FEDERAL UNIVERSITY OF RIO GRANDE DO SUL

INSTITUTE OF FOOD SCIENCE AND TECHNOLOGY

GRADUATE PROGRAM IN FOOD SCIENCE AND TECHNOLOGY

DOCTORAL THESIS

Tailoring chitosan-genipin complex for β-galactosidase immobilization: porosity modification, structure characterization, and bioprocessing applications

Elí Emanuel Esparza Flores

Porto Alegre

July – 2023

Elí Emanuel Esparza Flores

Master in Food Science and Technology (UFRGS)

Tailoring chitosan-genipin complex for β-galactosidase immobilization: porosity modification, structure characterization, and bioprocessing applications

Thesis presented to the Graduate Program in Food Science and Technology of the Federal University of Rio Grande do Sul, as a partial requirement for obtaining a Doctorate degree in Food Science and Technology.

Advisors:

Prof. Plinho Francisco Hertz, PhD

Prof. Rafael Costa Rodrigues, PhD

Porto Alegre, July of 2023

CIP - Catalogação na Publicação

Flores, Elí Emanuel Esparza
Tailoring chitosan-genipin complex for β-galactosidase immobilization: porosity modification, structure characterization, and bioprocessing applications / Elí Emanuel Esparza Flores. -- 2023.
145 f.
Orientadores: Plinho Francisco Hertz, Rafael Costa Rodrigues.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciência e Tecnologia de Alimentos, Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Porto Alegre, BR-RS, 2023.

1. Imobilização enzimática. 2. Quitosano. 3. Genipina. 4. β-galactosidase . 5. Crosslinking. I. Hertz, Plinho Francisco, orient. II. Rodrigues, Rafael Costa, orient. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

Approval of the thesis:

Tailoring Chitosan-Genipin Complex for β-Galactosidase Immobilization: Porosity Modification, Structure Characterization, and Bioprocessing Applications

Submitted by Elí Emanuel Esparza Flores in partial fulfillment of the requirements for obtaining a Doctorate degree in Food Science and Technology at Federal University of Rio Grande do Sul, on July 6th, 2023.

Examining Committee Members:

Prof. Plinho Francisco Hertz, PhD (Advisor)

Federal University of Rio Grande do Sul (UFRGS)

Prof. Rafael Costa Rodrigues, PhD (Advisor)

Federal University of Rio Grande do Sul (UFRGS)

Prof. Adriano Brandelli, PhD

Federal University of Rio Grande do Sul (UFRGS)

Profa. Lorena Evelyn Wilson Soto, PhD

Pontifical Catholic University of Valparaíso (PUCV), Valparaíso – Chile

Prof. Juan Carlos Contreras Esquivel, PhD

Autonomous University of Coahuila (UAdeC), Saltillo – México

DEDICATORY

To those who always took care of me
and stood by my side on each step,
who never gave up and showed me that
hard work and persistence are key
to achieving everything I set out to do.

My parents: Rosy and Noé.

ACKNOWLEDGMENTS

La conclusión de esta tesis no sería posible sin un conjunto de personas que estuvieron a mi lado y que sin duda me ayudaron a que esta caminada fuera un poco más leve, a continuación, destaco algunas de las más importantes:

A mis grandes tutores, Prof. Plinho Hertz y Prof. Rafael Rodrigues, quienes fueron pieza clave para mi creciente madurez como científico y persona, siempre les seré grato por sus valiosos conocimientos, paciencia, comprensión, confianza y amistad.

A mi cotutor (no oficial) Prof. Patricio Santagapita, quien siempre con su mirada externa, acrecentó puntos valiosos para el mejoramiento de este trabajo.

A la Dra. Iliana Medina, quien amablemente me recibió en su laboratorio por un corto periodo y aportó valiosos conocimientos para mi formación.

A los Profesores Tania Costa, Edilson Benvenutti y Mercedes Perullini por sus valiosas aportaciones a mi trabajo.

A mis compañeros de laboratorio y posgrado, que, en esta caminada, se han convertido en grandes amigos. En especial a Fer Kuhn, mi fiel amiga que siempre estuvo para escucharme y darme bastantes consejos; a Mari, grande amiga y colega de apartamento, quien sin duda hizo mucho más leve esta jornada y siempre estuvo al pendiente para recordarme de fechas, plazos y requisitos; a Eduarda de quien aprendí tanto y siempre estuvo dispuesta a ayudarme con diversos análisis; a Fabíola, por esas largas y divertidas conversaciones. Por supuesto, a Fer Dias y a Lari, grandes colaboradoras y amigas. A Camila, que, a lo largo de toda esta jornada del posgrado fue una gran mentora, amiga y colaboradora. A mi gran amiga Nay, quien siempre me dio los consejos más acertados y me inyectó motivación.

A los profesores y técnicos del Instituto de Ciencia y Tecnología de Alimentos, quienes fueron pieza fundamental en mi formación, en especial a los Profesores Adriano y Eliseu, así como a las técnicos Ana Raísa y Michele.

A los laboratorios y personal de los departamentos de Química, Ciencia de los Materiales y Design de la UFRGS que pusieron a mi disposición diversos equipos para la realización de algunos experimentos.

A mi universidad, la UFRGS, por sus facilidades y por haberme adoptado como uno más de sus hijos científicos.

A los diversos órganos de fomento, como FAPERGS y CAPES del Gobierno de Brasil y a CONACyT del Gobierno de México, por los recursos financieros y materiales para la realización de mi doctorado.

Al CNPEM y todo su personal, por todas las facilidades para realizar diversos experimentos y permitirme formar parte de una de las primeras generaciones a usar el Sirius.

Por supuesto, a mis padres, Rosy y Noé, a quienes también les dedico este logro, pues han sido quienes desde mis primeras etapas de formación me han incentivado, motivado y apoyado para dar lo mejor de mí, ser perseverante y confiar en mis capacidades.

Finalmente, a Dios y las/sus fuerzas superiores, que de alguna u otra forma colocaron personas y circunstancias que me ayudaron para llegar hasta este grande y feliz momento.

EPIGRAPH

“A scientist in his laboratory is not a mere technician:

he is also a child confronting natural phenomena

that impress him as though they were fairy tales”.

- Marie Curie

ABSTRACT

Enzyme immobilization is a technique capable of increasing their stability and reusability. The use of the chitosan-genipin complex (CH-GE) as a support material for enzyme immobilization becomes highly attractive because crosslinking enhances the mechanical stability of the polymer and allows the formation of a complex polymeric network with high affinity for proteins. In this regard, the present study aimed to modify the porosity of chitosan sphere structures, crosslink them with genipin, and apply the spheres to immobilize β-galactosidase (E) from *Aspergillus oryzae*, thus enabling the application of the resulting biocatalyst in lactose hydrolysis and galactooligosaccharides (GOS) production. To achieve this, Na_2CO_3 was used as a porogenic agent to induce pore formation in the chitosan sphere polymer matrix. Subsequently, the spheres were crosslinked with genipin and used for enzyme immobilization, resulting in two biocatalysts: one unmodified (CH-GE-E) and one modified (PCH-GE-E, with added Na_2CO_3). The biocatalysts were characterized using conventional and unconventional methods such as low-angle X-ray scattering, dye adsorption, atomic force microscopy, scanning electron microscopy, compression force, N_2 adsorption/desorption, and thermogravimetric analysis. The results revealed modification of the support's porosity, showing larger and more interconnected pores, which also reduced its deformation resistance. The microstructure was also modified, increasing fractality, particularly at larger scales (~100 nm), and decreasing it at smaller scales (~1-10 nm). Additionally, the biocatalysts were applied in fixed-bed reactors, with PCH-GE-E demonstrating better operational stability in GOS production over 30 days. Subsequently, the enzyme immobilization process on the chitosan-genipin support was further studied in detail. β-galactosidase from *A. oryzae* was immobilized on the PCH-GE support using four different pH values (4.5, 6.0, 7.5, and 9.0), resulting in four biocatalysts: B4, B6, B7, and B9. The biocatalysts were characterized for their thermal stability, pH stability, and storage stability. Furthermore, operational stability was studied for lactose hydrolysis in continuous and batch reactors. It was found that all four biocatalysts exhibited superior thermal stability compared to free enzyme at 55 °C. Biocatalysts prepared under alkaline conditions performed better in acidic environments, while those prepared under acidic conditions showed greater stability in alkaline environments.

Moreover, they retained at least 95% of their initial activity after 20 weeks of storage at 4 °C. Finally, when applied for 50 cycles in lactose hydrolysis, all biocatalysts maintained at least 71% of their initial activity, with B6 standing out, retaining nearly 100% of its activity. On the other hand, in the continuous process, after 20 days, all biocatalysts demonstrated high stability and productivity, with B6 showing an increase in productivity from 41.3 to 48.1 g L^{-1} h^{-1} while retaining 78.1% of its initial activity. Thus, it can be concluded that the obtained biocatalysts improved the enzyme's stability, and the chitosan-genipin complex has great potential for application not only as a support for enzyme immobilization but also in other areas due to its plastic and dynamic properties, making it a sustainable and environmentally friendly alternative.

Keywords: chitosan, genipin, enzymatic immobilization, β-galactosidase, galactooligosaccharides.

RESUMO

A imobilização de enzimas é uma técnica capaz de aumentar sua estabilidade e capacidade de reuso. O uso do complexo quitosana-genipina (CH-GE) como material de suporte para imobilização enzimática torna-se bastante atrativo, pois o entrecruzamento aumenta a estabilidade mecânica do polímero e permite a formação de uma rede polimérica complexa que possui alta afinidade por proteínas. Neste sentido, o presente trabalho buscou modificar a porosidade da estrutura de esferas de quitosana, entrecruzar com genipina e aplicar as esferas para imobilizar a β-galactosidase (E) de *Aspergillus oryzae* e assim, poder aplicar o biocatalisador resultante na hidrólise de lactose e na produção de galactooligossacarídeos (GOS). Para isso, foi aplicado o Na_2CO_3 como agente porogênico para induzir a formação de poros na matriz polimérica de esferas de quitosana, depois, foram entrecruzadas com genipina e usadas para a imobilização da enzima, gerando dois biocatalisadores, um não modificado, CH-GE-E, e outro modificado, PCH-GE-E (adicionado de Na_2CO_3). Os biocatalisadores foram caracterizados por métodos convencionais e não convencionais, como espalhamento de raios-X a baixo ângulo, adsorção de corantes, microscopia de força atômica, microscopia eletrônica de varredura, força de compressão, adsorção/dessorção de N_2 e análise termogravimétrica. Os resultados constataram a modificação da porosidade do suporte, apresentando poros maiores e mais interconectados, o que também diminuiu sua resistência à deformação. A microestrutura também foi modificada, aumentando a fractalidade, especialmente a maiores escalas (~100 nm) e diminuindo-a a menores escalas (~1-10 nm). Além disso, os biocatalisadores foram aplicados em reatores de leito fixo e o PCH-GE-E demonstrou uma melhor estabilidade operacional na produção de GOS durante 30 dias. A seguir, o processo de imobilização da enzima em suporte de quitosana e genipina foi estudado mais detalhadamente. A β-galactosidase de *A. oryzae* foi imobilizada no suporte PCH-GE usando quatro diferentes pH (4,5, 6,0, 7,5 e 9,0), obtendo-se assim quatro biocatalisadores: B4, B6, B7 e B9. Os biocatalisadores foram caracterizados quanto às suas estabilidades térmica, ao pH e ao armazenamento. Ainda, foi estudada a estabilidade operacional para a hidrólise de lactose em reatores contínuos e batelada. Foi encontrado que os quatro biocatalisadores apresentaram estabilidade térmica superior à enzima livre a 55 °C. Os biocatalisadores preparados em condições

alcalinas tiveram melhor desempenho no meio ácido e os biocatalisadores preparados em condições ácidas mostraram maior estabilidade no meio alcalino. Além disso, mantiveram pelo menos 95% da sua atividade inicial, após 20 semanas armazenados a 4 °C. Por último, quando aplicados durante 50 ciclos para a hidrólise de lactose, todos mantiveram pelo menos 71% da sua atividade inicial, destacando o B6, que manteve quase o 100%. Por outro lado, no processo contínuo, após 20 dias, todos os biocatalisadores demonstraram alta estabilidade e produtividade, destacando o B6 que inclusive aumentou sua produtividade de 41,3 a 48,1 g L^{-1} h^{-1}, mantendo 78.1% da sua atividade inicial. Assim, pode se concluir que os biocatalisadores obtidos melhoraram a estabilidade da enzima e o complexo quitosana-genipina possui um grande potencial para aplicação não apenas como suporte para imobilização enzimática, mas também para aplicação em outras áreas devido à suas propriedades plásticas e dinâmicas, além de ser uma alternativa sustentável e ambientalmente amigável.

Palavras-chave: quitosana, genipina, imobilização enzimática, β-galactosidase, galactooligossacarídeos.

SUMMARY

INTRODUCTION **14**

OBJECTIVES **17**

BIBLIOGRAPHIC REVIEW **18**

CHAPTER 1 ERRO! INDICADOR NÃO DEFINIDO.

Chitosan-genipin complex: a promissory biomaterial for enzyme immobilization **Erro! Indicador não definido.**

RESULTS ERRO! INDICADOR NÃO DEFINIDO.

CHAPTER 2 ERRO! INDICADOR NÃO DEFINIDO.

Chitosan with modified porosity and crosslinked with genipin: a dynamic system structurally characterized **Erro! Indicador não definido.**

CHAPTER 3 ERRO! INDICADOR NÃO DEFINIDO.

Genipin crosslinked porous chitosan beads as robust supports for β-galactosidase immobilization: characterization, stability, and bioprocessing potential **Erro! Indicador não definido.**

CHAPTER 4 **128**

GENERAL DISCUSSION 128

CHAPTER 5 **133**

CONCLUSION AND PERSPECTIVES 133

BIBLIOGRAPHY **135**

APPENDIX 1 **138**

Supplementaray material of Chapter 2 138

APPENDIX 2 **138**

Supplementary material of Chapter 3 138

APPENDIX 3 **145**

Collaborations 145

INTRODUCTION

Chitosan is a polymer widely abundant in nature, obtained from the alkaline deacetylation of the chitin (QU; LUO, 2020; SABAB *et al.*, 2021; VIVEK *et al.*, 2013). Chitin is found in the cell wall of some fungi and yeast, as well as in the exoskeletons of crustaceous and the cuticles of insects (BIRANJE *et al.*, 2019; KUMARIHAMI *et al.*, 2022; MA *et al.*, 2022). Chitosan is known by its biodegradable, biocompatible and non-toxic properties, making it suitable for biomedical, chemical, agricultural and food technologies applications (KRAJEWSKA, 2004; LOHIYA; KATTI, 2022; OUERGHEMMI *et al.*, 2018; SADIQ *et al.*, 2021; ZUBAREVA *et al.*, 2017). Additionally, chitosan can produce diverse structures, like films, fibers, beads, hydrogels in different sizes and formats, from nano to macroscale (ISLAM *et al.*, 2022; KIANFAR *et al.*, 2019; PINTO *et al.*, 2020).

Chitosan can be modified to expand its possibly applications and overcome some limitations like low mechanical stability of acid solubility (SADY *et al.*, 2021). For this purpose, some modifications have been proposed depending on the final application (KESHVARDOOSTCHOKAMI *et al.*, 2021). One of them is the crosslinking, reaction between the polymer and a specific chemical specie that have at least two functional groups to react with the polymeric chains, forming strong and very complex nets (MUZZARELLI, 2009; SADIQ *et al.*, 2021).

Genipin, have been gained highlight in recent years as crosslinker agent, especially due its biocompatibility and antioxidant properties, attractive for biomedical and food applications (BRAUCH *et al.*, 2016; MENG *et al.*, 2021; ZAFEIRIS *et al.*, 2021). Genipin is an iridioid obtained from some fruits, highlighting the genipap from *Genipa americana*, plant originary from Central America, also founded in Brazil (BELLÉ et al., 2018; MIRAS et al., 2021).

The crosslinking process is strongly dependent on pH, temperature, reactional time, species concentration and presence of light and oxygen (CHEN et al., 2009; DELMAR; BIANCO-PELED, 2015; FLORES et al., 2019). This reaction has been widely studied (MI; SHYU; PENG, 2005; MUZZARELLI *et al.*, 2016), especially for tissue engineering. The reactional mechanism has been described by some authors, but till present, the formed complex

chitosan-genipin is treated as quite stable. This complex has higher mechanical stability and great protein affinity than the polymer itself, that is why is suitable for enzyme immobilization.

Enzyme immobilization is a technique that pursues to add an insoluble portion to the enzyme, making it reusable and suitable for continuous and discontinuous bioprocesses (CAO, 2005; CAO *et al.*, 2016; SHELDON; VAN PELT, 2013). The complex chitosan-genipin has been applied as enzyme immobilization support, especially for β-galactosidases from *Aspergillus oryzae* (FLORES *et al.*, 2019; KLEIN *et al.*, 2016; TAVARES *et al.*, 2020) and *Kluyveromyces lactis* (BELLÉ et al., 2018; LIMA et al., 2021), and some other proteins (CAVELLO; CONTRERAS-ESQUIVEL; CAVALITTO, 2014; CERÓN et al., 2023; IQBAL et al., 2022; LIU et al., 2023; MA et al., 2018; NATH et al., 2015; PHADUNGCHAROEN et al., 2019; YANG et al., 2011). The main objective of those studies was to find the best immobilization parameters and the characterization of the obtained biocatalyst by some common techniques.

This thesis is organized in chapters. In **CHAPTER 1**, it is provided a critical review focusing on chitosan and its modifications, genipin and its reactivity, and the chitosan-genipin complex and its applications, with particular emphasis on enzyme immobilization. In chapters 2 and 3 it is presented the experimental results obtained. In **CHAPTER 2**, the main objective is to characterize a modified chitosan support using Na_2CO_3 as a porogen agent. It was conducted a comprehensive characterization using both conventional and unconventional techniques to better understand of the complex structure and any potential modifications. Additionally, it was performed the immobilization of β-galactosidase from *Aspergillus oryzae* on the modified chitosan support. The resulting biocatalyst was then employed in a continuous bioprocess for the production of galactooligosaccharides.

In the subsequent step, the previous porous biocatalyst obtained was fully characterized in terms of immobilization (**CHAPTER 3**). Various stability tests, such as thermal stability, pH stability, storage stability and operational stability, were conducted. The operational stability for lactose hydrolysis was assessed in both continuous and discontinuous modes, providing insights into the performance and stability of the immobilized enzyme under different operational conditions.

The final part of the thesis is composed by a comprehensive discussion (**CHAPTER 4**) of all the experiments carried out. Subsequently, the main conclusions and perspectives (**CHAPTER 5**) will be provided as the closing chapter of this study.

OBJECTIVES

The main objective of this work was to develop a chitosan-based support with modified porosity, crosslinked with genipin, and apply it for enzymatic immobilization.

The specific objectives were:

- To produce a polymeric support from chitosan with a porosity modified using Na_2CO_3 as porogen agent.
- To use the genipin as crosslinker agent to modify the support obtained and immobilize the β-galactosidase from *Aspergillus oryzae*.
- To perfume the structural and physicochemical characterization of the support and obtained biocatalysts through conventional and unconventional techniques.
- To immobilize the β-galactosidase from *A. oryzae* in the prepared support under different conditions.
- To characterize the different stabilities of the obtained biocatalysts.
- To apply the biocatalysts in continuous and discontinuous bioprocess for lactose hydrolysis and GOS production.

BIBLIOGRAPHIC REVIEW

This section will be presented as review article, discussing chitosan, genipin and the complex chitosan-genipin as support material for enzyme immobilization. The review has already been submitted to the international peer-reviewed journal Biotechnology Advances.

CHAPTER 4

GENERAL DISCUSSION

The present doctoral thesis, pursued to modify the chitosan beads structure by the application of Na_2CO_3 as porogen agent and their subsequent application for enzyme immobilization. To achieve this objective, several methodologies were conducted to gain a deeper understanding of the proposed modification and its impacts on the properties of the enzymatic support and the enzymatic immobilization. Furthermore, the resulting biocatalysts were tested in different bioprocesses to evaluate the enzyme's performance and its correlation with the structural modifications implemented during the bioprocesses.

Initially, a comprehensive review was conducted to gather more information about our material support. This review encompassed three main components: chitosan, genipin, and the chitosan-genipin complex as an enzymatic immobilization support. Several key points emerged from this review. Firstly, it was noted that the primary application of this complex lies in the field of biomedical sciences, particularly in tissue engineering, drug release, and wound healing. While a limited number of studies have explored its use for enzyme immobilization, most of them have focused on investigating enzyme stability rather than the structure itself and the interactions with the immobilized enzyme or protein.

Furthermore, it was observed that modifications to the structure of chitosan provided various alternatives to overcome certain limitations of the polymer, such as its low mechanical stability and solubility in acid. Crosslinking emerged as one of the most employed techniques to enhance the mechanical stability and prevent acid solubility of chitosan. Notably, genipin gained attention due to its unique properties and its ability to create diverse structures when interacting with chitosan under different pH conditions.

In contrast, physical modifications offered the opportunity to create a different format for the immobilization support, resulting in improved stability of the immobilized enzyme. Enhanced porosity, in particular, appeared to be an attractive feature as it allowed for the immobilization of enzymes not only on the surface of the support but also within the porous internal structure. This

internal immobilization provided greater protection to the protein against changes in the reaction medium.

Subsequently, a new support material was fabricated using Na_2CO_3 as a porogen agent. This material was crosslinked with genipin and applied for the immobilization of β-galactosidase from *A. oryzae*, resulting in the biocatalyst PCH-GE-E. Extensive characterization of this biocatalyst was conducted using both conventional and non-conventional techniques, as this particular type of investigation had not been previously performed, particularly in the context of enzyme immobilization.

The results demonstrated a notable increase in porosity. This macrostructural modification was confirmed through dye adsorption experiments, with the PCH biocatalyst exhibiting a higher dye adsorption capacity (36.1 and 31.9 mg g^{-1} for PCH and CH, respectively). The SEM images also supported this finding, revealing a porous biocatalyst with more interconnected pores and thinner layers. Furthermore, the microstructure underwent modifications, as evidenced by the results obtained from SAXS and AFM analyses. The addition of genipin resulted in higher fractality in the PCH-GE biocatalyst at all scales (1-100 nm).

However, the compression strength of the structure showed a decrease (22.5 N for CH and 17 N for PCH), which was expected due to the increased porosity. However, this weakening was partially overcome by reinforcing the polymer matrix with genipin crosslinking, resulting in compression strengths of 32 N for CH-GE and 28 N for PCH-GE. Interestingly, no significant differences were observed in the BET analysis among the systems, possibly due to the collapse of the structure during lyophilization, which modified the pore structure. Therefore, multiple techniques were employed, especially those allowing analysis with hydrated samples to preserve the original structure.

Among all the previous results, the pH was identified as the main factor influencing the structural modifications. It facilitated the creation of different conformations within the complex matrix, attributed to the high reactivity of genipin and the potential increase or decrease in crystallinity of chitosan chains through hydration or dehydration of the polymer. These modifications suggest that the chitosan-genipin complex is a plastic and dynamic system that can

undergo changes even after the crosslinking process, particularly when the pH fluctuates over time.

The immobilization of the enzyme showcased the superior properties of the porous biocatalyst. The PCH-GE-E biocatalyst exhibited a recovered activity of 53.8%, while the CH-GE-E biocatalyst demonstrated a recovered activity of 37.5%. This result can be attributed to the enhanced immobilization efficiency of the porous biocatalyst. The increased surface area and improved protein dispersion likely contributed to the better performance observed. Moreover, the enzyme could attach to the internal side of the porous structure, facilitating mass transfer and maintaining its activity. This was confirmed during continuous production of GOS for a duration of 30 days.

Initially, optimal operational conditions for GOS production were determined, including substrate flow rate and production rate. A direct relationship between flow rate and productivity was observed, with reduced flow rate and increased residence time resulting in higher conversion. However, very low flow rate led to lactose crystallization, forming large crystals that disrupted the flow. Consequently, a flow rate of 0.07 mL min^{-1} was chosen. Periodic system washes with 0.1 M acetate buffer at pH 4.5 were required to remove visible crystals that formed. Therefore, the most feasible solution was to use a 300 g L^{-1} lactose solution dissolved in 0.1 M acetate buffer at pH 4.5.

During the GOS production bioprocess, the PCH-CH-E biocatalyst maintained almost the same conversion yield (25.8%), while the CH-GE-E biocatalyst decreased to 11.9% after 30 days. Additionally, the porous biocatalyst exhibited higher productivity, more than twice that of the non-porous biocatalyst.

With the understanding of these phenomena, the study progressed, where the obtained biocatalyst was extensively tested as an enzymatic support. It was divided into two parts. In the first part, only the PCH-GE-E (or B4) biocatalyst was tested, while in the second part, three additional biocatalysts (B6, B7, and B9) were prepared and evaluated for their application in lactose hydrolysis bioprocesses.

Initially, different concentrations of Na_2CO_3 were tested for the fabrication of chitosan beads. It was observed that as the porogen concentration increased, the beads appeared more transparent probably due to the enhanced porosity. After the crosslinked and enzyme immobilization processes, was observed the increase on the immobilization yield, indicating an expanded surface area. However, the efficiency showed a decrease. In order to strike a balance and achieve good recovery activity, a concentration of 50 mM Na_2CO_3 was determined to be the optimal condition.

Furthermore, the enzyme load was investigated to determine if more protein could be immobilized on the increased surface area. However, in terms of yield and recovery activity (90.8% and 49.7%, respectively), it was found that using 2.73 mg P g^{-1} of chitosan was the most effective. It is possible that adding more protein caused diffusional restrictions, leading to a decrease in the overall performance of the biocatalyst.

During the analysis of B4, it was compared to the free enzyme in terms of optimal pH, and both showed similar behavior, exhibiting high stability between pH 4.0 and 6.0. However, the most intriguing finding was observed when all biocatalysts were exposed to pH-controlled solutions ranging from 4.0 to 8.0 for 5 h and then evaluated for its activity. It was discovered that all biocatalysts maintained at least 85.7% of its initial activity, indicating some reversible changes in the polymeric matrix did not adversely affect the immobilized enzyme performance.

Subsequently, the immobilization process was performed under four different pH conditions (4.5, 6.0, 7.5 and 9.0), resulting in the biocatalysts B4, B6, B7, and B9. Among these, B9 exhibited the lowest immobilization parameters, but it was chosen for further experiments. In terms of thermal stability, all biocatalysts demonstrated higher stability compared to free enzyme when tested at 55 °C under the same four pH conditions of the immobilization process. This indicates the beneficial effect of enzyme immobilization using different pH conditions, resulting in the stabilization of the enzyme.

The batch operational stability test revealed the high stability of both the enzyme and the biocatalysts, despite the decreased mechanical resistance resulting from the increased porosity. After 50 cycles of 3 h each, all biocatalysts retained at least 71% of their initial activity. Only B4

exhibited some fractures, with 44.4% of the beads remaining undamaged. To assess the mechanical resistance, tests were conducted before and after the bioprocess. It was confirmed that the support weakened after the stirred mode bioprocess, particularly for B6, B7, and B9. In contrast, B4 did not show significant differences as the beads already demonstrated lower mechanical resistance prior to the bioprocess. This suggests that the acidic pH effectively weakens the polymeric structure. These structural modifications were further confirmed through SEM analysis.

Lastly, in continuous lactose hydrolysis, the biocatalysts exhibited high stability, especially B6, B7, and B9, retaining over 78% of their initial activity after 20 days. Notably, B6 demonstrated increased productivity, from 41.3 to 48.1 g L^{-1} h^{-1}. This improvement could be attributed to the covalent attachment of the enzyme directly to the support during the immobilization process. The relaxation of the polymeric chains allowed for stable enzyme immobilization. Furthermore, the increased productivity was influenced by changes in the biocatalyst size and consequently, the residence time. These findings once again highlighted the plastic and dynamic nature of the polymeric matrix in the complex chitosan-genipin system.

CHAPTER 5

CONCLUSION AND PERSPECTIVES

In conclusion, the objectives of this thesis were successfully achieved by modifying the chitosan structure to fabricate a new support material, crosslinking with genipin, and immobilizing β-galactosidase from *Aspergillus oryzae*. The biocatalysts were thoroughly characterized structurally, tested for various stabilities, and applied in three different bioprocesses. Several conclusions can be drawn from the study:

- Genipin is a highly reactive compound influenced by various factors, with pH playing a significant role in creating different polymer structures when combined with chitosan, which acts as an amine source.
- Sodium carbonate (Na_2CO_3) is an inexpensive and easily manipulated porogen agent that induces the formation of CO_2 within the chitosan matrix during coagulation, resulting in a modified porous structure.
- Structural modifications of the polymer matrix should be analyzed comprehensively to understand potential changes at the micro and macrostructural levels.
- The structural properties of the biocatalysts strongly influence the performance and stability of the immobilized enzyme.
- The obtained biocatalysts demonstrated high stability, particularly in continuous and discontinuous bioprocesses for lactose hydrolysis and the production of GOS.

Overall, this thesis provides valuable insights into supports for enzyme immobilization based on the complex chitosan-genipin system, making it applicable to other enzymes and bioprocesses. The structural characterization of the complex offers new understanding of structural modifications during the crosslinking process and in systems where pH may vary over time, such as tissue engineering, drug release, and other bioprocesses and applications.

Several perspectives for future research are suggested, such as reinforcing the polymeric matrix using hybrids materials and other structures from the same polymer or from others, like nanofibers, to enhance mechanical stability for application in stirred and fluidized bioreactors. Additionally, testing enzymes with different sizes and conformations, as well as those that act on

larger substrates, would provide a better understanding of the benefits and drawbacks of immobilization within the internal pores of the support. It is also important to evaluate the allergenicity of the bioprocess products, particularly for food technology applications, as fragments of the support may be leached over time and could potentially cause reactions in individuals with shrimp allergies. Lastly, improvements should be made to enhance the thermal stability of both β-galactosidase and the support itself, making them suitable for application in thermal milk treatments.

BIBLIOGRAPHY

BELLÉ, A. S. *et al.* Efficient enzyme-assisted extraction of genipin from genipap (Genipa americana L.) and its application as a crosslinker for chitosan gels. **Food Chemistry**, [*s. l.*], v. 246, n. November 2017, p. 266–274, 2018. Disponível em: https://doi.org/10.1016/j.foodchem.2017.11.028.

BIRANJE, S. S. *et al.* Hemostasis and anti-necrotic activity of wound-healing dressing containing chitosan nanoparticles. **International Journal of Biological Macromolecules**, [*s. l.*], v. 121, p. 936–946, 2019.

BRAUCH, J. E. *et al.* Jagua blue derived from Genipa americana L. fruit: A natural alternative to commonly used blue food colorants?. **Food Research International**, [*s. l.*], v. 89, p. 365–372, 2016. Disponível em: http://dx.doi.org/10.1016/j.foodres.2016.08.029.

CAO, L. Immobilised enzymes : science or art ?. [*s. l.*], p. 217–226, 2005.

CAO, S. *et al.* Recent advances in immobilized enzymes on nanocarriers. **Cuihua Xuebao/Chinese Journal of Catalysis**, [*s. l.*], v. 37, n. 11, p. 1814–1823, 2016.

CAVELLO, I. A.; CONTRERAS-ESQUIVEL, J. C.; CAVALITTO, S. F. Immobilization of a keratinolytic protease from Purpureocillium lilacinum on genipin activated-chitosan beads. **Process Biochemistry**, [*s. l.*], v. 49, n. 8, p. 1332–1336, 2014. Disponível em: http://dx.doi.org/10.1016/j.procbio.2014.04.016.

CERÓN, A. *et al.* Study of stability, kinetic parameters and release of lysozyme immobilized on chitosan microspheres by crosslinking and covalent attachment for cotton fabric functionalization. **Process Biochemistry**, [*s. l.*], v. 128, n. October 2022, p. 116–125, 2023.

CHEN, M. C. *et al.* Mechanical properties, drug eluting characteristics and in vivo performance of a genipin-crosslinked chitosan polymeric stent. **Biomaterials**, [*s. l.*], v. 30, n. 29, p. 5560–5571, 2009. Disponível em: http://dx.doi.org/10.1016/j.biomaterials.2009.06.039.

DELMAR, K.; BIANCO-PELED, H. The dramatic effect of small pH changes on the properties of chitosan hydrogels crosslinked with genipin. **Carbohydrate Polymers**, [*s. l.*], v. 127, p. 28–37, 2015. Disponível em: http://dx.doi.org/10.1016/j.carbpol.2015.03.039.

FLORES, E. E. E. *et al.* Influence of reaction parameters in the polymerization between genipin and chitosan for enzyme immobilization. **Process Biochemistry**, [*s. l.*], v. 84, n. June, p. 73–80, 2019. Disponível em: https://doi.org/10.1016/j.procbio.2019.06.001.

IQBAL, M. *et al.* The effect of nano-calcium carbonate on β-glucosidase immobilized by alginate and chitosan. **Green Synthesis and Catalysis**, [*s. l.*], v. 3, n. 3, p. 265–271, 2022. Disponível em: https://doi.org/10.1016/j.gresc.2022.03.006.

ISLAM, M. T. *et al.* Fabrication and characterization of 3-dimensional electrospun poly(vinyl alcohol)/keratin/chitosan nanofibrous scaffold. **Carbohydrate Polymers**, [*s. l.*], v. 275, 2022.

KESHVARDOOSTCHOKAMI, M. *et al.* A review on the use of chitosan and chitosan derivatives as the bio-adsorbents for the water treatment: Removal of nitrogen-containing pollutants. [*S. l.*]: Elsevier Ltd, 2021.

KIANFAR, P. *et al.* Photo-crosslinking of chitosan/poly(ethylene oxide) electrospun nanofibers. **Carbohydrate Polymers**, [*s. l.*], v. 217, p. 144–151, 2019.

KLEIN, M. P. *et al.* Chitosan crosslinked with genipin as support matrix for application in food process: Support characterization and β-d-galactosidase immobilization. **Carbohydrate Polymers**, [*s. l.*], v. 137, p. 184–190, 2016.

KRAJEWSKA, B. Application of chitin- and chitosan-based materials for enzyme immobilizations: A review. **Enzyme and Microbial Technology**, [*s. l.*], v. 35, n. 2–3, p. 126–139, 2004.

KUMARIHAMI, H. M. P. C. *et al.* Application of chitosan as edible coating to enhance storability and fruit quality of Kiwifruit: A Review. [*S. l.*]: Elsevier B.V., 2022.

LIMA, P. C. *et al.* β-galactosidase from Kluyveromyces lactis in genipin-activated chitosan: An investigation on immobilization, stability, and application in diluted UHT milk. **Food Chemistry**, [*s. l.*], v. 349, n. January, 2021.

LIU, Y. *et al.* Papain immobilization on interconnected-porous chitosan macroparticles: Application in controllable hydrolysis of egg white for foamability improvement. **Food Hydrocolloids**, [*s. l.*], v. 139, n. February, p. 108551, 2023. Disponível em: https://doi.org/10.1016/j.foodhyd.2023.108551.

LOHIYA, G.; KATTI, D. S. Carboxylated chitosan-mediated improved efficacy of mesoporous silica nanoparticle-based targeted drug delivery system for breast cancer therapy. **Carbohydrate Polymers**, [*s. l.*], v. 277, p. 118822, 2022. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S0144861721012091.

MA, H. F. *et al.* Chitosan crosslinked with genipin as supporting matrix for biodegradation of synthetic dyes: Laccase immobilization and characterization. **Chemical Engineering Research and Design**, [*s. l.*], v. 132, n. 35, p. 664–676, 2018. Disponível em: https://doi.org/10.1016/j.cherd.2018.02.008.

MA, J. *et al.* Terrestrial insects as a promising source of chitosan and recent developments in its application for various industries. **Food Chemistry**, [*s. l.*], v. 373, p. 131407, 2022.

MENG, X. *et al.* Chitosan/alginate/hyaluronic acid polyelectrolyte composite sponges crosslinked with genipin for wound dressing application. **International Journal of Biological Macromolecules**, [*s. l.*], v. 182, p. 512–523, 2021.

MI, F. L.; SHYU, S. S.; PENG, C. K. Characterization of ring-opening polymerization of genipin and pH-dependent cross-linking reactions between chitosan and genipin. **Journal of Polymer Science, Part A: Polymer Chemistry**, [*s. l.*], v. 43, n. 10, p. 1985–2000, 2005.

MIRAS, J. *et al.* pH-responsive chitosan nanofilms crosslinked with genipin. **Colloids and Surfaces A: Physicochemical and Engineering Aspects**, [*s. l.*], v. 616, n. October 2020, p. 126229, 2021. Disponível em: https://doi.org/10.1016/j.colsurfa.2021.126229. Acesso em: 28 fev. 2023.

MUZZARELLI, R. A. A. Genipin-crosslinked chitosan hydrogels as biomedical and pharmaceutical aids. **Carbohydrate Polymers**, [*s. l.*], v. 77, n. 1, p. 1–9, 2009. Disponível em: http://dx.doi.org/10.1016/j.carbpol.2009.01.016.

MUZZARELLI, R. A. A. *et al.* Physical properties imparted by genipin to chitosan for tissue regeneration with human stem cells: A review. **International Journal of Biological Macromolecules**, [*s. l.*], v. 93, p. 1366–1381, 2016. Disponível em: http://dx.doi.org/10.1016/j.ijbiomac.2016.03.075.

NATH, S. D. eb *et al.* Chitosan-hyaluronic acid polyelectrolyte complex scaffold crosslinked with genipin for immobilization and controlled release of BMP-2. **Carbohydrate polymers**, [*s. l.*], v. 115, p. 160–169, 2015. Disponível em: http://dx.doi.org/10.1016/j.carbpol.2014.08.077.

OUERGHEMMI, S. *et al.* Synthesis and characterization of polyampholytic aryl-sulfonated chitosans and their in vitro anticoagulant activity. **Carbohydrate Polymers**, [*s. l.*], v. 196, n. May, p. 8–17, 2018. Disponível em: https://doi.org/10.1016/j.carbpol.2018.05.025.

PHADUNGCHAROEN, N. *et al.* Facile and green fabrication of biocatalytic chitosan beads by one-step genipin-mediated β-glucosidase immobilization for production of bioactive genistein. **Sustainable Chemistry and Pharmacy**, [*s. l.*], v. 14, 2019.

PINTO, R. V. *et al.* Glutaraldehyde-crosslinking chitosan scaffolds reinforced with calcium phosphate spray-dried granules for bone tissue applications. **Materials Science and Engineering C**, [*s. l.*], v. 109, 2020.

QU, B.; LUO, Y. Chitosan-based hydrogel beads: Preparations, modifications and applications in food and agriculture sectors – A review. **International Journal of Biological Macromolecules**, [*s. l.*], v. 152, p. 437–448, 2020. Disponível em: https://doi.org/10.1016/j.ijbiomac.2020.02.240.

SABAB, A. *et al.* The potential of chitosan-based haemostats for use in neurosurgical setting – Literature review. **Journal of Clinical Neuroscience**, [*s. l.*], v. 94, p. 128–134, 2021. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S0967586821005257.

SADIQ, A. C. *et al.* A decade development in the application of chitosan-based materials for dye adsorption: A short review. [*S. l.*]: Elsevier B.V., 2021.

SADY, S. *et al.* Quality assessment of innovative chitosan-based biopolymers for edible food packaging applications. **Food Packaging and Shelf Life**, [*s. l.*], v. 30, 2021.

SHELDON, R. A.; VAN PELT, S. Enzyme immobilisation in biocatalysis: why, what and how. **Chemical Society Reviews**, [*s. l.*], v. 42, n. 42, p. 6223–6235, 2013. Disponível em: http://xlink.rsc.org/?DOI=C3CS60075K.

TAVARES, L. *et al.* Effect of deacetylation degree of chitosan on rheological properties and physical chemical characteristics of genipin-crosslinked chitosan beads. **Food Hydrocolloids**, [*s. l.*], v. 106, p. 105876, 2020. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S0268005X19322799.

VIVEK, R. *et al.* PH-responsive drug delivery of chitosan nanoparticles as Tamoxifen carriers for effective anti-tumor activity in breast cancer cells. **Colloids and Surfaces B: Biointerfaces**, [*s. l.*], v. 111, p. 117–123, 2013.

YANG, Y. *et al.* Nerve conduits based on immobilization of nerve growth factor onto modified chitosan by using genipin as a crosslinking agent. **European Journal of Pharmaceutics and Biopharmaceutics**, [*s. l.*], v. 79, n. 3, p. 519–525, 2011.

ZAFEIRIS, K. *et al.* Additive manufacturing of hydroxyapatite–chitosan–genipin composite scaffolds for bone tissue engineering applications. **Materials Science and Engineering C**, [*s. l.*], v. 119, 2021.

ZUBAREVA, A. *et al.* Penetration and toxicity of chitosan and its derivatives. **European Polymer Journal**, [*s. l.*], v. 93, n. October 2016, p. 743–749, 2017. Disponível em: http://dx.doi.org/10.1016/j.eurpolymj.2017.04.021.

APPENDIX 3

Collaborations

Beyond the studies already presented, were stablished some collaborations with other fellow colligates and research groups. From those collaborations, 4 articles were published:

- Development of a biocomposite based on alginate/gelatin crosslinked with genipin for B-galactosidase immobilization: Performance and characteristics.

  Camila Regina Hackenhaar, Carolina Flores Rosa, **Elí Emanuel Esparza Flores**, Patricio Román Santagapita, Manuela Poletto Klein, Plinho Francisco Hertz.

  Carbohydrate Polymers

  Volume 291, 1 September 2022, 119483

  DOI: 10.1016/j.carbpol.2022.119483

- High performance biocatalyst based on β-D-galactosidase immobilized on mesoporous silica/titania/chitosan material.

  Natália Carminatti Ricardi, Leliz Ticona Arenas, Edilson Valmir Benvenutti, Ruth Hinrichs, **Elí Emanuel Esparza Flores**, Plinho Francisco Hertz, Tania Maria Haas Costa

  Food Chemistry

  Volume 359, 15 October 2021, 129890

  DOI: 10.1016/j.foodchem.2021.129890

- Batch synthesis of galactooligosaccharides from co-products of milk processing using immobilized β-galactosidase from *Bacillus circulans*

  Camila Regina Hackenhaar, Luiza Strapasson Spolidoro, **Elí Emanuel Esparza Flores**, Manuela Poletto Klein, Plinho Francisco Hertz

  Volume 36, September 2021, 102136

  DOI: 10.1016/j.bcab.2021.102136

- Effect of deacetylation degree of chitosan on rheological properties and physical chemical characteristics of genipin-crosslinked chitosan beads

  Loleny Tavares, **Elí Emanuel Esparza Flores**, Rafael Costa Rodrigues, Plinho Francisco Hertz, Caciano Pelayo Zapata Noreña

  Food Hydrocolloids

  Volume 106, September 2020, 105876

  DOI: 10.1016/j.foodhyd.2020.105876

Also, was established a collaboration with the laboratory of organometallic chemistry from the Autonomous University of Aguascalientes (México) for the development of the project: PIT20-3C, "*Fabricación de un prototipo para la desinfección de aire de interiores para diminuir la propagación de enfermedades respiratórias*". The participants of this project are:

- Autonomous University of Aguascalientes (México): Profa. Dra. Iliana Ernestina Medina Ramírez (project coordinator), Profr. Dr. David Masouka Ito, Profa. Dra. Yolanda Romo Lozano and Prof. Dr. Juan Jáuregui Rincón.
- Federal University of Rio Grande do Sul (Brasil): Prof. Dr. Plinho F. Hertz, Prof. Dr. Rafael C. Rodrigues e **MCTA Elí Emanuel Esparza Flores**.
- City University (Hong Kong): Prof. Dr. Juan Antonio Zaipen.

Our contribution was the fabrication of new materials based on perlite covered with chitosan crosslinked with genipin and titanium oxide, copper, and silver nanoparticles ($Ag@TiO_2$-Cu^{2+}) incorporated to the polymeric matrix. This material will be characterized and tested for its antimicrobial capacity against *E. coli* and *S. aureus*, afterwards will be developed an air filter prototype.