo, favorece uma orientação mais adequada da enzima no suporte, mantendo o sítio ativo mais acessível ao substrato [36]. A literatura também destaca que a imobilização covalente reduz a autólise comum em proteases em solução e limita a tendência natural de agregação, preservando a integridade estrutural da tripsina [37]. Outro aspecto relevante é o microambiente formado ao redor da enzima imobilizada, no qual a matriz do suporte e os grupos funcionais podem criar condições de pH, polaridade e hidratação mais favoráveis à conformação ativa da enzima. Além disso, considerando que a enzima utilizada não apresentava pureza absoluta, é possível que o processo de imobilização tenha ocorrido de forma preferencial sobre a protease, resultando em uma fração proteica imobilizada relativamente mais rica em enzima ativa quando comparada à solução livre. Esse efeito pode contribuir para o aumento da atividade enzimática específica observada no biorreator. Em conjunto, esses fatores favorecem a preservação da estrutura catalítica e a melhoria da estabilidade operacional do sistema [38,39].

3.2 Reutilização dos biorreatores

O biorreator foi avaliado acerca do seu desempenho em sucessivos ciclos de reutilização, os resultados estão apresentados na Figura 1.

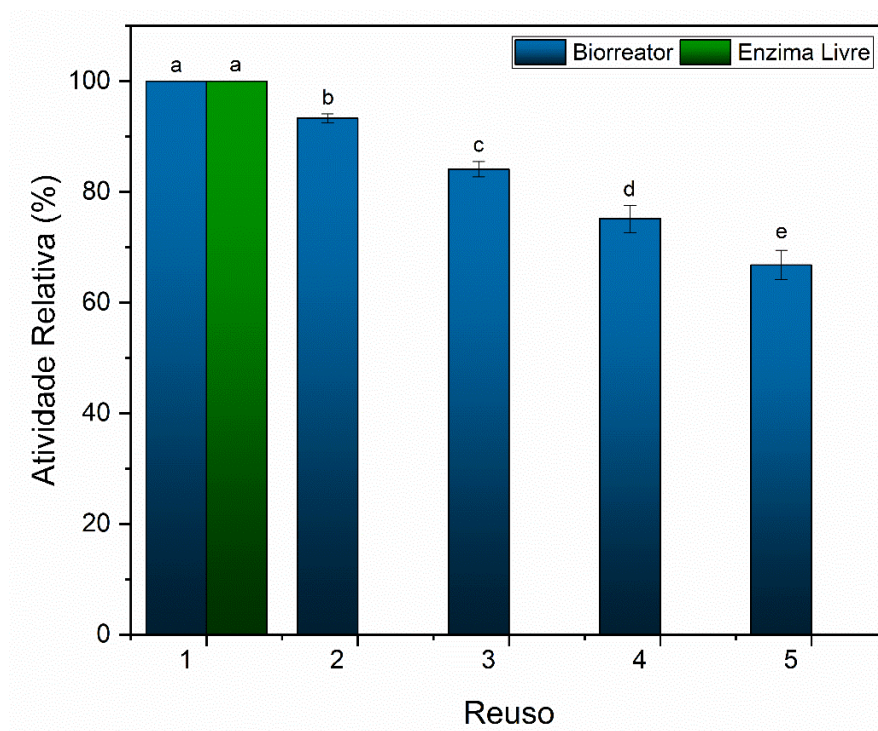


Figura 1. Atividade relativa da enzima livre e imobilizada sobre múltiplos ciclos de reuso

Médias seguidas da mesma letra não diferem significativamente de acordo ao teste Tukey ($p>0,05$)

A capacidade de reutilizar a enzima é uma das principais vantagens da imobilização, por isso avaliar seu desempenho ao longo de ciclos sucessivos é essencial. No caso da tripsina imobilizada, o reuso foi testado por cinco ciclos utilizando WP como substrato. No segundo ciclo, o biocatalisador manteve 93,96% de atividade relativa e, ao final do quinto ciclo, apresentou 68,25% em relação à atividade inicial. Esses resultados mostram que o biocatalisador reteve parte de sua atividade após múltiplos usos. No entanto, a queda de atividade observada pode estar relacionada, entre outros fatores, à lixiviação da enzima, um processo em que parte da proteína se desprende do suporte ao longo dos ciclos, indicando certa instabilidade das ligações entre suporte e enzima. Outros fatores também podem estar relacionados a desnaturação estrutural progressiva e bloqueio de sítios por agregação de peptídeos[33].

Os dados obtidos aqui estão alinhados com estudos anteriores. Aversa et al. [40] relataram retenção de cerca de 40% da atividade inicial da tripsina imobilizada em óxido de ferro após seis ciclos. Estudo envolvendo nanopartículas magnéticas modificadas com ácido tânico apontaram aproximadamente 60 % de atividade após cinco ciclos[41], enquanto sistemas magnéticos revestidos com PVA e ativados com glutaraldeído apresentaram cerca de 50% de retenção no mesmo número de usos[32]. Apesar da perda

gradual de atividade ser comum em sistemas imobilizados, o custo elevado das enzimas livres, aliado à dificuldade de recuperá-las do meio reacional, reforça a relevância de biocatalisadores imobilizados para aplicações industriais.

3.3 Perfil cromatográfico do soro de leite e peptídeos obtidos da hidrólise

A Figura 2 mostra os cromatogramas referentes ao soro de leite e aos peptídeos produzidos pela hidrólise enzimática nos tempos de reação estudados (0,5 a 4 h), obtidos por detecção a 280 nm.

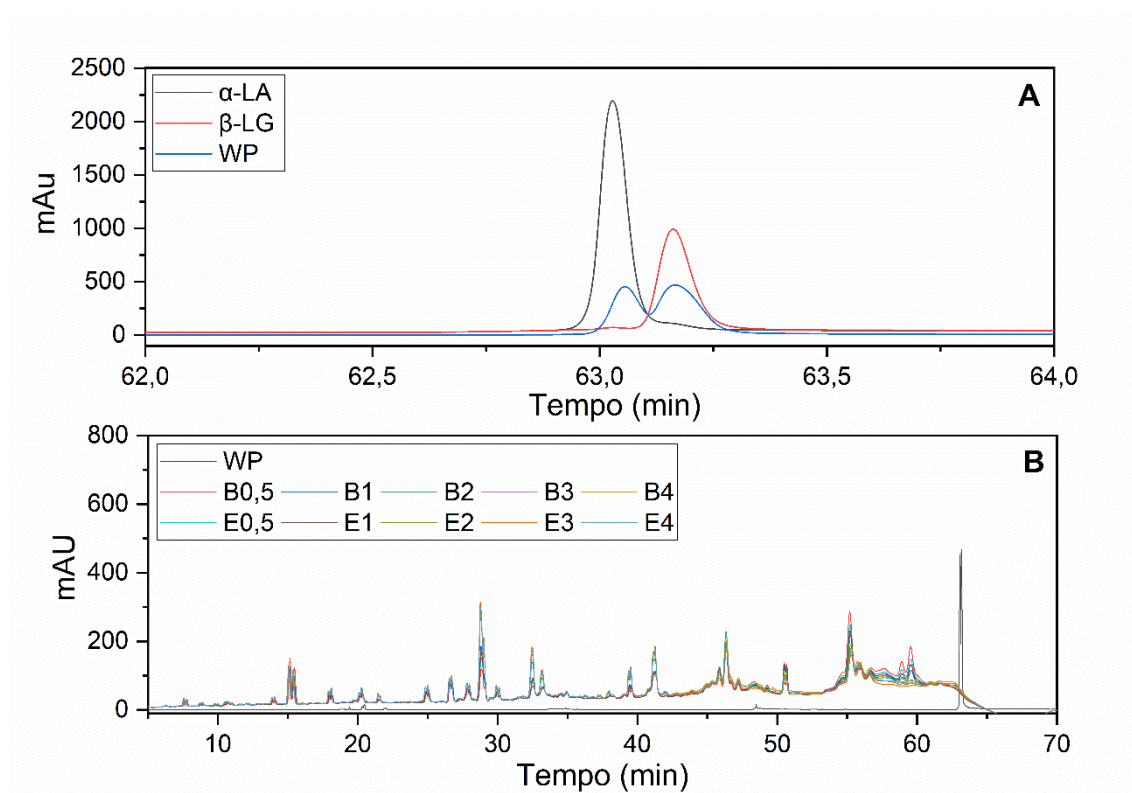


Figura 2. Perfil cromatográfico do WP e padrões de β -LG e α -LA (A); e peptídeos obtidos da hidrólise com tripsina livre e imobilizada livre e imobilizada (B) em diferentes tempos (0,5 a 4 h) (B)

Legenda: α -LA (α -lactoalbumina); β -LG (β -lactoglobulina); WP (Soro de Leite); E (Enzima Livre); e B (Enzima Imobilizada)

As amostras de soro de leite e peptídeos foram avaliadas em HPLC-RP para comparação dos picos por tempo de retenção. De maneira geral, ambas as formas da tripsina produziram perfis de peptídeos semelhantes ao decorrer do tempo de hidrólise, com distribuição de picos equivalente. O comportamento indica que o processo de imobilização preservou a estrutura da tripsina e o reconhecimento dos seus sítios de clivagem, que ocorrem preferencialmente após resíduos básicos de lisina e arginina [37].

Outro ponto relevante, é a distribuição de picos ao longo de toda a corrida cromatográfica em fase reversa. Nesse tipo de corrida, os peptídeos polares tendem a eluir nos primeiros minutos da corrida, enquanto os fragmentos hidrofóbicos são eluídos apenas com maior proporção de solvente orgânico, ou seja, ao final da corrida. A presença de picos em toda extensão do gradiente, revela que a ação da tripsina produziu um conjunto diversificado de peptídeos. Esse comportamento é compatível com estudos que abordam a hidrólise de proteínas como β -LG e α -LA, que apresentam regiões estruturais com características químicas variadas[10,42,43]. A capacidade da tripsina na forma imobilizada de produzir esses padrões reforçam sua estabilidade catalítica e confirma que a imobilização preservou a eficiência de hidrólise.

3.4 Avaliação do Grau de Hidrólise (DH) pelo ensaio o-ftaldialdeído (OPA)

A determinação do DH foi avaliada pelo ensaio espectrofotométrico por meio do reagente OPA. Os resultados podem ser observados na Figura 3.

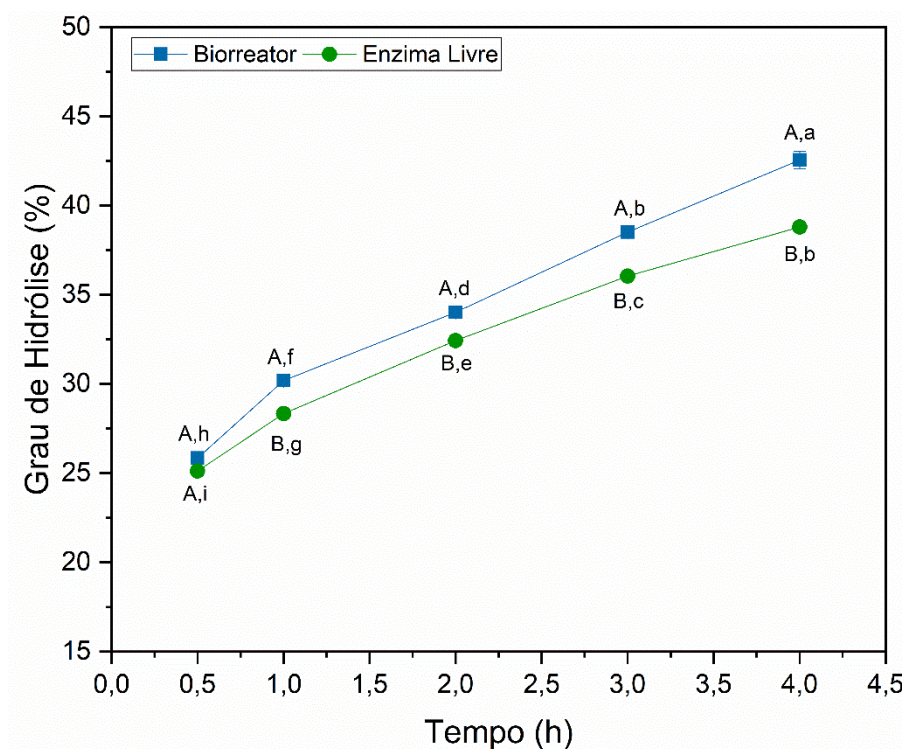


Figura 3. Grau de hidrólise dos peptídeos pelo ensaio espectrofotométrico o-ftaldialdeído. Médias seguidas da mesma letra maiúscula para amostras e minúscula para o tempo não diferem significativamente de acordo ao teste Tukey ($p > 0,05$).

No presente estudo, o ensaio de DH foi utilizado para monitorar a extensão da hidrólise do WP pela tripsina livre e imobilizada ao longo de 0,5 a 4 h de reação. Conforme mostrado na Figura 3, o DH da tripsina imobilizada variou de 25,84% $\pm$ 0,20 a 42,55% $\pm$ 0,49, enquanto a enzima livre apresentou valores entre 25,12% $\pm$ 0,20 e

38,80% $\pm$ 0,24. Observa-se um aumento progressivo do DH em ambos os sistemas, com maior extensão de hidrólise obtida após 4 h, especialmente para a forma imobilizada. Esse comportamento é coerente com estudos da literatura que investigaram a hidrólise do soro de leite por tripsina, os quais também relatam incremento gradual no DH com o avanço do tempo de hidrólise[43,44].

Como já foi abordado, a hidrólise de tripsina tem principal ação nas ligações peptídicas adjacentes à lisina e arginina[37]. No soro de leite, essas regiões tendem a estar acessíveis estruturalmente, favorecendo a ação da enzima. Além disso, o WP apresenta elevado teor de aminoácidos essenciais e um perfil proteico diversificado, o que contribui para a formação de peptídeos de diferentes tamanhos e características[45]. A presença significativa de lisina, um dos principais motivos de clivagem da tripsina [12,35], também favorece uma hidrólise mais eficiente, resultando em um conjunto amplo de peptídeos com potencial valor funcional e nutricional.

Vale ressaltar que o melhor desempenho observado para a tripsina imobilizada está de acordo com os resultados de atividade específica e com o perfil cromatográfico dos peptídeos. As pequenas variações no padrão de aminoácidos ao longo da reação indicam que a enzima manteve sua seletividade e sua conformação ativa durante a hidrólise, reforçando a estabilidade proporcionada pelo suporte. Além disso, a preservação do padrão de clivagem sugere que a imobilização ajudou a reduzir efeitos comuns da enzima na forma livre, como autólise e perda gradual de estrutura, permitindo uma atuação mais constante ao longo do tempo. Assim, além de manter a atividade catalítica, o sistema imobilizado apresentou estabilidade operacional, o que se refletiu no maior DH final e na formação de peptídeos com perfil semelhante ao obtido com a tripsina livre.

3.5 Determinação do potencial antioxidante dos peptídeos produzidos

Os peptídeos foram avaliados por diferentes ensaios antioxidantes com o objetivo de caracterizar seu potencial frente aos processos oxidativos. A Figura 4 apresenta os resultados de inibição do radical DPPH obtidos para os hidrolisados produzidos.

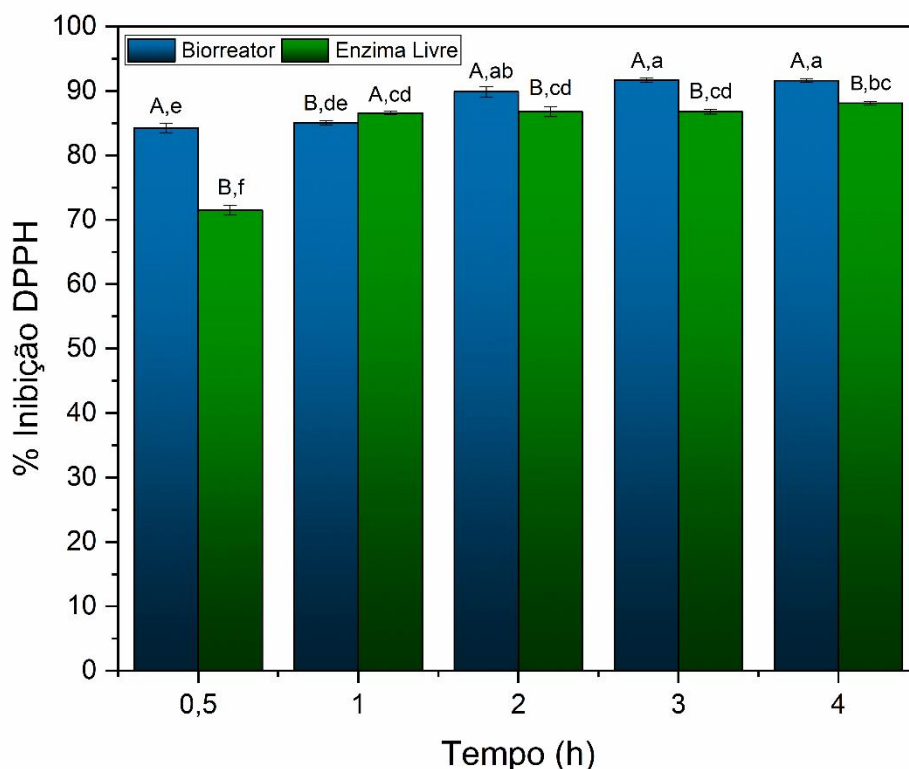


Figura 4. Potencial antioxidante *in vitro* dos peptídeos obtidos da hidrólise do soro de leite medidos pela capacidade de inibir o radical DPPH.

Médias seguidas da mesma letra maiúscula para amostras no mesmo tempo e minúscula para mesma amostra em diferentes tempos diferem significativamente de acordo com o teste Tukey ($p > 0,05$).

De acordo com os resultados apresentados na Figura 4 para a inibição do radical DPPH, os peptídeos produzidos pela tripsina imobilizada apresentaram valores significativamente superiores ($p > 0,05$) em todos os tempos avaliados. Esse comportamento reforça a estabilidade e a preservação da eficiência catalítica da enzima após a imobilização. Em relação ao tempo de hidrólise, observou-se um aumento gradual nos percentuais de inibição ao longo da reação. O valor inicial para a tripsina livre foi de 71,48% em 0,5 h, enquanto a tripsina imobilizada atingiu 91,59% no mesmo intervalo. Além disso, a partir de 3 h, os peptídeos gerados pela forma imobilizada mostraram tendência à estabilização, indicando um possível platô na capacidade antioxidante frente ao radical DPPH.

É visto que a capacidade antioxidante dos peptídeos frente ao radical DPPH pode estar relacionada à presença de determinados aminoácidos e ao mecanismo de transferência de hidrogênio. Estudos mostram que aminoácidos aromáticos, como tirosina e triptofano, possuem anéis eletronicamente conjugados capazes de estabilizar o radical formado após a doação de hidrogênio (HAT) ou elétron (SET)[46,47]. Assim como nos

compostos fenólicos, a estrutura aromática desses resíduos favorece a ressonância, distribuindo a carga e estabilizando o radical aminoacídico[48]. Além disso, aminoácidos hidrofóbicos como fenilalanina, e peptídeos contendo leucina, isoleucina e valina, podem facilitar o acesso ao DPPH, já que o radical é solúvel em meio orgânico e tende a interagir com moléculas menos polares[49]. Nesse contexto, a hidrólise do soro pela tripsina favorece a liberação desses aminoácidos e fragmentos peptídicos, o que ajuda a explicar o aumento da capacidade antioxidante observado ao longo do tempo de reação.

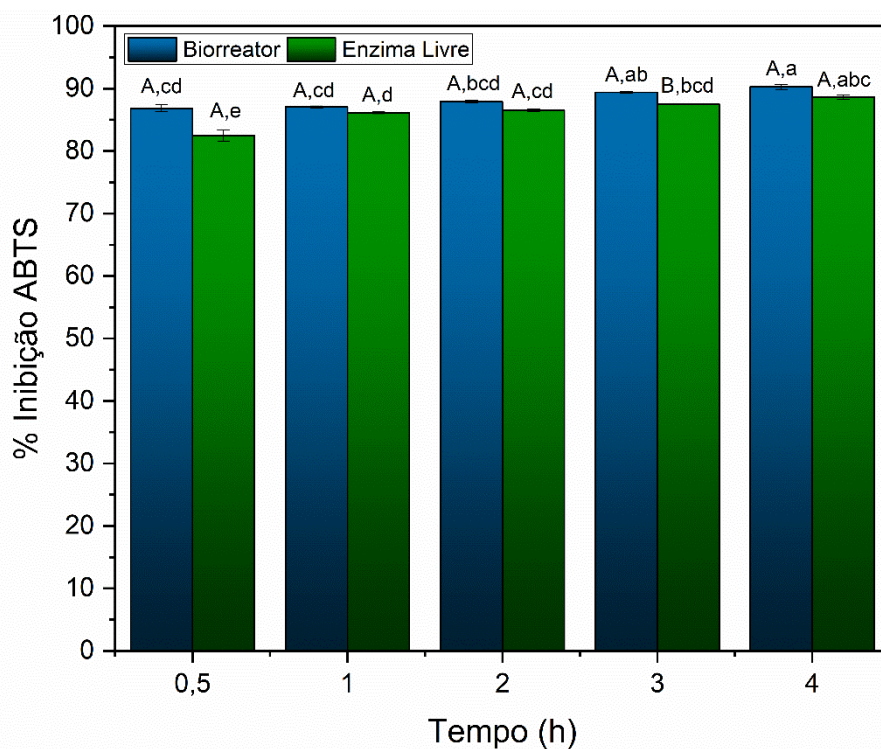


Figura 5. Potencial antioxidante *in vitro* dos peptídeos obtidos da hidrólise do soro de leite medidos pela capacidade de inibir o radical ABTS.

Médias seguidas da mesma letra maiúscula para amostras e minúscula para o tempo não diferem significativamente de acordo ao teste Tukey ($p > 0,05$).

O ensaio antioxidante com o radical ABTS foi realizado para avaliar a capacidade dos peptídeos produzidos em neutralizar radicais livres. Conforme apresentado na Figura 5, todos os valores de inibição foram superiores a 80%, indicando um elevado potencial antioxidante dos hidrolisados. De modo geral, não foram observadas diferenças significativas entre os peptídeos obtidos pela tripsina livre e imobilizada na maioria dos tempos avaliados, o que está de acordo com o perfil cromatográfico, que mostrou padrões de picos semelhantes para ambas as formas de hidrólise. O maior valor registrado foi de 90,26% após 4 h para a tripsina imobilizada, reforçando a boa manutenção da atividade

do biocatalisador ao longo da reação. Esses resultados sugerem que os hidrolisados possuem potencial para atuar na prevenção de danos oxidativos em sistemas biológicos.

Assim como no ensaio DPPH, o radical ABTS pode ser neutralizado por mecanismos de transferência de elétron (SET) e de hidrogênio (HAT), ambos influenciados pela presença de resíduos aromáticos, como triptofano e tirosina, que estabilizam o radical formado após a doação[45,50]. No entanto, o ABTS se diferencia por conseguir detectar tanto peptídeos hidrofílicos quanto hidrofóbicos, tornando-se um método mais abrangente do que o DPPH[51]. Essa característica foi refletida nos resultados, já que os valores de ABTS foram superiores aos de DPPH. Esse comportamento também é compatível com o perfil peptídico distribuído ao longo de todo o cromatograma, indicando a formação de peptídeos com diferentes polaridades e, consequentemente, diferentes capacidades de interação com o radical ABTS. O mesmo comportamento foi observado por Espindola et al. [42] quando avaliaram a hidrólise do soro de leite, e obtiveram 75,1 % para o radical DPPH, enquanto o radical ABTS demonstrou inibição de 99,5 %.

Abaixo, a Figura 6 apresenta os resultados para poder redutor (FRAP) dos hidrolisados do WP.

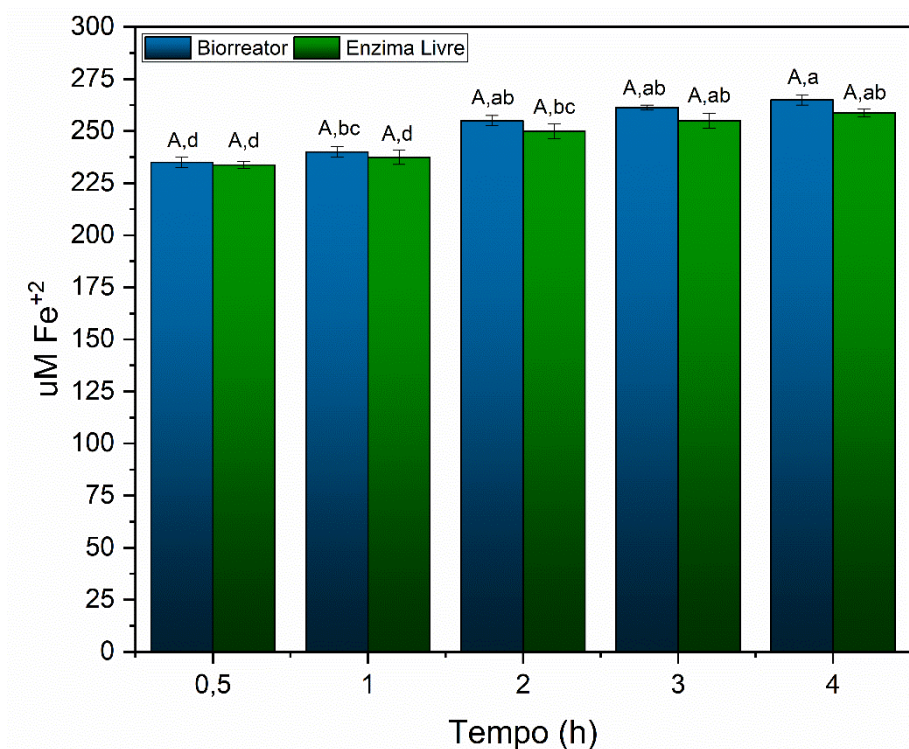


Figura 6. Potencial antioxidante *in vitro* dos peptídeos obtidos da hidrólise do soro de leite medidos ensaio de redução de ferro (FRAP)

Médias seguidas da mesma letra maiúscula para amostras no mesmo tempo e minúscula para mesma amostra em diferentes tempos diferem significativamente de acordo com o teste Tukey ($p > 0,05$).

O ensaio do método redutor FRAP foi realizado para avaliar a capacidade dos peptídeos em reduzir Fe(III) para Fe(II). De acordo com os dados apresentados na Figura 6, observa-se que os sistemas com tripsina imobilizada e livre atuaram da mesma forma, não apresentando diferença estatisticamente significativa ($p < 0,05$) dentro de cada tempo de reação. Além disso, o maior valor observado foi de 265 μM de Fe^{2+} , obtido com a tripsina imobilizada no tempo de reação de 4 h. De acordo Muley et al. [52] aumento do poder redutor em função do tempo de reação pode estar relacionado à maior disponibilidade de grupos funcionais presentes no hidrolisado. Esse comportamento está de acordo com os dados obtidos neste estudo, uma vez que o DH revelou aumento na concentração de peptídeos com o prolongamento do tempo de reação.

Diferentemente dos métodos de sequestro de radicais livres (DPPH e ABTS), o método FRAP avalia o poder redutor de um composto por meio do mecanismo de transferência de elétrons (ET)[53]. Um estudo indicou que grande parte da contribuição para o poder redutor de peptídeos está associada à presença de aminoácidos ácidos, como ácido aspártico e ácido glutâmico, devido à sua capacidade de doação de elétrons[54]. Ademais, grupos sulfidríla presentes em aminoácidos sulfurados, como cisteína e metionina, também contribuem significativamente para a elevada capacidade redutora[52].

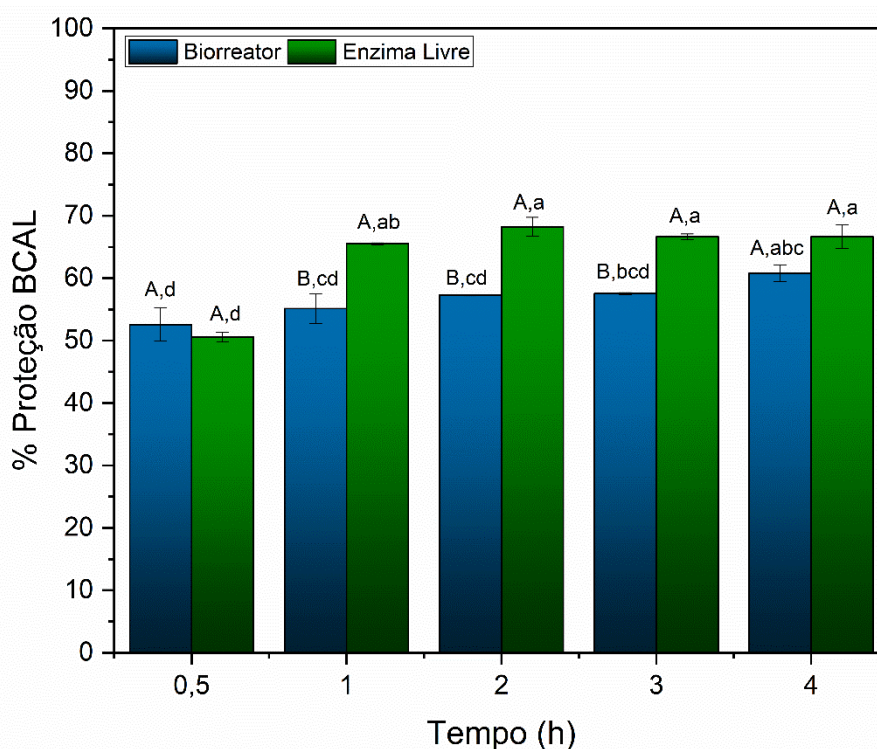


Figura 7. Potencial antioxidante *in vitro* dos peptídeos obtidos da hidrólise do soro de leite avaliados pela inibição da oxidação do sistema BCAL

Médias seguidas da mesma letra maiúscula para amostras no mesmo tempo e minúscula para mesma amostra em diferentes tempos diferem significativamente de acordo com o teste Tukey ($p > 0,05$).

Por fim, o sistema β -caroteno-ácido linoleico (BCAL) foi empregado para determinar o potencial antioxidante dos peptídeos obtidos do WP, com base na inibição da oxidação lipídica. De acordo com os resultados apresentados na Figura 7, a enzima na forma livre apresentou valores superiores de atividade antioxidante ($p > 0,05$) a partir de 1 hora de reação, indicando uma liberação mais eficiente de peptídeos associados a esse efeito. Assim como observado nos demais ensaios antioxidantes, o método BCAL demonstra melhor desempenho na presença de peptídeos hidrofóbicos[55], característica que pode ser corroborada pelos cromatogramas apresentados anteriormente. Também foi observado um efeito de platô na porcentagem de proteção ao longo do tempo, com valores máximos de 66,63 % para a enzima livre e 60,74 % para a forma imobilizada. Dessa forma, os resultados indicam que os peptídeos obtidos apresentam boa capacidade de neutralização dos radicais livres, retardando a oxidação do ácido linoleico e, consequentemente, preservando o β -caroteno no sistema avaliado.

De modo geral, os resultados obtidos nos diferentes ensaios antioxidantes indicam que o soro de leite hidrolisado apresenta elevado potencial antioxidante, evidenciando a eficácia da hidrólise enzimática na geração de peptídeos bioativos. Observou-se ainda que esse potencial é intensificado com o uso da tripsina na forma imobilizada, a qual favoreceu a obtenção de peptídeos antioxidantes. O desempenho satisfatório nos métodos avaliados, baseados em distintos mecanismos de ação, sugere que os peptídeos produzidos são capazes de atuar em múltiplas etapas do processo oxidativo, incluindo a neutralização de radicais livres, o poder redutor e a inibição da oxidação lipídica.

Resultados com desempenho antioxidante semelhantes têm sido amplamente descritos na literatura para peptídeos obtidos por hidrólise enzimática de fontes proteicas de maior valor agregado, como caseinato de sódio, proteínas isoladas do soro, colágeno e isolados proteicos comerciais[56,57]. Nesse contexto, a obtenção de atividade antioxidante comparável a partir do soro de leite, um coproduto da indústria láctea frequentemente tratado como resíduo e com elevado potencial poluidor, evidência sua capacidade de ser convertido em um ingrediente funcional de alto valor agregado, permitindo que esse material retorne à cadeia produtiva em uma aplicação mais nobre e tecnologicamente relevante.

4. CONCLUSÃO

Neste estudo, demonstrou-se a eficácia da tripsina imobilizada em nanopartículas magnéticas para a hidrólise enzimática do soro de leite, resultando na produção de peptídeos com comprovada capacidade antioxidante *in vitro*. O sistema imobilizado apresentou desempenho superior em relação à enzima livre, além de maior estabilidade operacional e reusabilidade, mantendo sua eficiência mesmo quando aplicado a uma matriz complexa como o soro de leite, o que reforça sua robustez e potencial de aplicação em condições industriais reais. Adicionalmente, a sustentabilidade do processo é evidenciada pela arquitetura do biocatalisador, com destaque para o uso da genipina como agente de ligação de origem natural e para a natureza magnética do suporte, que permite a recuperação eficiente da enzima e reduz o consumo de insumos e a geração de resíduos. Dessa forma, a estratégia proposta demonstra não apenas viabilidade técnica, mas também relevância tecnológica e ambiental na valorização do soro de leite, contribuindo para o desenvolvimento de processos alinhados aos princípios da economia circular e aos Objetivos de Desenvolvimento Sustentável, especialmente os ODS 3, 9 e 12.

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CRedit Statement

Jonathan Barbosa Santos: Investigation; Methodology; Writing – Original Draft; Validation. **Emilia Carmen da Silva:** Investigation; Methodology. **Arthur Pompílio da Capela:** Investigation; Methodology. **Débora Lemos da Silva:** Methodology. **Tanisa Andrade Araújo de Souza:** Methodology. **Mateus Pereira Flores Santos:** Supervision. **Leandro Soares Santos:** Supervision; **Sibelli Passini Barbosa Ferrão:** Supervision. **Rafael C. I. Fontan:** Funding acquisition; Resources; Supervision; Project administration; Writing – Review & Editing.

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CAPÍTULO 5

CONSIDERAÇÕES FINAIS

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A presente tese demonstrou o desenvolvimento de uma plataforma biotecnológica inovadora, baseada na imobilização de proteases em nanopartículas magnéticas (MNPs), estabelecendo um elo eficiente entre a nanotecnologia e a sustentabilidade na indústria de alimentos. A trajetória da pesquisa permitiu não apenas superar limitações catalíticas, mas também propor soluções alinhadas aos princípios da economia circular.

A etapa inicial de revisão evidenciou que, embora as MNPs possuam um potencial para a imobilização enzimática, a transição para a escala industrial dependia criticamente da superação de desafios como a toxicidade de agentes de reticulação e a complexidade dos métodos de funcionalização. Em resposta a essas lacunas, o desenvolvimento de um suporte magnético funcionalizado com genipina, um agente de origem natural e biocompatível, revelou-se uma estratégia promissora aos agentes sintéticos. Os resultados experimentais confirmaram que essa abordagem verde não apenas garantiu a integridade estrutural das enzimas tripsina e bromelina, mas também proporcionou estabilidade operacional, permitindo a reutilização dos biocatalisadores por múltiplos ciclos.

A eficácia dessa tecnologia foi validada na produção de peptídeos bioativos. A hidrólise da caseína e a valorização do soro de leite, demonstraram o desempenho dos biorreatores magnéticos. A obtenção de peptídeos com elevada capacidade antioxidante (superando 90% de inibição de radicais livres) confirma que a imobilização não compromete a especificidade enzimática, mas sim potencializa a geração de compostos de alto valor agregado.

Por fim, a aplicação do sistema na hidrólise do soro de leite representa o fechamento de um ciclo tecnológico e ambiental. Ao transformar um subproduto de alto impacto poluidor em uma fonte de ingredientes funcionais, esta tese oferece uma alternativa viável para a realocação de resíduos industriais. Conclui-se, portanto, que o uso de nanopartículas magnéticas funcionalizadas com agentes verdes constitui uma ferramenta poderosa para a bioeconomia, promovendo processos industriais mais limpos, econômicos e tecnicamente eficientes, em consonância com os Objetivos de Desenvolvimento Sustentável (ODS), em especial o ODS 12, que trata do estabelecimento de padrões sustentáveis de consumo e produção, o ODS 9, voltado à promoção da inovação e de infraestruturas industriais mais sustentáveis, e o ODS 3, relacionado ao desenvolvimento de produtos e tecnologias com potencial contribuição para a promoção da saúde e do bem-estar.