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Hidrólise da quitosana: obtenção de um extrato enzimático e caracterização do produto hidrolisado

Belém - PA

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CLEIDIANE GONÇALVES E GONÇALVES

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RESUMO

A quitina, amplamente encontrada em exoesqueletos de crustáceos, insetos e micro-organismos, possui utilidade limitada devido sua baixa solubilidade em solução aquosa, sendo necessário sua desacetilação parcial para obtenção da quitosana. A despolimerização da quitosana tem atraído atenção considerável, pois seus oligômeros apresentam alta solubilidade em água, biocompatibilidade e não toxicidade, além de propriedades benéficas, tais como, antimicrobiana, antioxidante e antitumoral. Por esse motivo, nesse estudo, foi produzido um artigo de revisão (Capítulo I) baseado nos principais métodos de hidrólise da quitosana, além de analisar os parâmetros que influenciam na obtenção e características dos produtos de hidrólise, de forma eficaz e com menor custo. Entre os métodos estudados, a hidrólise enzimática se destacou por ser de fácil controle e atuar sob condições mais brandas, sendo possível utilizar enzimas de baixo custo pertencentes ao grupo dos glicosídeos hidrolases. Deste modo, foi definida a hidrólise enzimática como técnica para obtenção de quitosana de tamanhos variados (Capítulo II) por meio da produção de um extrato enzimático (extrato enzimático integral - EEI) a partir de uma linhagem de fungo filamentoso. A identificação das enzimas presentes no EEI deu destaque para exo-quitinases, endo-quitinase e celobiohidrolase. Considerando as mesmas condições reacionais, o EEI apresentou maior eficiência em comparação com a enzima comercial (Celluclast 1,5 L®), que foi utilizada como parâmetro devido ser uma enzima capaz de clivar a ligação β-1,4-glicosídica da quitosana - similar à quitosanase, além apresentar menor custo. O EEI reduziu o peso molecular da quitosana em 47,80; 75,24 e 93,26 % em 2,0; 5,0 e 24 h, respectivamente. Através da análise de FTIR, foi observado menor absorbância dos sinais espectrais dos oligômeros de quitosana e a cristalinidade foi reduzida a partir do tempo de 3,0 h de hidrólise. Com base nesse estudo, podemos inferir que a hidrólise enzimática, nas condições estabelecidas, foi eficaz para a obtenção de quitosana de menor peso molecular utilizando extrato bruto não purificado.

Palavras-chave: quitosana, hidrólise, fungo filamentoso, enzimas.

ABSTRACT

Chitin, extensively found in crustaceans exoskeletons, insects, and microorganisms, has limited usage due to its low solubility in aqueous solution, requiring its partial deacetylation to obtain chitosan. Chitosan's depolymerization has attracted considerable attention, as its oligomers have high water solubility, biocompatibility, and non-toxicity, as well as beneficial properties such as antimicrobial, antioxidant, and antitumor properties. For this reason, in this research, a review article was produced (Chapter I) based on the main methods of chitosan hydrolysis, besides analyzing the parameters that influence the acquisition, and characteristics of hydrolysis results, effectively and at a lower cost. Among the approaches studied, enzymatic hydrolysis excels due to its control ease and performance under milder conditions, making it possible to use low-cost enzymes belonging to the glycoside hydrolases group. Thus, enzymatic hydrolysis was defined as a technique for various sizes of chitosan acquisition (Chapter II) through the production of an enzymatic extract (integral enzymatic extract - IEE) from a filamentous fungus strain. The enzyme identification present in the IEE showed exo-chitinases, endo-chitinase, and cellobiohydrolase. Considering the same reaction conditions, the IEE showed greater efficiency than the commercial enzyme (Celluclast 1.5 L®), which was used as a parameter because it is an enzyme capable of cleaving the β-1,4-glycosidic bond of chitosan - similar to chitosanase, besides presenting a lower cost. The IEE reduced the molecular weight of chitosan by 47.80; 75.24 and 93.26% at 2.0; 5.0, and 24 h, respectively. Through the FTIR analysis, a lower absorbance of the spectral signals of chitosan oligomers was detected, and the crystallinity reduced after 3.0 h of hydrolysis. Based on this study, we can infer that enzymatic hydrolysis, under established conditions, is effective at obtaining lower molecular weight chitosan using unpurified crude extract.

Keywords: chitosan, hydrolysis, filamentous fungus, enzymes.

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ESTRUTURA DA TESE

O presente estudo foi estruturado em 2 capítulos descrito a seguir:

Capítulo I – "Produção de quitosana de baixo peso molecular e quito-oligossacarídeo (COS): uma revisão": esse capítulo é um artigo de revisão sobre os principais métodos de hidrólise da quitosana (ácida, radiação gama, micro-ondas, oxidativa e enzimática).

Capítulo II – "Obtenção de oligômeros de quitosana a partir de extrato enzimático integral produzido por gênero Aspergillus": este capítulo teve como objetivo a obtenção de quitosana de baixo peso molecular a partir de um extrato enzimático produzido por fungo filamentoso.

1 INTRODUÇÃO GERAL

A quitina é o segundo polissacarídeo mais abundante na natureza, após a celulose, constituído principalmente de unidades de N-acetil-D-glicosamina (GlcNAc). Seu valor econômico está associado a sua atividade biológica, aplicações industriais e biomédicas. As principais fontes de quitina são principalmente os crustáceos, com destaque para o camarão e caranguejo, além de ser encontrada também, em menor quantidade, em insetos e micro-organismo (KUMARI, et al., 2015).

Apesar da quitina ser abundante e ter características funcionais excepcionais, como biocompatibilidade, bioatividade, biodegradabilidade e alta resistência mecânica, ela possui utilidade limitada devido à sua fraca solubilidade. Isso faz com que a quitina não seja muito útil e seja utilizada a quitosana, principal derivada da quitina (BEDIAN et al., 2017).

A quitosana é um polissacarídeo linear, obtido pela desacetilação parcial da quitina em condições alcalinas, que contém copolímeros de D-glicosamina (unidades desacetiladas) e N-acetil-D-glucosamina (unidades acetiladas) interligados por ligações glicosídicas β (1→4). (HAMED; ÖZOGUL e REGENSTEIN, 2016).

A quitosana é solúvel em soluções ácidas e nessas condições seus grupos amina tornam-se protonados, melhorando a sua atividade antibacteriana em uma ampla faixa de micro-organismos, incluindo bactérias gram-negativas, bactérias gram-positivas e fungos (GOY; BRITTO e ASSIS, 2009; SAMAR et al., 2013, YOUNES et al., 2014).

Devido aos seus grupos funcionais, este biopolímero também exibe atividade antioxidante, incluindo capacidade de eliminação de radicais hidroxila e capacidade quelante de íons ferrosos (YEN; YANG e MAU 2008; SAMAR et al., 2013; YOUNES et al., 2014). A quitosana também exibe atividade anticancerígena (YOUNES et al., 2014; SRINIVASAN; VELAYUTHAM e RAVICHANDRAN, 2018) e tem grande potencial como conservante de alimentos para prolongar a vida útil dos produtos de peixe (BONILLA et al., 2018).

As propriedades da quitosana podem ser afetadas pelo seu grau de desacetilação e peso molecular (LIU; XIA e ZHANG, 2008). A despolimerização da quitosana tem atraído atenção considerável e os produtos obtidos apresentam alta solubilidade em água, biocompatibilidade e não toxicidade. A quitosana com grau de polimerização menor que 20 e peso molecular menor que 3900 Da são chamados oligômeros de quitosana, quito-oligômeros ou quito-oligossacarídeos (KIM et al., 2013).

A hidrólise ácida surgiu como o método conveniente para despolimerizar polissacarídeos. No entanto esse método apresenta algumas desvantagens, incluindo a dificuldade em obter oligossacarídeos com baixo grau de polimerização e controlar a extensão

da hidrólise, o que resulta frequentemente em hidrolisados contendo uma proporção elevada de monossacarídeos. Além disso, as condições de reação exigidas, temperaturas elevadas e altas concentrações de reagentes, podem causar problemas ambientais (KIM e RAJAPAKSE, 2005).

Além da hidrólise ácida (SANTOSO et al., 2020), a forma de obtenção dos oligômeros de quitosana podem ser por hidrólise oxidativa (XING et al., 2017), micro-ondas (HE et al., 2018), radiação gama (MULEY et al., 2019) e enzimática (XU et al., 2020).

O uso de enzimas, na despolimerização da quitosana, tem recebido grande interesse devido ao fato de não agredir o meio ambiente, ser de fácil controle e apresentar menor variação na obtenção dos oligossacarídeos em comparação com a despolimerização química (SÁNCHEZ et al., 2017). O método enzimático tem vantagem sobre as reações químicas devido as enzimas atuarem sob condições mais brandas, apresentarem alta especificidade e não modificarem a estrutura do anel de glicose (LI et al., 2005). No entanto, esse método tem as desvantagens de apresentar alto custo, disponibilidade limitada de enzimas adequadas, além da ação lenta em soluções de polímero viscoso, para isso são necessárias baixas concentrações de substrato e maiores quantidade de enzimas caras (SÁNCHEZ et al., 2017).

A quitosanase e a quitinase são as enzimas específicas responsáveis pela hidrólise da ligação glicosídica ligada ao β-1,4 no interior da cadeia da quitosana, liberando uma mistura de quito-oligossacarídeos (VIENS; LACOMBE-HARVEY e BRZEZINSKI, 2015). No entanto, são enzimas caras e sua disponibilidade é limitada, dessa forma, enzimas que não possuem substratos específicos e têm maior disponibilidade, podem ser úteis. A papaína, a lisozima e a celulase são enzimas de baixo custo e podem clivar a ligação β-1,4-glicosídica da quitosana (LIN; LIN e CHEN, 2009).

A maioria das celulases produzidas por diferentes tipos de micro-organismos podem degradar a quitosana devido serem enzimas promíscuas. Isso se deve a semelhança estrutural de quitina, quitosana e celulose, ou seja, todos são polímeros de D-glicose ligados por ligações β-1,4-glicosídicas (XIA; LIU e LIU, 2008).

Dessa forma, o objetivo do presente trabalho foi obter um extrato enzimático bruto a partir de um fungo filamentoso e empregá-lo na hidrólise da quitosana para produzir oligômeros funcionais, comparando sua eficiência com a celulase comercial.

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3 OBJETIVOS

3.1 OBJETIVO GERAL

Utilizar extrato enzimático integral obtido a partir de fungo filamentoso para produzir oligômeros de quitosana.

3.2 OBJETIVOS ESPECÍFICOS

- Produzir um artigo de revisão referente às diferentes técnicas de hidrólise da quitosana e a sua influência no rendimento, característica do produto e eficiência do processo;
- Obter extrato enzimático integral através de fungo filamentoso;
- Identificar as enzimas presentes no extrato;
- Comparar a eficiência do extrato enzimático integral e da celulase comercial na hidrólise da quitosana;
- Caracterizar a quitosana hidrolisada por meio dos espectros FTIR;

HIPÓTESE

Este estudo baseia-se na hipótese de que é possível produzir um extrato enzimático integral por meio de um fungo filamentoso com capacidade de hidrolisar a quitosana, com eficiência equivalente a uma enzima comercial.

CAPÍTULO I

Production of low molecular weight chitosan and chitooligosaccharides (COS): A Review

Autores: Cleidiane Gonçalves, Nelson Ferreira and Lúcia Lourenço

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Review

Production of Low Molecular Weight Chitosan and Chitooligosaccharides (COS): A Review

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Abstract: Chitosan is a biopolymer with high added value, and its properties are related to its molecular weight. Thus, high molecular weight values provide low solubility of chitosan, presenting limitations in its use. Based on this, several studies have developed different hydrolysis methods to reduce the molecular weight of chitosan. Acid hydrolysis is still the most used method to obtain low molecular weight chitosan and chitooligosaccharides. However, the use of acids can generate environmental impacts. When different methods are combined, gamma radiation and microwave power intensity are the variables that most influence acid hydrolysis. Otherwise, in oxidative hydrolysis with hydrogen peroxide, a long time is the limiting factor. Thus, it was observed that the most efficient method is the association between the different hydrolysis methods mentioned. However, this alternative can increase the cost of the process. Enzymatic hydrolysis is the most studied method due to its environmental advantages and high specificity. However, hydrolysis time and process cost are factors that still limit industrial application. In addition, the enzymatic method has a limited association with other hydrolysis methods due to the sensitivity of the enzymes. Therefore, this article seeks to extensively review the variables that influence the main methods of hydrolysis: acid concentration, radiation intensity, potency, time, temperature, pH, and enzyme/substrate ratio, observing their influence on molecular weight, yield, and characteristic of the product.

Keywords: hydrolysis; chitosan; molecular weight; chitooligosaccharides

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1. Introduction

Chitin is the second most abundant polysaccharide in nature after cellulose. Chitin is found mainly in the exoskeleton of crustaceans and insects, in addition to bacteria, fungi, and mushrooms [1]. The partial deacetylation of chitin promotes the attainment of chitosan, and the difference between them is in the acetyl group. Chitin contains mainly units of N-acetyl-D-glucosamine (GlcNAc), while chitosan consists mainly of D-glucosamine (GlcN). The units that form both chitin and chitosan

are linked by β (1 → 4) glycosidic bonds, Figure 1. In this context, it is understood that the greater the number of deacetylated units (GlcN) in chitosan, the greater the degree of deacetylation [1,2].

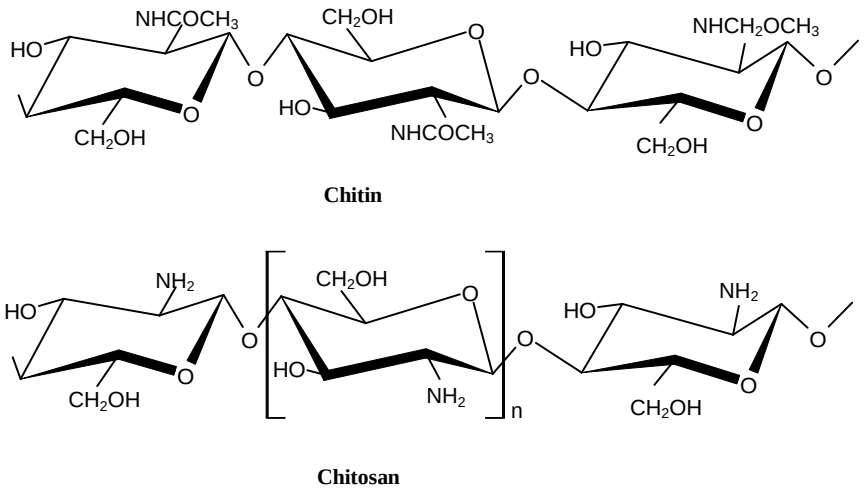

Figure 1. Chemical structure of chitin and chitosan. Reprinted from Vo et al. [1] with permission from John Wiley and Sons and Copyright Clearance Center.

Chitosan has received attention as a functional biopolymer due to its cationic nature, biocompatibility, biodegradability, non-toxicity, and adsorption properties [2]. Its main characteristics are the molecular weight (Mw) and the degree of acetylation (DA) or degree of deacetylation (DDA), which correspond to the molar fractions of GlcNAc and GlcN. Most commercial chitosans have molecular weights ranging from 50–2000 kDa, with an average DDA of 50–100% (commonly 80–90%) [3]. Based on molecular weight, chitosan can be grouped into low molecular weight (<100 kDa), medium molecular weight (100–1000 kDa), and high molecular weight (>1000 kDa) [4].

The high molecular weight and the high degree of polymerization (DP) of chitosan result in low solubility at neutral pH. The high viscosity in solution is the main limitation in the food, cosmetics, agriculture, and health industry [5]. Therefore, to obtain chitosan with a more uniform molecular size and easy solubility, it is necessary to convert chitosan into oligomers. Chitosan with DP <20 and a molecular weight less than 3.9 kDa is called chitosan oligomers, chitooligomers, or chitooligosaccharides (COS) [3].

Chitooligosaccharides are the products of the hydrolysis of chitosan, and because they are soluble in water, they have several applications, such as antimicrobial, antioxidant, anti-tumor, and agricultural purposes [6–12].

Oligomers obtained from the hydrolysis of chitosan can be classified into homo-chitooligosaccharides, which are formed exclusively by GlcN or GlcNAc units, and also hetero-chitooligosaccharides. These latter are formed by units of GlcN

and GlcNAc with varying degrees of deacetylation. Additionally, they can have different degrees of polymerization (number of monomer units within an oligomer) [13]. Regarding solubility, hetero-chitooligomers with DP <10 are considered soluble in water. However, water solubility with DP greater than 10 depends on the degree of deacetylation and the pH solution [14].

The way to obtain chitooligosaccharides can be by acid hydrolysis [4,15], oxidative [16,17], microwaves [18,19], gamma radiation [11,20], and enzymatic methods [21,22]. These hydrolysis methods provide chitosan with different molecular weights and degrees of deacetylation, which influences its composition, yield, and functionality. These methods have advantages and limitations, described in Figure 2.

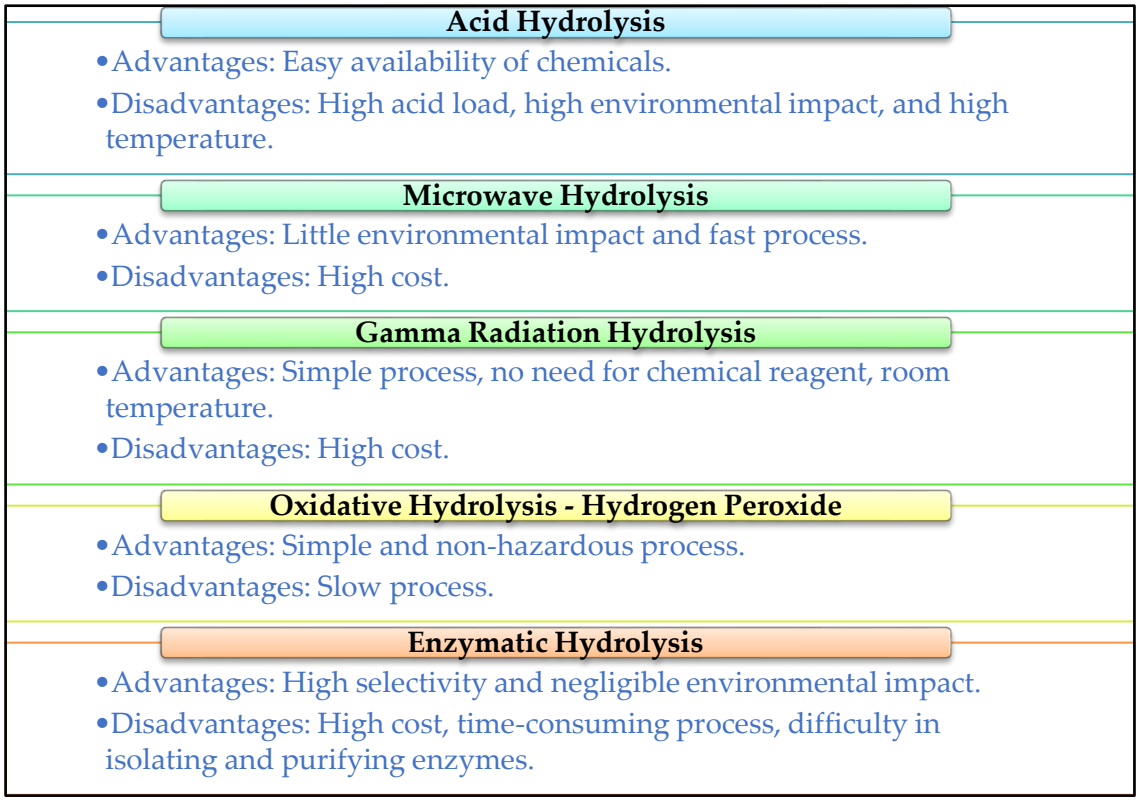

Figure 2. Advantages and disadvantages of the main chitosan hydrolysis methods.

Among hydrolysis techniques, acid hydrolysis has emerged as a convenient method for depolymerizing polysaccharides. However, acid hydrolysis generally requires severe treatment, as the rigid crystalline region in the chitosan granules inhibits acid penetration. For this reason, a high concentration of acid, ranging from 5 to 12 M, is used in most studies [9,23–25]. However, excessive acid loading can cause glucosamine degradation, which significantly reduces yield and generates major waste deposition problems [15,26]. In recent studies, a smaller amount of acid only made it possible to obtain medium molecular weight chitosan in a short hydrolysis time [4]. In high time, low molecular weight chitosan and chitooligosaccharides were obtained. However, yield decreased due to soluble products such as glucosamine

monomers and dimers [15,27]. Therefore, current studies seek to develop the technique using different types and concentrations of acids associated with ionic liquids, induced electric fields, or other hydrolysis methods.

Oxidative hydrolysis, using hydrogen peroxide, is considered an easy and non-dangerous method. Because it is a slow method, most studies use this type of hydrolysis associated with other techniques [17,20]. In addition, to reduce the production cost, process variables are also studied [12,16].

The hydrolysis of chitosan by radiation has gained considerable attention because it is a relatively simple process, does not need to use a chemical reagent, is carried out at room temperature, and can be applied on a large scale [8,28]. This type of hydrolysis can have a high production cost if high doses of radiation are used. In this case, studies seek to use radiation associated with other reagents, especially hydrogen peroxide and acetic acid, to increase the efficiency of the process [11,29].

Otherwise, microwave hydrolysis is considered less efficient when compared to other high-energy radiation, such as ultraviolet and gamma. However, due to the lower environmental impact, microwave hydrolysis presents a viable alternative to obtain polysaccharides with molecular weights of interest [30]. In addition, due to the cost of the process, it is necessary to use less power and less hydrolysis time. For this, studies have associated this method with the use of hydrochloric acid, acetic acid, or ionic liquid [17–19]. However, it is necessary to be careful with the addition of these solvents in high concentrations, as they can generate residues and, consequently, a more significant environmental impact.

Enzymatic hydrolysis has received much attention because of its lower environmental risk. However, despite the enzymatic process taking place under mild conditions, the hydrolysis rate is slow in viscous solutions. Thus, a low substrate concentration (1% m/v) is used in most studies. The low substrate concentration causes an increase in the solution's volume and the need for more enzymes. Additionally, specific enzymes have high prices and limited availability. Alternatively, low-cost enzymes from the hydrolases group can degrade chitosan [31–33].

Enzymatic hydrolysis is the only method that has association restrictions with other types of hydrolysis due to the sensitivity of the enzymes related to pH, high temperature, and radiation. Thus, most studies seek to optimize process variables (enzyme/substrate ratio, temperature, pH, and hydrolysis time) as an alternative to reduce costs [6,22,31,34]. Despite the disadvantages, enzymatic hydrolysis still seems to be the best alternative due to its selectivity. This selectivity favors the obtaining of chitosan chains with a specific average molecular weight [33].

Global interest in chitosan hydrolysis products has been steadily increasing. Based on the references cited in this review,

the diversity of countries in which this theme has been highlighted was verified (Appendix A).

This review will present the main hydrolysis techniques of chitosan and analyze the main factors that influence the obtaining and characteristics of low molecular weight chitosan and chitooligosaccharides effectively at a lower cost.

2. Acid Hydrolysis

The acid hydrolysis of glycosidic bonds involves the following steps: 1) protonation of oxygen in the glycosidic bond through the connection of a proton (H_3O^+) to the glycosidic bond; 2) adding water to the final reducing sugar group; 3) decomposition of protonated glycosidic bonds (Figure 3). In this case, catalytic protons may be present in the water contained in the samples, and the protonated amino group of chitosan is also likely to act as a proton donor in catalysis [24].

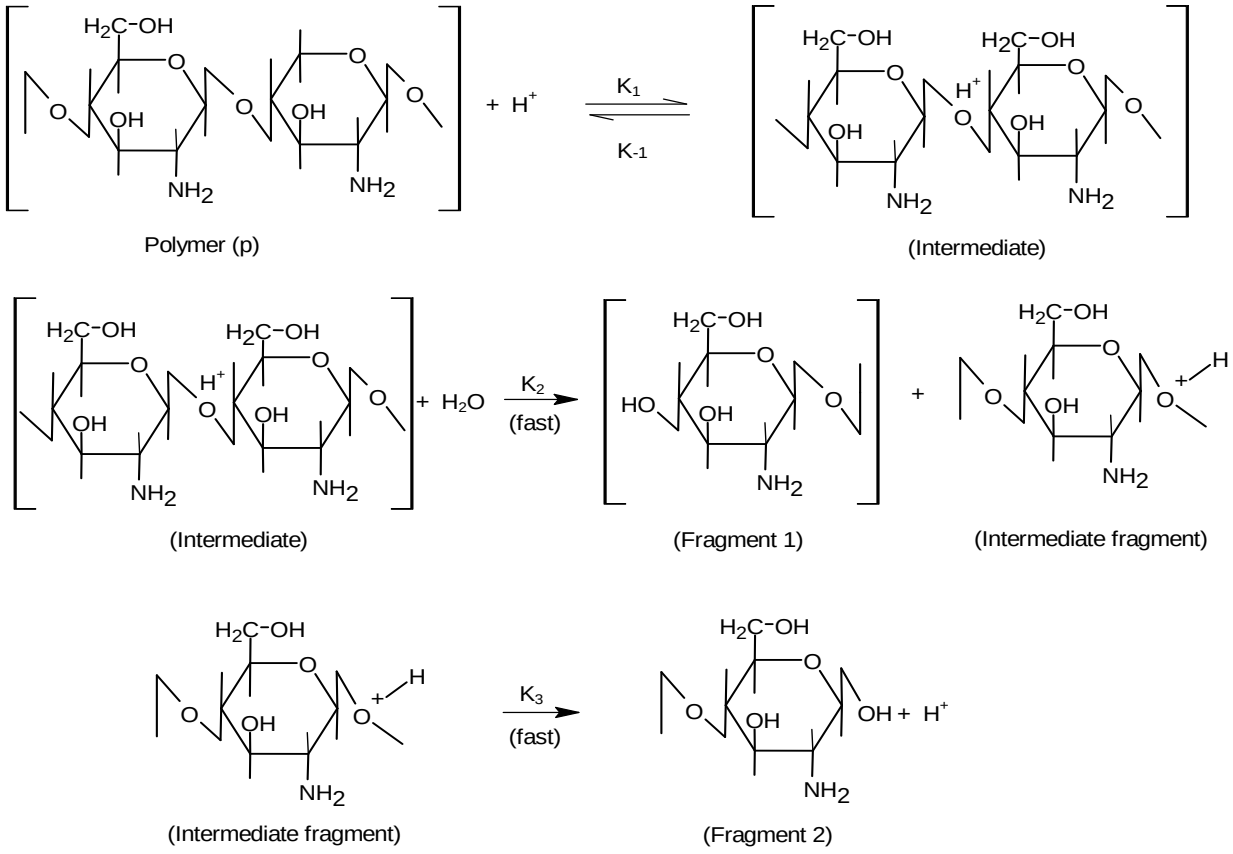

Figure 3. Mechanism proposed for acid hydrolysis of chitosan. Reprinted from Kasaai et al. [24] with permission from Creative Commons Attribution.

2.1. Principal Acids for Hydrolysis

Acid hydrolysis is commonly performed by hydrochloric (HCl), acetic (CH_3COOH), phosphoric (H_3PO_4), sulfuric (H_2SO_4), nitric (HNO_3), and lactic ($C_3H_6O_3$) acids (Table 1).

Table 1. Parameters for chitosan acid hydrolysis.

Reference	Chitosan	Solvent	Solvent Concentration	Temperature	Time	Mw/DP
[4]	5%	CH_3COOH	1–5%	30–60 °C	30–90 min	166.3–592.8 kDa
[9]	3.33%	HCl	6 M	70 °C	2 h	2–12 DP
[15]	0.8%	HCl	0.15 and 1.5 M	25–45 °C	0–60 h	20–90 kDa
[23]	2%	HCl	6.27 M	56 °C	3 h	-
[24]	1%	HCl	0.1–5 M	65 °C	13–2160 min	73.8–1076 kDa
[25]	0.5%	HCl	8, 10, and 12 M	90 and 105 °C	0–10 h	-
[26]	255 mg	([C4mim] Cl [1] or [C4mim]Br [2]) + (H_2SO_4 or HCl or HNO_3) + (Water)	(4g) + (290–600 mg)+ (27–108 mg)	100 °C	190–540 min	-
[27]	1%	HCl	2 M	-	0.5–24 h	1.5–29 kDa
[35]	1%	$C_3H_6O_3$	1%	8, 22, and 37 °C	10, 20, 30, 60, and 90 days	-
[36]	5%	H_3PO_4	85%	Ambient; (40, 60, and 80 °C)	35 days; (1–15 h)	19–164 kDa
[37]	0.2%	HCl:H_3PO_4	100:0, 75:25, 50:50, 25:75, 0:100 (6M H^+) 75:25 (4, 6, 8, and 10 M H^+)	110 °C	0–36 h	-

[1] [C4mim]Cl: 1-butyl-3-methylimidazolium chloride; [2] [C4mim]Br: 1-butyl-3-methylimidazolium bromide.

Among the acids studied, HCl is the most widely used due to its efficiency in the hydrolysis of the glycosidic bond (depolymerization) and the N-acetyl bond (deacetylation). HCl makes it possible to obtain fractions of the trimer, pentamer, hexamer, and heptamer-decamer. In this sense, chitooligosaccharides with DP ranging from 2 to 12 can be obtained from chitosan with a molecular weight of 658 kDa and 82% DDA, hydrolyzed with 6 M HCl at 70 °C, for 2 h [9].

In the study by Li et al. [15], there was a marked decrease in the molecular mass of chitosan (85.9% DDA) from 230 to 90 kDa in 12 h of hydrolysis with 1.5 M HCl using an induced electric field and temperature variation between 25 to 45 °C. In another study using chitosan of 200 kDa (90% DDA) hydrolyzed with HCl 2 M for 12 h, chitooligosaccharide of 2 kDa was obtained, with a yield of 85.2% [27].

In the kinetic study of acid hydrolysis, chitosan was quantitatively hydrolyzed to glucosamine in 6 h with 10 M hydrochloric acid (HCl) at 105 °C or 12 M HCl at 90 °C [25]. Despite being more used, the hydrolysis of chitosan with hydrochloric acid requires excess acid, complex reactors, and presents problems of deposition of residues. Excessive acid treatments result in the breakdown of glucosamine, which significantly reduces yield [26,37].

Thus, when chitosan is hydrolyzed in an HCl-H_3PO_4 solution, 75:25 in a molar ratio, a significantly higher efficiency (p <0.05) is

verified compared to HCl. This efficiency can decrease dramatically by increasing the proportion of H_3PO_4 [37]. However, when this comparison is made with other organic acids, hydrochloric and nitric acids require longer reaction times to obtain yields like those catalyzed with sulfuric acid [26].

2.2. Effect of Acid Concentration

In most studies, the types of acids and their concentrations are carefully established to hydrolyze chitosan (break glycosidic bonds) and minimize GlcN degradation (deacetylation). In this sense, the concentrations of sulfuric, hydrochloric, and nitric acids are directly proportional to the concentration of total reducing sugars, which are products of the hydrolysis of chitosan [26].

The concentration of the acid is also directly proportional to the rate of hydrolysis. Chitosan with a medium molecular weight (198.64 kDa) was obtained from hydrolysis with 5% acetic acid for 30 min. At concentrations of 1% and 3%, the molecular weight was 592.89 and 281.98 kDa, respectively [4]. In this case, it is observed that the lower acid concentration and short hydrolysis time enabled the production of only medium molecular weight chitosan.

In another study with HCl-H_3PO_4 acid solution (maintaining the molar ratio of 75:25) and 4 M [H +], the yield of 64.6% was observed due to incomplete hydrolysis of chitosan. Otherwise, 6 M [H +] showed an ideal recovery of 98.8%. However, by increasing the concentration to 9 M [H +], 90.3% was obtained due to the degradation of GlcN [37]. In this case, the increase in the acid concentration favored hydrolysis, but there is an upper limit to prevent GlcN degradation.

An alternative to decrease the high acid concentration is the association with other solvents, such as ionic liquids. The depolymerization of chitosan carried out by hydrochloric acid in the presence of ionic liquids shows a higher yield of total reducing sugars when compared to an aqueous system using only hydrochloric acid [26]. Additionally, chitosan is highly dissolved in ionic liquids, which facilitates the hydrolysis process [38,39].

2.3. Effect of Time

Studies have shown that increasing the hydrolysis time decreases the molecular weight, resulting in low molecular weight chitosan and chitooligosaccharides in the first hours. Even so, it is necessary to use a high concentration of acid. When using chitosan with a molecular weight of 214 kDa (96% DDA), hydrolyzed in 85% phosphoric acid solution (60 °C), a high decrease was obtained in the first 4.0 h (74 kDa), and, after 15 h, molecular weight reached 19 kDa [36]. In another study with a 2 M HCl solution, the molecular weight of chitosan was reduced from 200 to 29, 17, and 3.0 kDa in 30 min, 1.0 h, and 5.0 h, respectively [27]. In chitosan 2038 kDa, hydrolyzed in 5 M HCl at

65 °C, the molecular weight after 5.0 h was 156 kDa, reaching 74 kDa in 36 h [24].

In addition, the yield of the hydrolyzate product may decrease when the reaction time is prolonged. In this case, more chitosan is hydrolyzed to soluble products such as glucosamine monomers and glucosamine dimers. When phosphoric acid at 85% is used, the yield in 1 and 15 h is 89.3% and 49.2%, respectively [36]. In a 2 M hydrochloric acid solution, the yield is 92.1% and 80.1% over 0.5 and 24 h, respectively [27]. A longer hydrolysis time in HCl-H_3PO_4 solution (4.5 : 1.5 M) will be necessary to combine the two acids. However, it shows an increase in the relative yield with the hydrolysis time up to 24 h. After that time, glucosamine degrades slightly, decreasing from 97.7% in 24 h to 90.0% in 36 h. In addition to the decrease in yield, the solution may darken with the hydrolysis time for more than 24 h [37].

2.4. Effect of Temperature

The hydrolysis temperature also influences the molecular weight and yield of chitosan. Thus, chitosan with 214 kDa and hydrolyzed in a phosphoric acid solution (85%) at temperatures of 40, 60, and 80 °C for 8.0 h produced low molecular weight chitosan of 37, 35, and 20 kDa, respectively. This shows that the higher the temperature, the lower the molecular weight of chitosan. However, the yield will be lower. In this case, the yield was 86%, 71%, and 61%, respectively [36].

Chitosan of initial molecular weight of 726 kDa (medium molecular weight) and 28 kDa (low molecular weight) hydrolyzed in lactic acid (1%) at temperatures of 8, 22, and 37 °C also showed an increase in the rate of hydrolysis with the increasing temperature. However, there was an influence of the initial molecular weight of chitosan on the hydrolysis rate in this case. This rate increased from 25% to 50% with the increase in temperature in medium molecular weight chitosan. The effect of temperature was less on low molecular weight chitosan. In these conditions, rates of 5% to 10% were observed [35].

When the three most essential variables in acid hydrolysis (acid concentration, time, and temperature of hydrolysis) were evaluated in the same process, the yield of glucosamine reached 98% in the condition of 10 M HCl and 105 °C for 6 h, a recovery also being observed such as using 12 M HCl and 90 °C for 6 h. In this case, it was observed that the acid concentration had a better influence on the reaction [25]. However, when analyzing only the effect of time and temperature of hydrolysis in acetic acid solution, it was observed that these variables have the same influence [4].

2.5. Influence of the Degree of Deacetylation and Initial Molecular Weight of Chitosan

The degree of deacetylation is inversely proportional to the rate of hydrolysis. It was observed that chitosan with a deacetylation degree of 85%, 67%, and 53% showed a decrease in viscosity of 40%, 50%, and 60%, respectively, after 10 days of hydrolysis in 1% lactic acid [35]. This behavior was confirmed in a study with chitosan with an initial molecular weight of 230 kDa and a degree of deacetylation of 86% and 55%. It was observed that after 12 h of hydrolysis, there was a decrease in molecular weight to 90 and 30 kDa, respectively [15].

The initial molecular weight influences the viscosity reduction rate. Chitosan hydrolyzed in 1% lactic acid with medium molecular weight (726 kDa) showed a 90% decrease in viscosity in 180 days. This decrease is most evident during the first 10 days. In low molecular weight chitosan (28 kDa), the viscosity decreases by only 51% in 180 days [35].

2.6. Antimicrobial and Antioxidant Properties of Chitosan Oligomers

The hydrolysis product of chitosan under conditions (HCl 6.7 M; 56 °C; 3 h) had an effect against Gram-positive bacteria, *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 43300, *Bacillus subtilis,* and *Bacillus cereus,* and Gram-negative bacteria, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium*, *Vibrio cholerae*, *Shigella dysenteriae*, *Prevotella melaninogenica,* and *Bacteroides fragilis* [23].

Another characteristic of the product of chitosan hydrolysis is the antioxidant activity. Fractions with different degrees of polymerization were obtained by hydrolysis with 6M HCl. In this sense, trimer, tetramer-pentamer, pentamer, hexameter, and heptamer-decamer showed activity against hydroxyl radicals. The activity was greater with the decrease in the degree of polymerization due to the chito-oligomers having shorter chains and weak intramolecular and intermolecular hydrogen bonds. This fact allows the hydroxyl and free amino groups to be activated, which helps to promote antioxidant activity [9].

3. Gamma Radiation Hydrolysis

The hydrolysis of chitosan by gamma radiation (γ) has gained considerable attention due to its advantages. Among these advantages, there is the possibility of carrying out the process at room temperature and applying it on a large scale [40]. This type of hydrolysis is mainly influenced by the intensity of the radiation dose and the hydrolysis solution (Table 2).

Table 2. Parameters of chitosan hydrolysis by gamma radiation.

Reference	Chitosan	Solvent	Solvent Concentration	Radiation Doses (kGy)	Rate (kGy/h)	Mw (kDa)
[7]	-	$(NH_4)_2S_2O_8$ or $K_2S_2O_8$ or H_2O_2	10%	20 to 200	6.7	130–3,000
[8]	dry/wet/solution	wet (H_2O)/solution (CH_3COOH/H_2O_2)	1%	15 to150	1.02	10–180
[11]	2%	CH_3COOH	1%	25 to 100	-	82.2–337.73
[20]	1:6 (chitosan:water)	H_2O_2	1–5%	6	-	8–14
[28]	2%	CH_3COOH	1%	2, 10 and 20	3.35	2.1–35.2
[29]	5%	CH_3COOH/H_2O_2	0.2 M/1%	0 to 150	3	6–100
[40]	5%	$C_3H_6O_3/H_2O_2$	3%/1%	4 to 16	1.33	2.7–8.6
[41]	2%	CH_3COOH	2%	2 to 200	-	-
[42]	20%	H_2O_2	2%, 10%, and 30%	10 to 100	10	1–300
[43]	0,1–2%	CH_3COOH	0.1 M	0.5 to 200	10	0.97–67

The radiation causes changes in the polymer chain structure, inducing the formation of radicals and breaking the chain. The mechanism of hydrolysis of chitosan by radiation is described by Kim et al. [44]:

$$R\text{–}H \xrightarrow{h\nu} H\cdot\ (C_4\text{–}C_6) + H$$

$$R\text{–}H + H\cdot \rightarrow R\cdot\ (C_1\text{–}C_6) + H_2$$

$$R\cdot\ (C_1\text{–}C_6) \rightarrow F_1\cdot + F_2\ (\text{Scission})$$

$$R\text{–}NH_2 + H\cdot \rightarrow R\cdot\ (C_2) + NH_3$$

where R–N and $R\text{–}NH_2$ are chitosan macromolecules, and R· (Cn) is a chitosan macroradical located on a Cn carbon atom. F1· and F2 are fragments of the main chain after scission.

3.1. Effect of Radiation Intensity

The increase in radiation intensity will directly influence the decrease in the molecular weight of chitosan. Although intensity is an important parameter, there is a limit on the radiation dose to make the process effective.

When 0.5–200 kGy radiation was used, 50 kGy was needed to obtain the chitooligosaccharides with a decrease in molecular weight from 67 to 0.97 kDa [43]. In another study using chitosan irradiated between 2–200 kGy, there was a decrease in viscosity with up to 10 kGy. In this case, the increase in radiation only increased the monomer, dimer, and trimer amounts [41]. In chitosan irradiated with 20–200 kGy, the molecular weight decrease occurred rapidly up to 120 kGy. Subsequently, the rate of degradation was low [7].

In the study by Dung et al. [29], the chitosan of 193 kDa, 80% DDA, showed a decrease in molecular weight to 11.4 kDa with irradiation up to 75 kGy and then slowly decreased with an increase in irradiation up to 150 kGy. Using chitosan 210 kDa after

hydrolysis with radiation of 2, 10.0, and 20 kGy, low molecular weight chitosan and chitooligosaccharide of 35, 6.0, and 2.0 kDa, respectively, were obtained [28].

The variation in the radiation dose required to hydrolyze chitosan is due, among other factors, to the initial molecular weight of chitosan. In chitosan with an initial molecular weight of 338 kDa, 82% DDA, irradiated at 100 kGy, there was a significant decline in molecular weight to 82 kDa [11]. In Choi et al. [41], chitosans with molecular weights of 61 and 110 kDa showed, after hydrolysis with irradiation of 10 kGy, a decrease in viscosity to 99.8% and 95.7%, respectively. These studies show that the initial molecular weight of chitosan is directly proportional to the radiation dose required for hydrolysis.

3.2. Hydrolysis Solution

The intensity of radiation is also influenced by the environment/solution of chitosan during hydrolysis. In aqueous solutions, the influence occurs mainly by water radiolysis due to the formation of transient products that react with the solute. The subsequent reaction of the macroradicals can be chain scission, hydrogen transfer, inter-and intramolecular recombination, and disproportionation macroradicals [43]. These reactions lead to the creation of hydroxyl radicals, which can bind strongly to the β-1-4 glycosidic bonds, favoring hydrolysis [44].

Hydrolysis of chitosan by radiation can occur under different conditions. These conditions can be identified as dry, moist, and in solution (acetic acid/H_2O_2). Chitosan with 220 kDa, 73% DDA, in dry and moist form showed a reduction in molecular weight to 180 and 160 kDa with 15 kGy, respectively. However, from 25 kGy onwards, a similar trend was observed in the reduction of molecular weight. This process was more efficient in the moist form due to the radiolysis of the absorbed water that initiated the fission reactions in the polymer chain. More efficiently, chitosan in H_2O_2 solution showed a significant reduction in molecular weight to 40 kDa, even at lower doses such as 15 kGy. This hydrolysis is enhanced due to the combined action of water radiolysis and H_2O_2, reaching 10 kDa in 150 kGy against 50 kDa of chitosan in the wet form [8].

The same trend was obtained when using chitosan with a molecular weight of 90 kDa, 70% DDA, irradiated in the presence of H_2O_2 (1%). The irradiation showed a highly effective result even in low doses compared to previous studies, with a decrease in molecular weight to 8.6 and 2.7 kDa, with 4 and 16 kGy, respectively. The results indicate that the molecular weight obtained after hydrolysis was much lower when there was a combination of radiation with H_2O_2 [40]. In another study with chitosan 173 kDa, 96.9% DDA, it was also evident that lower radiation of 6 kGy associated with hydrogen peroxide 1%, 3%, and 5% reduced the molecular weight to 14, 11, and 8 kDa,

respectively. Less efficient results of hydrolysis were observed without the addition of H_2O_2 [20].

Chitosan with 400 kDa and 90.5% DDA showed a higher rate of hydrolysis with the combination of gamma radiation (50 kGy) and hydrogen peroxide (2%). The reduction in molecular weight reached 6 kDa and was more efficient when using only radiation. In this case, the molecular weight of the hydrolyzed chitosan was 100 kDa [42].

When the chitosan was hydrolyzed by radiation in the presence of H_2O_2, hydroxyl radicals formed due to the water radiolysis. Hydroxyl radicals are efficient oxidants that react with chitosan to abstract hydrogen linked to carbon. Subsequently, the resulting carbohydrate radicals cause the breakdown of glycosidic bonds by rearrangement, which reduces the molecular weight of chitosan very effectively [29,42].

The use of other initiators can also increase the rate of hydrolysis. Chitosan with 10^7 Da molecular weight, irradiated at 80 kGy, showed a higher rate of hydrolysis in the presence of initiators such as potassium persulfate and ammonium persulfate when compared only with ionizing radiation. In this context, ammonium persulfate was the most effective initiator in hydrolysis in chitosan. It was also observed that the use of ammonium persulfate with 40 kGy decreased the molecular weight by 98%, while the sample hydrolyzed by radiation had a reduction of 25% [7].

Based on these results, it can be inferred that the presence of initiators is an alternative to decrease the radiation dose required to hydrolyze chitosan. As previously mentioned, this dose may be higher with the increase in the initial molecular weight of chitosan. From an economic point of view, high doses of radiation may not be accepted due to the high cost.

3.3. Macroscopic Changes

In addition to the change in molecular weight due to the cleavage of the β-(1-4) glycosidic bond, a positive characteristic of radiation hydrolysis is that the irradiated chitosan maintains its degree of deacetylation, regardless of the radiation load. A study based on chitosan with an initial deacetylation degree of 91.5% shows that deacetylation was 91% and 91.2% at doses of 10 and 20 kGy, respectively [28].

Another characteristic of irradiated chitosan is the change in color from creamy yellow to dark brown when the radiation dose is above 100 kGy. The color change can occur because of the oxidation of chitosan at the molecular level and C = O due to the chain fission reaction [11,41].

3.4. Antioxidant and Antimicrobial Properties of Chitosan Oligomers

The radiation intensity directly influences the antioxidant activity of chitosan due to the change in molecular weight,

because the lower the molecular weight, the greater the antioxidant activity [11]. In a study with different radiation doses between 2 and 20 kGy, the percentage of elimination of hydroxyl radical from 41% to 64% was observed. Additionally, at 20 kGy, the elimination of 74% of the superoxide radical was observed. From these results, radiation at 20 kGy was observed, providing sufficient hydrolysis to increase chitosan activity due to the decrease in molecular weight. In this sense, chitosan with high molecular weight has a compact structure. This compacted structure makes intramolecular hydrogen bonds more effective. Consequently, there will be a decrease in the hydroxyl and amino groups [28].

The microbial activities against Gram-positive and Gram-negative bacteria are also influenced by the molecular weight and, consequently, the radiation intensity. Thus, chitosan with medium and low molecular weight of 220, 120, 75, and 52 kDa have high antibacterial activity with less molecular weight and a higher radiation dose. Positive activity against *Staphylococcus* sp, *Pseudomonas aerogonisa*, *Escherichia coli*, and *Staphylococcus aureus* was evaluated. The main reason may be related to chitosan with lower molecular weight to access the microbial cell more easily and alter cellular metabolism [8].

4. Microwave Hydrolysis

The use of microwave irradiation to accelerate organic reactions has increased interest due to being environmentally positive. This hydrolysis mechanism is a complex process and probably involves two separate paths, such as shear forces induced mechanically by the oscillation of molecules (breaking the main polymer chain) and thermal degradation (heat-induced hydrolysis) [30]. Chito-oligosaccharides formed mainly by disaccharides, trisaccharides, tetrasaccharides, pentasaccharides, and less hexasaccharide can be obtained [17]. This type of hydrolysis is mainly influenced by the power used, solution, and hydrolysis time (Table 3).

4.1. Microwaves versus Conventional Heating

The use of microwave irradiation can reduce the reaction time compared to the conventional method of heating in a thermostatic bath or shaker incubator. Chitosan with an initial molecular weight of 560 kDa, 85% DDA, 25 min, showed a higher hydrolysis rate (105 kDa) with microwaves when compared to conventional heating (270 kDa) at the same temperature (100 °C) for 2 h [47].

In another study with chitosan 66 kDa, 95% DDA, hydrolyzed with 100 W for 20 min and equilibrium temperature of 89 ± 2 °C, chitosan of 26 kDa was obtained. Otherwise, in conventional hydrolysis, in a water bath, at 89 °C, for 20 min, the hydrolysis was significantly lower (54 kDa). These aspects led to the conclusion that thermal effects are not the only degradation

mechanisms involved. The most likely simultaneous process is mechanical shear induced by molecular vibrations [30].

In addition to the reaction time, the product yield is higher in microwave hydrolysis. In the study with microwave irradiation (800 W) in chitosan of 560 kDa, 90% DDA, at 155 °C for 2 min, a yield of reducing sugars of 87% was observed, while in conventional heating, in an oil bath at 150 °C, was 38.9% for 5 h [18].

Table 3. Parameters of chitosan hydrolysis by microwave.

Reference	Chitosan	Solvent	Solvent Concentration	Other Solutions	Power	Time	Temperature	Mw (kDa)
[17]	3%	CH_3COOH; H_2O_2	2%, 1%	-	800 W	25 min	80 °C	1.46
[18]	3%	AmimCl[a]: HmimCl[b]	(9:1)	15 mg (H_2O) + DMSO[c] e SFILs[d]	160, 320, 640, 800 W	30, 60, 90, 120 s	120, 125, 130, 146, and 155 °C	0.45–24
[19]	1%	HCl	3 M	-	80–100 W	5, 10, and 15 min	80 and 100 °C	-
[30]	1%	CH_3COOH	0.1 M	-	10–100 W	5–80 min	-	25–42
[45]	3 e 4%	H_2O_2	5, 10 e 15%	-	700 W	3, 4, and 5 min	-	0.9–1
[46]	-	acidified	-	-	650 or 390 W	10, 20, 30, and 60 min	98–100 °C	79.2–142.2
[47]	-	CH_3COOH or HCl	2%	NaCl, KCl, $CaCl_2$ (0,15 mol/L [Cl^{-1}]	480–800 W	0,5–25 min	100 °C	30–105

[a] AmimCl: 1-Ally-3-methylimidazolium chloride; [b] HmimCl: 1-H-3-methylimidazolium chloride.; [c] DMSO: Dimethyl Sulfoxide.; [d] SFILs: Sulfonic acid-functionalized ionic liquids.

4.2. Hydrolysis Solution

The hydrolysis of chitosan through microwave irradiation can be accelerated by adding inorganic salts. Thus, chitosan with a molecular weight of 5.6×10^5 Da decreased to 10^4 (without adding salt) and 3×10^4 Da (with adding salt). This is due to the salt's ability to cause the solution to overheat. Microwave heat involves direct interaction with certain classes of absorbent molecules that can lead to the introduction of energy and raise the temperature of the solution. Additionally, the addition of salts to solvents can increase conductivity and influence the rate of heating. The presence of salts in polar solvents also improves dielectric loss and the microwave coupling of the solvents [47].

Another way to accelerate the rate of hydrolysis is through the hydrogen peroxide associated with the microwave. In this case, the concentration of hydrogen peroxide is proportional to hydrolysis, obtaining a recovery rate of chitosan dissolved in water of 52% and 92% when using 10% and 15% of this reagent, respectively. Additionally, chitosan with a molecular weight of 220 kDa, 92% DDA, hydrolyzed in a microwave (700 W), in a 15% hydrogen peroxide solution for 4 min, had a molecular weight of

0.9 kDa [45]. When using 658 kDa chitosan, hydrolyzed with 800 W for 25 min, in a 1% hydrogen peroxide solution, chitooligosaccharide with a molecular weight of 1.46 kDa was obtained [17].

Most studies of hydrolysis of chitosan through microwave irradiation use acids to dissolve chitosan. These solvents leave an environmental liability and promote the corrosion of the equipment. However, the hydrolysis of chitosan can be carried out using "green solvents". An alternative is to use ionic liquids as suitable solvents to hydrolyze polysaccharides. Thus, when the chitosan hydrolysis is carried out with microwave irradiation (640 W), using ionic liquids, it is observed that the molecular weight of the chitosan decreases from 560 to 0.88 kDa in 90 s. In this case, the yield of reducing sugars was 84% and can reach 92% in 2 min. Additionally, to reduce the viscosity of the reaction system, dimethyl sulfoxide can be added as a co-solvent, forming a homogeneous solution. Polar materials in a microwave field can quickly reach high temperatures and a higher reaction rate [18].

4.3. Effect of Time

The reaction time also influences the molecular weight of microwave-hydrolyzed chitosan. The hydrolyzed chitosan with 100 W (without additives and initiators) showed a significant decrease in molecular weight from 66 to 42, 34, and 26 kDa in times of 5, 10, and 20 min, respectively. After this time, there was no change in molecular weight [30].

The increase in hydrolysis time with the inorganic salt also favors the decrease in viscosity and consequently in molecular weight [47]. However, the rate of hydrolysis in inorganic salt is low compared to ionic liquids. Chitosan of 560 kDa, 90% DDA, hydrolyzed with microwave (640 W) and ionic liquids showed a decrease in molecular weight, reaching 24, 0.88, and 0.45 kDa for 30, 90, and 120 s, respectively. This indicates that the combination of microwave irradiation in the presence of ionic liquids attacks 1,4-β-glycosidic bonds very efficiently [18].

4.4. Power During Hydrolysis

Another variable that influences the efficiency in breaking glycosidic bonds is the potency during hydrolysis. In chitosan with a molecular weight of 175 kDa, 83% DDA, in the power of 390 W (30 min) and 650 W (10 min), low molecular weight chitosan of 103 and 79 kDa were obtained, respectively [46]. In other studies, with 80–100 [19] and 10–100 W, the same behavior was also observed because a higher power has a higher shear force between molecules, causing a faster decrease in molecular weight [30].

This behavior was also observed in the study with powers of 160, 320, 480, 640, and 800 W, where it was verified that the final temperature reached 120, 125, 130, 146, and 155 °C, respectively,

and the corresponding reducing sugar yields were 35%, 67%, 74%, 93%, and 87%. In this case, the highest power, 800 W, showed a lower sugar yield than the 600 W power attributed to the decomposition of the product, caused by high temperature [18].

5. Oxidative Hydrolysis with Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is used for the hydrolysis of polysaccharides because it is easy to handle, readily available, and environmentally friendly [16]. This method is based on the formation of reactive hydroxyl radicals by dissociating hydrogen peroxide. Hydroxyl radicals, which are robust oxidizing species, can attack β-D- glycosidic bonds (1 → 4), resulting in the hydrolysis of chitosan [48].

The depolymerization of chitosan by hydrogen peroxide provides a breakdown of the 1,4-β-D-glucoside bonds of the polysaccharide chain, leading to a decrease in molecular weight [49]. Chito-oligosaccharides obtained by oxidative hydrolysis are mainly composed of monosaccharides to pentasaccharides. The most significant amount found is disaccharides and trisaccharides, with or without acetylation [17]. Tian et al. [49] present the hydrolysis mechanism described below:

$$R–NH_2 + H^+ \boxed{\leftrightarrow} R–NH_3^+$$

$$H_2O_2 \boxed{\leftrightarrow} H^+ + HOO^-$$

$$H_2O_2 + R–NH_2 + H^+ \boxed{\leftrightarrow} R–NH_3^+ + HOO^- + H^+$$

The hydroperoxide anion is very unstable and is easily decomposed into a highly reactive hydroxyl radical (HO·).

$$HOO^- \rightarrow OH^- + O$$

$$H_2O_2 + HOO^- \rightarrow HO\cdot + O_2^{\cdot -} + H_2O$$

Eventually, the hydroxyl radical (OH ·) attacks the glycosidic bond of chitosan to produce the chitosan oligomer according to the reactions below:

$$(GlcN)_m – (GlcN)_n + HO\cdot \rightarrow (GlcN\cdot)_m – (GlcN)_n + H_2O$$

$$(GlcN\cdot)_m–(GlcN)_n + H_2O \rightarrow (GlcN)_m + (GlcN)_n$$

During hydrolysis, R-NH_2 reacts preferentially with H^+ to produce R-NH_3^+, which causes a decrease in H^+ and an increase in pH. Additionally, HOO^- is rapidly decomposed into HO ·, which means that H_2O_2 is continuously decomposed. These radicals undergo other reactions quickly to form low molecular weight water-soluble oxidation products.

This type of hydrolysis is mainly influenced by the concentration of hydrogen peroxide, time, and temperature of hydrolysis, Table 4.

Table 4. Parameters of chitosan hydrolysis by hydrogen peroxide.

Reference	Chitosan	Solvent	Solvent Concentration	Temperature	Time	Mw/DP
[12]	1%	H_2O_2; CH_3COOH	0.5–3%; 1%	50–75 °C	1–6 h	2–7 DP
[16]	1%	H_2O_2; CH_3COOH; $H_3PW_{12}O_{40}$	0,5–3%; 1%; 0,04–0,14%	50–75 °C	10–60 min	7 DP
[17]	3%	H_2O_2; CH_3COOH	3%; 2%	80 °C	180 min	1.36 kDa
[46]	1%	H_2O_2; CH_3COOH	5.7, 2.8, 1.9, and 1.7 (molar ratio)	50 °C	8 h	6.61–9.97 kDa
[48]	2%	H_2O_2; CH_3COOH	2%; 1%	40 °C	30–180 min	-
[49]	2%	H_2O_2; HCl	(0.5, 1.0, 1.5, 2.0 M)/0.5%	25, 40, 50, 70 °C	1, 2, 3 h	11–1200 kDa
[50]	7.5%	H_2O_2; $H_3PW_{12}O_{40}$	4.5%; 0.1%	70 °C	30–120 min	4.3–4.7 kDa
[51]	-	H_2O_2; HCl	0–5%; 0–9%	10–90 °C	0.5–8 h	5–200 kDa

5.1. Concentration of Hydrogen Peroxide

The concentration of hydrogen peroxide is directly proportional to the rate of hydrolysis. However, its high concentration can influence the elimination of hydroxyl radicals and reduce the reaction efficiency. In this sense, the concentration of 2% H_2O_2 is ideal for chitosan hydrolysis [48].

In addition to the hydrolysis rate, the yield is also maximum (62%) when the H_2O_2 concentration reaches 2%. From this concentration, the yield decreases to 58% and 55% at concentrations of 2.5% and 3%, respectively. This decrease can be attributed to the oligosaccharides production with a shallow degree of polymerization that makes ethanol precipitation difficult [12].

The concentration of reducing sugars is also maximum (14.8%) when using 2% H_2O_2. However, from the concentration of 2.5% and 3%, it was observed that the amount of reducing sugars reduced to 14% and 12%. This occurred due to the aldehyde oxidation when there is an excess of H_2O_2 [16].

5.2. Association with Other Types of Hydrolysis

The formation of radical groups is practically inefficient when hydrogen peroxide is used alone. For this reason, to improve the efficiency of hydrolysis, several studies associate hydrogen peroxide with other patterns of degradation. Among them are some works previously discussed using gamma radiation [7,8,20,40,42] and microwaves [17,45].

In addition to these methods, chitosan is also effectively hydrolyzed by hydrogen peroxide under ultraviolet irradiation. In this case, when chitosan is hydrolyzed only in the presence of hydrogen peroxide, viscosity decreases by 20% and 63% at 30 and 180 min, respectively. However, when hydrogen peroxide is combined with ultraviolet irradiation, viscosity decreases by 84% and 92% for the same analyzed times [48].

5.3. Association with Other Reagents

Another way to increase the efficiency of the process is to hydrolyze chitosan using hydrogen peroxide under the catalysis of phosphotungstic acid. Phosphotungstic acid is a heteropolytic acid that presents simple preparation and high reactivity and is non-corrosive, in addition to having acid resistance and relatively high thermal stability [16,50]. The rate of hydrolysis of chitosan at 70 °C for 30 min without the catalyst was only 43%. This indicates that degradation is inefficient when H_2O_2 is used alone. However, when 0.1% phosphotungstic acid was used, the rate was 99.32% [50].

The concentration of phosphotungstic acid influences the hydrolysis product. There is a sharp increase in the concentration of reducing sugars with a higher concentration of phosphotungstic acid from 0.04% to 0.1%, without an additional increase from 0.1% [16].

When associated with acetic acid, its molar ratios influence the recovery of chitooligosaccharides. Therefore, the higher the concentration of hydrogen peroxide about acetic acid, the higher the yield of chitooligosaccharides (dimers–decamers), with a yield of 36%, 22%, 18%, and 14%, for molar ratios of 5.7, 2.8, 1.9, and 1.7 respectively, in addition to obtaining chitosan with lower molecular weight, 6.61, 7.7, 9.29, and 9.03 kDa, respectively [46].

5.4. Effect of Time

As with other types of hydrolysis, time will also influence when using hydrogen peroxide. In chitosan hydrolyzed by H_2O_2 in HCl solution (0.9%) for 0.5–8 h, there was a reduction in molecular weight with the increase in hydrolysis time. A rapid decrease from 480 to 50 kDa in 0.5 h has been shown, with a 95% yield [51]. In the study using hydrogen peroxide and acetic acid, the yields of chitooligosaccharides increased considerably with increasing hydrolysis time, reaching an optimal yield of 62.42% in 4 h [12].

The reaction rate is higher when H_2O_2 hydrolyzes the chitosan under the catalytic action of phosphotungstic acid with an ideal time of 30 min of hydrolysis. In these conditions, a higher concentration of reducing sugars was obtained in 30 min and no further increase after 40 min [16]. Additionally, there is a reduction in the molecular weight of chitosan from 700 to 4.7 and 4.3 kDa in 30 and 120 min, respectively. This shows that after 30 min, under the catalysis of phosphotungstic acid (0.1%), there will be no significant reduction in molecular weight [50].

5.5. Effect of Temperature

Temperature is another variable that influences the reaction, since the temperature is directly proportional to the hydrolysis rate. This behavior was observed in 498 kDa chitosan, hydrolyzed in H_2O_2 solution (0.3%) for 2 h at 30, 50, and 90 °C. The

obtainment of medium and low molecular weight chitosan of 200, 25 Da, and 5 kDa, respectively, was observed [51].

Additionally, the yield of chitosan after H_2O_2 hydrolysis is proportional to the increase in temperature, with a maximum concentration of reducing sugars at 65 °C. However, when the reaction temperature exceeds 65 °C, browning occurs in the reaction mixture and a decrease in the concentration of reducing sugars due to oxidation of the aldehyde. Therefore, the most suitable maximum reaction temperature is 60 °C [12,16,49].

6. Enzymatic Hydrolysis

Hydrolysis of chitosan catalyzed by enzymes is more specific compared to other types of hydrolysis. The specificity allows better control of the extent of the reaction and the size of the oligomer. Thus, enzymatic hydrolysis tends to be an attractive alternative [32]. Enzymes act specifically in reactive sites, either internally fragmenting the molecule or acting from one end, releasing monomers or dimers sequentially [31].

Chitosanases are the specific enzymes intended for the hydrolysis of chitosan. However, these enzymes have reduced commercial availability and, consequently, have high commercial value, presenting limited industrial use [32]. Therefore, as a lower-cost alternative, other commercially available enzymes capable of hydrolyzing polysaccharides in large quantities are studied (Table 5). These discoveries enable the development of efficient and economically viable industrial processes for the hydrolysis of chitosan.

Table 5. Parameters of chitosan enzymatic hydrolysis.

Reference	Enzime	Enzyme Concentration [1]	Chitosan [2]	Solution	pH	Temperature	Time	Mw/DP
[6]	Cellulase	10 U/g of chitosan	1%	Sodium acetate 0.5 M	5.2	55 °C	1–24 h	2.2–156 kDa
[10]	Not identified	20% [2]	1%	Phosphate 0.05 M	5.0	37 °C	1–24 h	1–6 DP
[21]	Chitosanase	0.95 U/mg		Sodium acetate	5.6	55 °C	1.5 h	1–100 kDa
[22]	Pepsin + papain	4%	1%	Sodium acetate 0.2 M	4.8	50 °C	0–24 h	0.6–150 kDa
[31]	Celuzyme® XB (cellulase/xylanase/β-glucanase)	0–2.5 × 10^{-3} mg/mL	0.5%	Acetic acid-sodium acetate 0.2 M	3.5–6.5	25–75 °C	25–250 min	64–152 kDa
[32]	Chitosanase, cellulase, hemicellulase, papain, bromelain, pepsin, protease type XIV, lysozyme, and lipase A.	0.1–10%	1%	Sodium acetate 0.1 M	3.0–5.0	30–50 °C	0–20 h	-
[33]	Cellulase	5–20%	0.5–4%	Acetic acid	2.0–6.0	40–60 °C	5 min–24 h	85.8–1.13 kDa
[46]	Pectinase + cellulase + papain	15+15+2%	2%	Phosphate 1M	5.3	39 °C	24 h	14.6–34 kDa
[52]	Cellulase	10 U/g of chitosan	-	Acetate-bicarbonate 0.5 M	5.2	55 °C	1–18 h	3.3–156 kDa
[34]	Papain/lysozyme/cellulase	0.003%	1%	Sodium acetate 0.1 M	4.0	40/30/37 °C	1–16 h	4.3–800 kDa
[53]	Chitinase, cellulase, or lysozyme	10% [2]	2.2%	Sodium acetate 0.1 M	4.0	42 °C	0–180 h	6–38 kDa
[54]	Cellulase	0.5 mL	1%	Sodium acetate 0.02 M	5.2	60 °C	0.5–12 h	2–4 DP
[55]	Papain	0.08–0.12 g/g of chitosan	6–10 g/L	Sodium acetate 0.2 M	4.0–5.0	40–50 °C	15–120 min	35–155 kDa
[56]	Chitosanase	1,5 U/mL	1%	Sodium acetate 0.02 M	5.5	30 °C	0,5–6 h	2–7 DP
[57]	Not identified	0.05 U/mL	1%	Sodium acetate 0.05 M	5.0	60 ºC	-	2–5 DP
[58]	Cellulase	10 U/g of chitosan	4.5%	Acetic acid-bicarbonate 0.5 M	5.2	55 °C	18 h	12 kDa/2–8 DP
[59]	Cellulase	20%	5%	Acetic acid 1%	5.6	50 °C	24 h	3–11 DP

[1] Regarding the substrate; [2] Regarding the solution.

6.1. Hydrolysis of Chitosan by Different Enzymes

Different commercial enzymes were tested to decrease the viscosity of chitosan solutions and release the reducing ends of the polymer. Among these enzymes, cellulase, pepsin, and lipase proved to be more suitable for the hydrolysis of chitosan at a level comparable to that obtained by chitosanase, with a decrease in

viscosity in 69%, 80%, 82%, and 65%, respectively. It was observed that some hydrolases might also have the ability to act on the reducing ends of chitosan in the first hour of hydrolysis. This is possible because of the cleavage specificities of exohydrolases [32].

In addition to the hydrolytic activity exo-1,4-β-D-glucanases, which cleaves glycosidic bonds present in the reducing ends of polymers, hydrolysis can also occur in endo-1,4-β-glucanases, which cuts off the β- 1,4-internal glycosides [60]. The performance of the endogluganase enzyme studied by Roncal et al. [32] could be observed through kinetics about viscosity. A decrease in viscosity was observed in the first 20 min. Thus, the greatest reduction in viscosity is better observed in the large chitosan chains than in the smaller chains.

The exact amount of unit of activity (100 U/mL) for each enzyme, in the hydrolysis of chitosan (371.5 kDa), presented a more efficient catalyst capacity in 96 h (<6 kDa) than chitinase (11.2 kDa) and lysozyme (22.2 kDa) [53]. Thus, due to the high rate of cellulase hydrolysis, in addition to greater availability and lower cost, most studies are increasingly focusing on the use of cellulases for the hydrolysis of chitosan.

The species that produce cellulases can influence hydrolysis rates. The purified cellulases obtained from *Trichoderma viride*, *Trichoderma reesei*, and unpurified cellulase obtained from *Aspergillus niger* have hydrolytic activity. However, *A. niger* and *T. reesei* may have high hydrolysis rates, while *T. viride* cellulase may be lower. In this case, the cellulase efficiency of the Aspergillus genus is possibly due to its better β-glycosidase activity compared to the Trichoderma genus [33].

Aspergillus cellulase has a good capacity for enzymatic hydrolysis, being a viable alternative to replace chitinase and chitosanase, which are enzymes of more excellent economic value [33,59]. Cellulase can hydrolyze chitosan 84% DDA and molecular weight from 518 to 35 kDa in the first hour of hydrolysis at 50 °C [33].

Although cellulase has a non-specific hydrolytic action on chitosan, it can be observed in previous studies that this enzyme has excellent hydrolysis capacity. This is due to the structural similarity of chitosan with cellulose (Figure 4). Cellulose is formed by D-glucose polymers joined by β-1,4-glycosidic bonds. In chitosan, the C-2 hydroxyl groups are replaced by amino groups, and apparently, the enzyme does not efficiently recognize the C2 position group in the glucose or glucosamine residues. For the same reasons, chitosanase and cellulases also exhibit high homogeneity, and it is not uncommon for them to be found in the same microorganism [60].

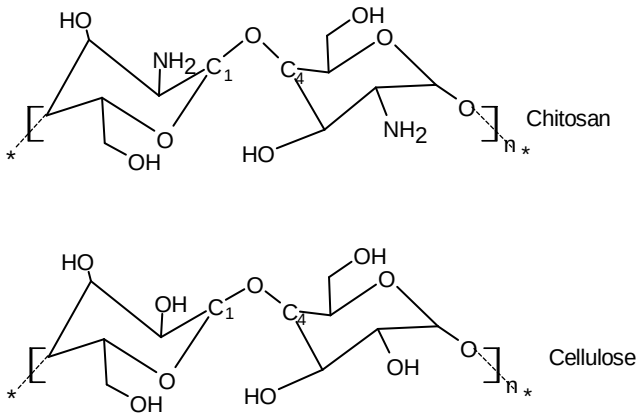

Figure 4. Chemical structure of chitosan and cellulose. Reprinted from Omari et al. [61] with permission Royal Society of Chemistry provided by Copyright learance Center.

6.2. Products of Enzymatic Hydrolysis

The structures of the chitooligosaccharides formed also depend on the specificity of the enzymes used in the enzymatic pool. In this sense, the enzymatic hydrolysis of chitosan at 39 °C, pH 5.3, for 24 h, from the mixture of pectinase from *Rhizopus oryzae*, cellulase from *Aspergillus niger* and papain, resulted in chitosan fractions with a molecular weight of 34 and 14.6 kDa. The content of chitooligosaccharides obtained was 33% of dimers–octamers and 54% of dimers–tetramers [46]. When only the *Aspergillus niger* cellulase enzyme was used at 50 °C, pH 5.6, for 24 h, the products were formed mainly by chitooligosaccharides with a degree of polymerization from 3 to 11 [59]. Otherwise, the use of papain alone resulted in a mixture of oligosaccharides and monomers (GlcN and GlcNAc), with four main components GlcNAc, GlcN, (GlcN)3, and (GlcN)4 [55].

Glucosamine (GlcN) and N-acetyl-glucosamine (GlcNAc) oligomers were identified as products of the depolymerization of chitosan when commercial cellulase was used. This result indicates that the enzyme cleaved the GlcN-GlcN and GlcNAc-GlcN bonds. Thus, from hydrolysis, 60 °C, pH 5.2, for 0.5–12 h, chitobiose $(GlcN)_2$, chitotriose $(GlcN)_3$, chitotetraose $(GlcN)_4$, and some chitooligosaccharides with long-chain length were obtained. Shorter D-glucosamine oligomers with increased hydrolysis time have also been produced [54].

An enzymatic solution of *Bacillus amyloliquefaciens* was used to obtain oligomers through the hydrolysis of chitosan at 37 °C at pH 5. The quantities of oligomers (GlcNAc)n, n = 1–6 increased with the hydrolysis time between 1 and 12 h. After hydrolysis for 12 h, the quantities of all oligomers increased, but (GlcNAc)6 was the lowest among the oligomers (GlcNAc)n, n = 1–6 in the composition of the hydrolysates. After hydrolysis for 24 h, the hydrolysates composition was almost all (GlcNAc) n, n = 1–3. The

amount of monomer that is the most found in the composition of the hydrolysates was GlcNAc [10].

Some bacteria also produce enzymes of interest for the hydrolysis of chitosan. The enzymatic extract obtained from a commercial preparation of *Bacillus thuringiensis* efficiently hydrolyzed chitosans with a deacetylation degree of 81% and 90% and showed a higher reaction rate for the more deacetylated chitosan. This made it possible to obtain partially acetylated GlcNAc-(GlcN)1-3 and deacetylated (GlcN)2-5 oligosaccharides. Chitosan with 90% deacetylation was converted to oligosaccharides in 55 h. Most products were 16% chitobiose, 17% chitotriose, 50% chitotetraose, and 14% chitopentaose [57].

The hydrolysis products resulting from the action of cellulase, pepsin, lipase, and chitosanase on chitosan were divided into two fractions. The first fraction is insoluble (low molecular weight chitosan), with a yield of 49%, 48%, 50%, and 45%, respectively. The second is the soluble fraction, formed by oligosaccharides and monomers. Oligosaccharides had yields of 46%, 52%, 42%, and 46%, respectively. The yield of the GlcN and GlcNAc monomers was not identified for pepsin. However, for the other enzymes, the yield was 5%, 7%, and 9%, respectively. This particular property of pepsin allowed to produce considerably more oligosaccharides than other enzymes. In this case, we can infer that pepsin is an excellent alternative for obtaining oligosaccharides due to the rapid decrease in viscosity from short and medium-chain oligosaccharides without monomer generation [32].

A new chitosanase (GsCsn46A) from *Gynuella sunshinyiii* showed high hydrolytic activity when producing oligosaccharides in a moderate reaction condition (pH 5.5) at 30 °C/30 min, where it was obtained mainly $(GlcN)_2$ to $(GlcN)_7$. After 2 h of incubation $(GlcN)_2$, $(GlcN)_3$, $(GlcN)_4$, and a smaller amount of $(GlcN)_5$ were obtained. However, only $(GlcN)_2$ and $(GlcN)_3$ were detected after 6 h of reaction. The yield rates of chitooligosaccharides ranged from 71% to 94% from 30 min to 6 h [56].

6.3. Effect of Temperature on Enzymatic Hydrolysis of Chitosan

The temperature on enzyme activity is related to two established thermal parameters. The first is the Arrhenius activation energy, which relates to the effect of temperature on the catalytic reaction. The second is thermal stability, which shows the effect of temperature on thermal inactivation [62].

The ideal value in the hydrolysis rate depends on the type of enzyme. In the study using the enzymatic complex Celuzyme® XB, formed by cellulase, xylanase, and β-glucanase, the highest hydrolysis rate was reached at 51 °C, reaching a relative activity of 90%. Additionally, the increase in temperature tends to decrease its catalytic activity. This occurs due to the modification of the tertiary structure that may lose its active and functional

conformation [31]. This behavior was also observed when using pepsin, which showed an increase in hydrolysis activity by increasing the temperature to 45 °C, with a relative enzymatic activity of 75% [32].

Cellulase acted significantly at 50 °C with a decrease in viscosity above 90% in 15 min. However, hydrolysis was lower at 60 °C, where the reduction was 20%. In this case, cellulase was relatively stable at a temperature below 55 °C and was quickly inactivated at higher temperatures [33].

6.4. Effect of Time on Enzymatic Hydrolysis of Chitosan

The molecular weight of chitosan decreases with increasing hydrolysis time. However, the intensity of the effect may vary according to the synergistic effect of different enzymes. Thus, enzymes that exhibit endo hydrolysis characteristics may present a higher rate of hydrolysis at the beginning of the reaction. Chitosan hydrolyzed by papain, cellulase, and lysozyme showed a rapid decrease in molecular weight in the first hour of hydrolysis, with a reduction of 194 kDa (90% DDA) to 41, 80, and 150 kDa, respectively. After 8 h of hydrolysis, the values were 5, 12, and 16 kDa, respectively. There was also no significant reduction after that time [34]. However, pepsin and papain, used in combination at pH 4.8 to 50 °C, also showed a molecular weight decrease. The molecular weight in the initial hydrolysis phase was 500 kDa (91% DDA), and after hydrolysis, it was reduced to 150 kDa after 1 h of reaction. The molecular weight continued to reduce up to 75 kDa in 24 h [22].

When papain (pH 4.5 at 45 °C) was used, the molecular weight decreased rapidly at the beginning of hydrolysis, initially from 350 to 82 and 40 kDa at 30 and 45 min, respectively. From this time on, stability was observed without a significant increase [55]. Otherwise, Celuzyme® XB showed a reduction in molecular weight in the first 135 min and became constant after four hours of reaction due to the decrease in enzymatic activity limiting depolymerization and obtaining glycoside units [31].

Chitosan 371.5 kDa (92% DDA) hydrolyzed by the enzymes lysozyme, chitinase, and cellulase showed a significant decrease in molecular weight in the first three hours hydrolysis to 38, 22, and 15 kDa, respectively. After 20 h, the reduction went to 26, 15, and 7 kDa, respectively. However, there was no significant reduction after that time [53]. Already under the conditions of 50 °C, pH 5, and cellulase to chitosan ratio 1: 5, there was a reduction in the molecular weight of chitosan from 518 kDa to 35, 12, 6, and 1.13 kDa in the times of 1, 4, 8 and 24 h, respectively [33].

6.5. Effect of pH on Enzymatic Hydrolysis of Chitosan

The stability and functionality of the enzyme depend on its spatial structure. Chemical changes, such as pH, can modify the structure of an enzyme. The bonds that make up the tertiary

structure of the enzyme are altered, which prevents its catalytic function from being efficiently performed. In this way, the pH influences the enzyme activity, where the best pH value or range will be according to the type of enzyme used in hydrolysis. Additionally, chitosan is also influenced by pH because its solubility is low at pH close to 7.0 [31,32].

Under the study conditions with Celuzyme® XB (cellulase/xylanase/β-glucanase) and chitosan, the activity was maximum at pH 4.8. From this pH, the performance of the system tended to decrease gradually. Favorably, the three enzymes are highly stable at acidic pH and, likewise, their efficiency decreases at pH close to neutral [31]. However, in another study using only the cellulase enzyme, the effect was not observed with pH values of 2.0 to 6.0. The enzyme was activated at other pH values, promoting the hydrolysis of chitosan determined by the decrease in viscosity by up to 97% in 1 h [33].

Regarding pepsin, the peak activity in the hydrolysis of chitosan was at pH 4.5. Although the protein pH activity is generally lower, the variability will depend on the specific protein substrate and its native or denatured state [32].

6.6. Influence of the Enzyme/Substrate Ratio

In addition to the other parameters already shown, the reaction rate also depends on the enzyme concentration and substrate (chitosan). The reaction rate is better in a higher enzyme/substrate ratio [32,33].

In a constant condition concentration of chitosan, the final viscosity is influenced by the change in enzyme concentration. In this case, the biopolymer degradation increases directly in a higher enzyme concentration. Concentrations of the enzyme Celuzyme® XB greater than 1×10^{-3} mg mL^{-1} caused the maximum degradation up to a limit of 2.15×10^{-3} mg mL^{-1}. After this value, the concentration of reducing sugars is constant [31].

In the study of pepsin, the viscosity of the chitosan solution decreases as the pepsin concentration increases. For hydrolysis times longer than 1 h, an enzyme/substrate ratio of 1: 100 is sufficient to achieve the maximum hydrolysis degree. From this concentration, the reduction in viscosity remains at approximately 80% [32].

A decrease in the hydrolysis rate was observed when the cellulase concentration was kept constant and the chitosan concentration was increased. After 10 min of hydrolysis, the 1: 5 ratio (enzyme: substrate) showed a more significant decrease in viscosity (95%), while the 1:20 ratio was 82% [33]. That is because the viscosity of the system increases with a higher concentration of chitosan. This effect significantly reduces the diffusion of chitosan to the active center of the enzyme. This will decrease enzyme activity [55].

6.7. Properties of Chitosan Oligomers

The hydrolytic product of chitosan has more excellent application due to its better solubility and functional characteristics when compared to integral chitosan. The DPPH antioxidant activity was higher in chitosan with a molecular weight below (72 kDa), with a reduction potential of up to 90% with chitooligosaccharide of 2.2 kDa and 38% with medium molecular weight chitosan of 300 kDa. In addition to the molecular weight, it was found that the concentration of chitosan is directly proportional to the antioxidant activity. In chitosan of 2.2 kDa in different concentrations of 1 and 5 mg mL^{-1}, the reduction of DPPH was 50% and 65%, respectively [6].

In the study with chitosan at a concentration of 1.2 mg mL^{-1} (152 kDa), there was a 15% reduction in DPPH. However, while in chitosan of 64 kDa, the reduction was 75%. Chitosan with higher molecular weight has a compact structure, which promotes the high viscosity of the solution. In these conditions, the strength of the intramolecular hydrogen bond reduces the activity of the hydroxyl and amine groups, limiting the reduction capacity of these groups [31].

Chitosan hydrolyzed with cellulase also had a more significant effect on antimutagenicity. This positive effect was observed at lower molecular weight [6] and evidences a potential use for reducing hypercholesterolemia through binding with bile acids [22]. Chitosan hydrolyzed by a crude enzyme from *Bacillus amyloliquefaciens* V656 also showed antitumor activity after 12 h of hydrolysis. This effect has been related to the presence of hexamer/$(GlcNAc)_6$ [10].

Several studies have analyzed the effect of chitosan's molecular weight on antibacterial activity. Some studies concluded that antibacterial activity increased when there was a reduction in molecular weight. This was observed in 12 kDa low molecular weight chitosan compared to 228 kDa medium molecular weight chitosan against *Bacillus cereus*, *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella* sp, and *Saccharomyces cerevisiae* [58]. It was also observed in 64 kDa chitosan compared to 152 kDa chitosan against *Staphylococcus aureus*, *Pantoea ananatis*, *Pseudomonas aeruginosa*, *Raoultella planticola*, and *Escherichia coli* [31].

Some reports have shown that chitosan of medium molecular weight (194 kDa) has more significant antibacterial activity than chitosan of lower molecular weight (41, 14, and 5 kDa) against Gram-positive microorganisms due to its greater thickness of the peptidoglycan layer, preventing the access of the chitooligosaccharides to the cell. Although the peptidoglycan layer prevents the chitooligosaccharides from accessing the cell, the antimicrobial effect of the higher molecular weight chitosan is due to the formation of a chitosan film. This film can inhibit the absorption of nutrients by the microorganism [34]. Another study,

with chitosan from 300 to 3.3 kDa, showed that the pH could influence differently according to the molecular weight of the chitosan, being observed that under acid pH conditions, the chitosan activity increased with the increase of the molecular weight, regardless of the temperature and the bacteria tested. However, at neutral pH, chitosan activity increased as the molecular weight decreased [52].

The antimicrobial activity of low molecular weight chitosan was compared with standard antibiotics. The inhibitory activities of molecular weight between 10 and 100 kDa were as efficient as the antibiotics Flomox® and Kluacid® against *Bacillus cereus*. The specific growth rate of *Candida albicans* was inhabited entirely by chitooligosaccharides of molecular weight less than 1 kDa at a concentration of 0.11 mg mL^{-1} and was more efficient than that of Kluacid® at a concentration of 0.42 mg mL^{-1}. These results show that chitosan and low molecular weight chitooligosaccharides can be considered possible alternative antimicrobial agents or additives in pharmaceutical compositions [21].

7. Conclusions

From the studies demonstrated, the choice of the type of hydrolysis of chitosan remains a challenge. Several factors influence the process, compromising the yield, cost, and characteristics of the hydrolyzed product. This hydrolyzed product (medium and low molecular weight chitosan and chitooligosaccharides) is responsible for its bioactive properties. New technologies and studies focused on using chitosan hydrolysates have been shown continuously in the scientific literature. However, one point that seems essential is related to the need to develop more efficient hydrolysis technologies to obtain oligomers with specific sizes. Thus, several alternatives of technologies are proposed in isolation or combined to improve process efficiency. The choice of technology to be used depends mainly on the type of application for which the hydrolysis product will be used.

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Appendix A. Diversity of countries and articles referenced in this review.

Country	Articles (Amount of Participation)
Algeria	1
Canada	3
China	27
Czech Republic	1
Egypt	2
France	1
India	3
Indonesia	1
Iran	1
Japan	1
Korea	1
Malaysia	2
Mexico	1
Pakistan	1
Republic of Korea	1
Romania	1
Russia	1
Saudi Arabia	1
South Korea	1
Spain	2
Switzerland	1
Taiwan	5
Thailand	1
Turkey	1
United Kingdom	3
United States	4
Vietnam	2

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CAPÍTULO II
OBTENÇÃO DE OLIGÔMEROS DE QUITOSANA A PARTIR DE EXTRATO ENZIMÁTICO INTEGRAL PRODUZIDO POR GÊNERO ASPERGILLUS

Resumo

Este estudo tem como objetivo a utilização de um extrato enzimático integral (*EEI*), produzido por um fungo filamentoso, eficiente na hidrólise da quitosana, para obtenção de oligômeros com massas moleculares variadas. As enzimas presentes no *EEI* foram identicadas por LC-MS/MS, com destaque para as endo-quitinases (E.C 3.2.1.14), exo-quitinases (E.C 3.2.1.52) e celobiohidrolase (E.C 3.2.1.91). Também foram identificadas as alfa-L-arabinofuranosidase (E.C 3.2.1.55) e pectina liase (E.C. 4.2.2.10). A linhagem do fungo foi identificada como *Aspergillus* sp pela amplificação da região ITS1-5.8S-ITS2 do rDNA comparada à base de dados GenBank, e através da análise metaproteômica onde o micro-organismo foi identificado como *Aspergillus nidulans*. Deste modo, as enzimas excretadas foram identificadas com cobertura de sequência superior a 84 % em relação às enzimas de *A. nidulans*. Ensaios de hidrólise da quitosana foram realizados comparando o *EEI* com a enzima comercial (Celluclast 1,5 L®). Os resultados mostraram que o *EEI* apresentou maior eficiência na hidrólise da quitosana em relação à celulase comercial, sendo capaz de reduzir o peso molecular inicial da quitosana em 47,80, 75,24 e 93,26 % após 2,0 5,0 e 24 h, respectivamente. As análises de FTIR revelaram menor absorbância dos sinais espectrais dos oligômeros de quitosana e sua cristalinidade foi reduzida a partir do tempo de 3 h de hidrólise. Com base nestes resultados, podemos concluir que o extrato enzimático integral apresentou um importante potencial tecnológico. Além disso, este estudo mostra que é possível utilizar extrato enzimático não purificado para obtenção de oligômeros de quitosana.

Palavras chave: quitosana, hidrólise, fungo filamentoso, enzima.

1 Introdução

A quitosana é um polissacarídeo linear, obtido pela desacetilação parcial da quitina em condições alcalinas, que contém copolímeros de ᴅ-glucosamina (unidades desacetiladas) e N-acetil-ᴅ-glucosamina (unidades acetiladas) interligados por ligações glicosídicas β (1→4) (Hamed, Özogul, & Regenstein, 2016) e possui propriedades especiais de biocompatibilidade, biodegradabilidade, bioatividade e não toxicidade.

Além dessas características positivas, também é necessário atender condições tecnológicas e necessidades específicas que são potencializadas em função do tamanho do

oligômero obtido por meio da hidrólise da quitosana. O tamanho do oligômero é traduzido em função do seu respectivo peso molecular de modo diretamente proporcional (Naveed et al., 2019). Com base no peso molecular, a quitosana pode ser agrupada em baixo peso molecular (<100 kDa), médio peso molecular (100-1000 kDa) e alto peso molecular (> 1000 kDa) (Santoso, Adiputra, & Soerdirga, 2020).

Os oligômeros de quitosana apresentam menor peso molecular, melhor solubilidade em água e maiores atividades fisiológicas envolvendo: atividade antimicrobiana e antioxidante (Águila-Almanza, Salgado-Delgado, Vargas-Galarza, García-Hernández, & Hernández-Cocoletzi, 2019; Laokuldilok et al., 2017; Yin, Du, Zhao, Han, & Zhou, 2020); propriedades hipocolesterolêmicas (Xu, Mohan, Pitts, Udenigwe, & Mason, 2020); antimutagenicidade (Chang, Wu, & Tsai, 2018); diminuição na formação de acrilamida em soluções de glicose/ frutose-asparagina (Chang, Zeng, & Sung, 2020); e inibição do crescimento de células tumorais (Qin et al., 2004).

A despolimerização da quitosana utilizando o método tradicional pode ser realizada por meio da hidrólise ácida. No entanto, esse método apresenta algumas desvantagens, incluindo a dificuldade em obter oligossacarídeos com baixo grau de polimerização e de controlar a extensão da hidrólise, o que resulta frequentemente em hidrolisados com uma quantidade elevada de monossacarídeos (Xing et al., 2017). Além disso, as condições reacionais exigem temperaturas elevadas e altas concentrações de reagentes, que frequentemente resultam na formação de oligossacarídeos quimicamente modificados (Kim & Rajapakse, 2005).

O uso de enzimas, na hidrólise da quitosana, tem recebido atenção por apresentar menor variação na obtenção dos oligossacarídeos em comparação com a hidrólise química (Sánchez et al., 2017). Além disso, o método enzimático tem vantagem sobre as reações químicas devido as enzimas atuarem sob condições mais brandas, apresentarem alta especificidade e não modificarem a estrutura do anel de glicose (Li et al., 2005). No entanto, esse método tem como desvantagem o alto custo, disponibilidade limitada de algumas enzimas, além da ação lenta em soluções viscosas. Para soluções com característica viscosas, são necessárias baixas concentrações de substrato e maiores quantidade de enzimas (Sánchez et al., 2017).

As quitosanases e as quitinases são enzimas específicas responsáveis pela hidrólise da quitosana. As quitosanases (E.C 3.2.1.132) são glicosil hidrolases que catalisam a endo-hidrólise de ligações β-1,4-glicosídicas de quitosana. Enquanto que as quitinases, classificadas de acordo com seu modo de ação em endoquitinases (E.C 3.2.1.14) e

exoquitinases (E.C 3.2.1.52), catalisam a hidrólise interna e externa da quitosana, respectivamente (Aranaz et al., 2021).

As enzimas não específicas ou promíscuas também são capazes de hidrolisar a quitosana. Essas enzimas pertencem à família das celulases, hemicelulases, pectinases, papaína, pepsina, proteases, bromelaína, lisozima. São enzimas de menor custo e podem clivar a ligação β-1,4-glicosídica da quitosana (Laokuldilok et al., 2017; Li et al., 2019; Pan, Zeng, Foua, Alain, & Li, 2016; Roncal, Oviedo, Armentia, Fernández, & Villarán, 2007; Santos-Moriano et al., 2018; Wang et al., 2020).

Essas enzimas são industrialmente importantes e têm sido tradicionalmente obtidas a partir de fermentação submersa devido à facilidade de manuseio e maior controle de fatores ambientais, como temperatura e pH (Sirohi et al., 2019). Os fungos filamentosos são preferidos para a produção dessas enzimas, pois a quantidade produzidas é maior do que aquelas obtidas de leveduras e bactérias (Mrudula & Murugammal, 2011)

A busca de novas fontes produtoras de enzimas e novos mecanismos de despolimerização enzimática são objetos de considerável interesse científico e tecnológico. Dessa forma, esse trabalho teve como objetivo produzir e utilizar um extrato enzimático eficiente com poucas etapas *downstream* capaz de hidrolisar a quitosana para obtenção de oligômeros.

2 Materiais e Métodos

2.1 Quitosana

A quitosana utilizada foi obtida da empresa Polymar/Brasil (cód. PB2112), apresentando grau certificado de desacetilação de 85 %.

2.2 Enzima comercial

A celulase comercial utilizada foi a Celluclast 1,5 L® (Novozymes, Paraná, Brasil), produzida por *Trichoderma reesei*.

2.3 Seleção do micro-organismo

A linhagem do micro-organismo (MIBA0666) utilizada nesse trabalho é pertencente à micoteca do Laboratório de Investigação Sistemática em Biotecnologia e Biodiversidade Molecular (LabISisBio), Universidade Federal do Pará. Este micro-organismo é um fungo filamentoso, onde a partir de testes preliminares, foi selecionado em função da maior atividade endoglucanásica.

2.4 Identificação do micro-organismo

2.4.1 Isolamento e sequenciamento de DNA

A biomassa fúngica foi obtida a partir do cultivo em meio líquido Czapek Dox (Sigma ®) com adição de 10 % de extrato de levedura e posterior incubação a 30 °C por 5 dias. O DNA foi extraído a partir da biomassa micelial de acordo com as instruções do fabricante do kit de extração e purificação do DNA genômico (Axygen®, ref. AP-MN-MS-GDNA-50). Foi utilizado o par de *primer* ITS-1f (5´-TCC GTA GGT GAA CCT GCG G-3´ e *primer* ITS-4r (5'-TCC TCC GCT TAT TGA TAT GC-3') (Chan, Cohen, & Bell, 2018; Chen et al., 2001), concentração de 10 pmol. Para realização das etapas de PCR, usou-se termociclador automático (Amplitherm®) programado para desnaturação inicial a 95 ºC/5', 35 ciclos de 94 ºC/1', 55,5 ºC/2', 72 ºC/2' (fases de desnaturação, anelamento e extensão, respectivamente) e extensão final a 72 ºC/10' (Chen et al., 2001).

2.4.2 Análise de sequência

A anotação das sequências obtidas foi realizada no programa Geneious® (versão 9.1.5) e, em seguida, foram comparadas à base de dados do GenBank por meio do BLAST (Basic Local Alignment Search Tool). Assim, a porcentagem de similaridade da sequência correspondente à região ITS dos fungos isolados neste estudo foi obtida em relação às sequências já depositadas no Genbank. Uma sequência > 98% foi considerada para identificar a espécie do fungo filamentoso.

2.5 Extrato enzimático integral (*EEI)*

2.5.1 Seleção do meio de cultivo

Na seleção do melhor meio de cultivo para a produção do *EEI* foram utilizados dois meios diferentes: Czapeck modificado (*Czm*): carboximetilcelulose (CMC) 10 g $L^{-}1$, nitrato de sódio 3 g L^{-1}, fosfato de potássio 1 g L^{-1}, cloreto de potássio 0,5 g L^{-1}, sulfato de magnésio 0,5 g L^{-1} e sulfato ferroso 0,01 g L^{-1}; e *CPY*: carboximetilcelulose (CMC) 10 g L^{-1}, peptona 1 g L^{-1} e extrato de levedura 20 g L^{-1}.

O fungo foi cultivado em frascos cônicos de 500 mL contendo 250 mL dos meios *Czm* e *CPY*. Em cada meio de cultivo foram adicionados 5 discos de fragmentos de micélio do fungo com 5 mm de diâmetro e incubado a 30 °C, sob agitação constante de 120 rpm, durante 13 dias. Os ensaios foram realizados em triplicata e durante este período foi determinada a atividade endoglucanásica. As condições de ensaio foram selecionadas a partir de testes preliminares (dados não apresentados).

2.5.2 Cultivo submerso para a produção do extrato enzimático integral

O extrato enzimático integral, utilizado na hidrólise da quitosana, foi produzido a partir do meio de cultivo que proporcionou maior atividade enzimática, selecionado no item 2.5.1. O cultivo foi realizado em frascos cônicos de 1000 mL com adição de 500 mL de meio de cultivo e 10 discos de fragmentos de micélio de fungo com diâmetro de 5 mm. O ensaio foi realizado em triplicata. A mistura foi incubada com agitação a 30 °C e 120 rpm. Foram retiradas alíquotas para verificar o tempo necessário para alcançar a maior atividade enzimática no meio de cultivo selecionado. Alíquotas de 2 mL de cultivo, foram filtradas com algodão empacotado em uma seringa para eliminação do micélio. O filtrado foi utilizado para a determinação da atividade endoglucanásica.

Com o objetivo de aumentar o volume de extrato enzimático, após selecionado o tempo de cultivo que apresentou maior atividade enzimática, foi realizado um novo cultivo, mantendo-se as condições anteriores com um volume total de 2000 mL dividido em 4 frascos. O cultivo final foi filtrado em disco de papel de filtro quantitativo com funil de Buchner sob vácuo, para a remoção da biomassa. A fase líquida foi concentrada por liofilização até reduzir 95 % do volume inicial.

2.5.3 Determinação do teor de proteínas pelo método Bradford

A quantidade de proteínas totais presente no extrato enzimático integral e celulase comercial foi determinada de acordo com a metodologia de Bradford (1976), com modificações. Foram retirados 200 μL do extrato enzimático filtrado e adicionado a 1800 μL de solução do reagente de Bradford a 0,1 mg mL^{-1}. O tempo reacional foi de 5 min e em seguida a leitura foi realizada em espectrofotômetro a 595 nm. O branco reagente foi preparado nas mesmas condições substituindo o extrato enzimático por água destilada.

2.5.4 Determinação da atividade da enzima endoglucanásica (CMCase)

A atividade enzimática (U/mL) foi determinada por meio da detecção de açúcares redutores pelo método do ácido 3,5-dinitrosalicílico (DNS) (Miller, 1959), com modificações, onde os açúcares redutores liberados (ARL) foram determinados por meio da curva de quantificação. Em microtubo de 1,5 mL foram adicionados 500 μL do extrato enzimático e 1000 μL de carboximetilcelulose (CMC) a 0,5 % diluído em tampão citrato de sódio (0,1 M; pH 4,8). O sistema reacional foi incubado a 50 °C durante 10 min em banho seco (Thermomixer ® compact, Eppendorf) com agitação fixada em 400 rpm. Após o processo reacional, foi transferido para um tubo de vidro 100 μL da mistura reacional, 200 μL de água e 300 μL do reagente DNS, incubado a 100 °C, em banho-maria, durante 5 min. Em seguida,

a mistura foi resfriada em água corrente e adicionado 1000 µL de água destilada. O branco reagente foi determinado nas mesmas condições da amostra, substituindo o CMC por água destilada. A leitura foi realizada em espectrofotômetro a 540 nm. A atividade enzimática foi determinada através da Eq. 1.

$$\text{Atividade enzimática } \left(\frac{\text{U}}{\text{mL}}\right) = \frac{\text{ARL}\left(\frac{\text{mg}}{\text{mL}}\right) \times \text{V}_\text{t}(\text{mL})}{\text{T}_\text{H}(\text{min}) \times 0{,}18 \times \text{V}_\text{E}(\text{mL})} \qquad \text{Eq. (1)}$$

Onde:

ARL: açúcares redutores liberados

V_t: volume reacional

T_H: tempo de hidrólise

V_E: volume do extrato enzimático

A atividade específica foi determinada através da Eq. 2.

$$\text{Atividade específica } \left(\frac{\text{U}}{\text{mg}}\right) = \frac{\text{Atividade enzimática}}{\text{Teor de proteína}} \qquad \text{Eq. (2)}$$

2.6 Identificação das proteínas presentes no extrato enzimático integral

2.6.1 Precipitação das proteínas

O extrato enzimático concentrado por liofilização foi submetido ao procedimento de precipitação de acordo com o método desenvolvido por Wessel & Flugge (1984). As proteínas foram precipitadas através da adição de metanol, clorofórmio e água na proporção de 1:4:3 (v/v/v), respectivamente. A solução foi agitada em vórtex, seguida de centrifugação a 12.000 g por 5 min a 25 °C. A fase superior foi descartada, mantendo-se o disco formado entre as fases superior e inferior. Em seguida, o metanol foi adicionado na proporção de 3 ml de metanol para 1 ml do volume inicial da amostra e a solução submetida a centrifugação a 12.000 g por 5 min a 25 °C. O sobrenadante foi descartado e o sedimento seco à temperatura ambiente. O pellet compreende as proteínas segregadas de interesse que foram lavadas por 5x a 12.000 g por 1 h (cada lavagem) com 50 mM de bicarbonato e amônio em Amicon Ultra 3 K MWCO.

2.6.2 Eletroforese unidimensional de proteínas (SDS PAGE)

Para analisar o perfil proteico do extrato enzimático, as proteínas foram submetidas a SDS PAGE conforme Laemmli (1970). 10 µg de proteínas foram imersas em tampão (Tris-HCl 0,2 M pH 6,8), SDS 40 % (v / v), β-mercaptoetanol 20 % (v / v) e azul de bromofenol 0,5 % (v / v), aquecidas a 100 °C durante 3 min e então submetidas a gel SDS-PAGE 12 %, 7 cm,

1,0 mm, a 30 V por 30 min e 100 V até o final da corrida. Para a obtenção das imagens, os géis foram corados com prata com o kit Silver Staining (GE Healthcare), de acordo com as instruções do fabricante.

2.6.3 Digestão das proteínas

Após as lavagens com 50 mM de bicarbonato e amônio em Amicon Ultra 3 K MWCO, item 2.6.1, a digestão das proteínas foi realizada de acordo com Cryar (2015). Cada amostra de proteína foi homogeneizada com 50 ml de bicarbonato de amônio 50 mM e DTT 10 mM e 0,25 % de rapigest. As amostras foram diluídas em 50 ml de bicarbonato de amônio e atingiram a concentração final de 0,1% rapigest. A digestão ocorreu a partir da incubação das amostras de proteínas com tripsina, enzima:proteína (1:50), a 37 °C por 16 h. As possíveis interferências presentes nas amostras foram removidas com a adição de ácido fórmico 0,1 %, e incubação a 60 °C por 60 min. Os detergentes insolúveis foram removidos das amostras por centrifugação a 10.000 g durante 30 min. O sobrenadante foi coletado e congelado a -80 °C para posterior identificação das proteínas por LC/MS.

2.6.4 Identificação por LC-MS/MS

As proteínas foram identificadas utilizando o sistema cromatográfico nanoElute nanoflow, da Bruker Daltonics, Bremen, Germany, acoplado online, a um espectrômetro de massa *hybrid trapped ion mobility spectrometry-quadrupole time-of-flight mass spectrometer-timsTof Pro* (Bruker Daltonics). Uma alíquota (1µL) de amostra, equivalente a 200 ng de peptídeos digeridos foi injetada em uma coluna Bruker FIFTEEN C18 column (1.9µm, 150 mm x 75 µm), da Bruker. Um gradiente típico RP (Solvente A: 0.1% AF, 99.9 % H_2O; Solvente B: 0.1 % AF, 99.9 % CH_3CN) foi estabelecido em um sistema de nanofluxo de cromatografia líquida e separado a uma taxa de fluxo de 500 $nL.min^{-1}$. A temperatura da coluna foi mantida a 50 °C. A corrida cromatográfica foi de 60 min (2 % a 30 % do Solvente B durante 55 min; elevada a 95 % aos 56 min; mantida nessa porcentagem de Solvente B por mais 4 min). A coluna foi acoplada, online, um timsTOF-Pro com uma fonte de íons CaptiveSpray, ambos da Bruker Daltonik GmbH. A temperatura da linha de transferência capilar de íons foi ajustada para 180 °C. O acúmulo de íons e a separação por mobilidade foram obtidos com uma rampa de potencial de entrada de -160V a -20V dentro dos 123s. Durante à aquisição, para habilitar o método PASEF, i.e., o acúmulo paralelamente à fragmentação dos íons, as informações de precursor de m/z e mobilidade foram primeiro derivadas de um experimento de full scan Tims-MS, com uma faixa de m/z de 100-1700. Precursores monocarregados foram excluídos por sua posição no plano m/z-ion de

mobilidade, e os precursores que atingiram o valor alvo de 20.000 a.u. foram dinamicamente excluídos por 0,4 min. O modo operacional do TIMS-TOF, MS e PASEF foram controlados e sincronizados com o auxílio do software OtofControl 5.1 de controle instrumental da Bruker Daltonik.

2.6.5 Parâmetros de processamento e busca em banco de dados públicos

O processamento dos dados, a identificação de proteínas e as análises de quantificação relativa foram realizadas utilizando-se o Software PEAKS studio, Version 10.6, Bioinformatics Solutions Inc., Waterloo, ON. Os parâmetros de processamento incluíram: carbamidometilação da cisteína como modificação fixa de aminoácidos. Já a oxidação da metionina e acetilação da região N- terminal foram consideradas como variações variáveis. A tripsina foi utilizada como enzima proteolítica, com o máximo de 2 possíveis erros de clivagem. A tolerância de desvio de massa de íons para peptídeos e fragmentos foi ajustada para 20 ppm e 0,05 Da, respectivamente. Uma máxima taxa de falso positivos (FDR) de 1 % foi utilizada para identificação de peptídeos e proteínas, considerando-se como critério, ao menos um peptídeo único para identificação de proteínas. Todas as proteínas foram identificadas com um grau de confiança ≥ 95 %, utilizando-se o algoritmo do PEAKS Software e busca dentro da base de dados de *Aspergillus* sp, pelo banco de dados Uniprot (http: //www.uniprot.proteomes/).

As proteínas identificadas foram agrupadas em categorias funcionais de acordo com suas funções e pesos moleculares utilizando o banco de dados (http: //www.uniprot.proteomes/).

2.6.6 *Análise da taxonomia do micro-organismo baseada em peptídeos*

Os peptídeos identificados foram analisados por ferramenta de bioinformática destinados à metaproteômica (https://unipept.ugent.be/) para verificar a taxonomia do micro-organismo estudado.

Após a identificação do micro-organismo foi feito a análise das proteínas identificadas contra o banco de dados desse micro-organismo (https://blast.ncbi.nlm.nih.gov/).

2.7 Hidrólise enzimática da quitosana

2.7.1 Extrato enzimático integral

A concentração de quitosana na mistura reacional foi de 1 % em relação ao tampão acetato de sódio (pH 4,5; 0,1M). A quantidade de proteína presente no extrato adicionada na mistura reacional, foi de 0,14 mg de proteína/g de quitosana. A hidrólise foi realizada de

acordo com o método de Roncal, Oviedo, Armentia, Fernández, & Villarán (2007), com modificações. Foi adicionado 1000 μL de extrato enzimático (*Ee*) em 100 mL de tampão acetato de sódio, em seguida foi adicionado 1000 mg de quitosana e incubado a 45 °C sob agitação (120 rpm) em diferentes tempos: 1; 2; 3; 4; 5; e 24 h. A reação de hidrólise foi interrompida através do aquecimento da mistura a 100 °C durante 5 min, para a inativação da enzima, e a amostra foi ajustada para pH 7, resultando em um precipitado de quitosana. O precipitado foi filtrado e seco a 60 ºC.

2.7.2 Enzima comercial

Foi utilizada a enzima Celuclast 1,5L como padrão de comparação com o extrato produzido nesse estudo.

A mistura reacional foi estabelecida em 1 % de quitosana em relação ao tampão acetato de sódio (pH 4,5; 0,1M). A quantidade de proteína presente na Celuclast 1,5L, adicionada na mistura reacional, foi de 0,51 mg de proteína/g de quitosana. A hidrólise foi realizada de acordo com o método de Roncal, Oviedo, Armentia, Fernández, & Villarán (2007), com modificações. Foi adicionado 20 μL da enzima Celuclast 1,5L (*Ec*) em 100 mL de tampão acetato de sódio, em seguida foi adicionado 1000 mg de quitosana e incubado a 45 °C, sob agitação (120 rpm) em diferentes tempos de incubação: 0,5; 1; 2; e 3 h. A reação foi interrompida através do aquecimento da mistura a 100 °C durante 5 min, para a inativação da enzima, e a amostra foi ajustada para pH 7, resultando em um precipitado de quitosana. O precipitado foi filtrado e seco a 60 ºC.

2.8 Caracterização da quitosana e seus oligômeros

2.8.1 Determinação da massa molecular

A massa molecular da quitosana (*Qt*) e da quitosana hidrolisada (*Qh*) foi determinada por viscosimetria conforme o método descrito por Garcia et al. (2018), com modificações. As amostras de *Qt* e *Qh* foram preparadas com concentração de 0,005 g/mL em solução tampão ácido acético 0,3 M e acetato de sódio 0,2 M, pH 4,5 e incubadas a 30 °C, sob agitação, 120 rpm, durante 24 h.

Para a determinação da viscosidade intrínseca, [η], as soluções de *Qt* e *Qh* foram diluídas nas concentrações de 0,004, 0,003, 0,002 e 0,001 g/mL e os tempos de escoamento das soluções foram determinados em viscosímetro capilar Canon Fensk (Schott AVS 350) a 25 °C. A viscosidade específica ($\eta_{sp)}$ foi determinada utilizando a Eq. 3.

$$\eta_{sp} = (t - t_0) / t_0 \qquad \text{Eq. (3)}$$

Onde: t é o tempo de escoamento da solução da quitosana e t_0 é o tempo de escoamento do solvente.

A viscosidade reduzida (η_{red}) é obtida através da relação entre a viscosidade específica e a concentração de quitosana (C), Eq. 4.

$$\eta_{red} = \eta_{sp} / C \quad \text{Eq. (4)}$$

A viscosidade intrínseca, [η], é definida como viscosidade reduzida, extrapolada para uma concentração de quitosana (C) de zero, Eq. 5.

$$[\eta] = (\eta_{sp} / C)_{c \to 0} = (\eta_{red})_{c \to 0} \quad \text{Eq. (5)}$$

A partir da viscosidade intrínseca foi calculado a massa molecular da quitosana através da equação de Mark-Houwink, Eq. 6.

$$[\eta] = KM_v^a \quad \text{Eq. (6)}$$

Onde: M_w é a massa molecular média da viscosidade; e K e α são constantes que dependem da polidispersão da quitosana e do sistema solvente utilizado. Os valores dessas constantes foram previamente determinados como sendo K = 0,074 e a = 0,76.

2.8.2 Espectroscopia de Infravermelho com Transformada de Fourier

As amostras de *Qt* e *Qh* foram analisadas por espectroscopia no infravermelho com Transformada de Fourier e Reflectância Total Atenuada (FTIR-ATR) na faixa de 4000 cm^{-1} a 400 cm^{-1}, resolução de 4 cm^{-1} e 32 varreduras. O equipamento utilizado foi o Cary 360 (Agilent) com cristal de seleneto de zinco (ZnSe).

2.8.3 Grau de desacetilação (GD)

O grau de desacetilação da *Qt* e *Qh* foi determinado por FTIR-ATR. As medidas das áreas dos sinais do espectro de infravermelho foram obtidas na absorção das amostras correspondentes aos grupos funcionais de amina (1350 cm^{-1}) e CH_2 (1465 cm^{-1}), o *GD* foi obtido utilizando-se a integração das áreas das bandas específicas, conforme motodologia descrita por Barragán, Fornué, & Ortega (2016), a integração foi realizada usando a linha de base dos picos calculado no software Spectragryph (v. 1.2.14 / 2020), estabelecendo os valores para os cálculos do grau de acetilação, por meio da Eq. 7.

$$A_{1350} / A_{1465} = 0{,}3822 + 0{,}0313\ GA \quad \text{Eq. (7)}$$

Onde:

GA: Grau de acetilação; A_{1350}: Área sob a curva da banda do espectro infravermelho com número de ondas de 1350 cm^{-1}; e A_{1465}: Área sob a curva da banda do espectro infravermelho com número de ondas de 1465 cm^{-1}. Os valores 0,3822 e 0,0313 foram obtidos por regressão linear (Brugnerotto et al., 2001).

O grau de desacetilação (GD) foi determinado através da Eq. 8.

Grau de desacetilação (%GD) = 100 - GA Eq. (8)

3 Resultados e discussão

3.1 Identificação do fungo filamentoso

A cepa foi identificada como *Aspergillus* sp (GenBank ID número de acesso MT135987) com similaridade maior que 97 %. A identificação desta cepa como gênero Aspergillus também corrobora os resultados do cultivo em meio líquido neste estudo.

Em vários estudos foi observado que o *Aspergillus* se destaca na produção de enzimas utilizadas na hidrólise da quitosana: *Aspergillus niger*, celulase (Xie, Wei, & Hu, 2010; Laokuldilok et al., 2017), hemicelulase (Roncal et al., 2007) e pectinase (Kittur et al., 2005); *Aspergillus nidulans*, quitina desacetilase (Liu et al., 2017); e *Aspergillus fumigatus*, quitosanase (Cheng et al., 2006).

Através da análise metaproteômica o micro-organismo foi identificado como *Aspergillus nidulans*, por meio da relação dos peptídeos identificados no extrato enzimático por LC-MS/MS, *item 2.6.6*, com a taxonomia do micro-organismo.

3.2 Extrato enzimático integral (*EEI*)

3.2.1 Seleção do meio de cultivo

A semelhança estrutural entre a celulose e a quitosana, polímeros de D-glicose unidos por ligações β-1,4-glicosídicas, possibilitou o uso da carboximetilcelulose como substrato nos dois meios de cultivos, *Czm* e *CPY*, para a produção de enzimas que serão posteriormente utilizadas na hidrólise da quitosana, sendo observado na Fig. 1 que o *Aspergillus nidulans* produziu enzimas com capacidade de hidrolisar a carboximetilcelulose, indicado pela atividade endoglucanásica.

Entre os meios de cultivo estudados, o czapeck modificado (*Czm*) apresentou maior atividade enzimática, Fig. 1. Além disso, observa-se o aumento da atividade enzimática até o 8º dia de cultivo, seguido de diminuição nos dias posteriores, sendo definido 8 dias de cultivo como o ideal, em meio *Czm*, para a produção do extrato integral nas condições estabelecidas.

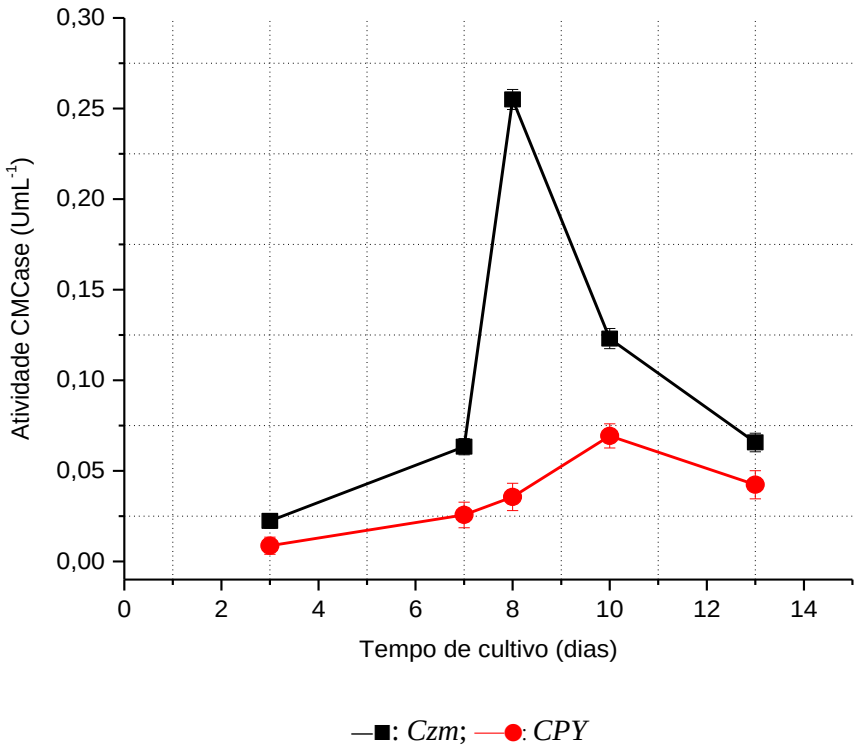


Figura 1. Perfil da atividade enzimática em diferentes meios de cultivo.

3.2.2 Extrato enzimático integral concentrado

O extrato enzimático produzido por *Aspergillus nidulans* em meio *Czm* durante 8 dias de cultivo apresentou atividade enzimática específica de 13,3 U mg^{-1}. Após a concentração por liofilização, o extrato enzimático apresentou atividade de 15,9 U mg^{-1}. A Tabela 1 mostra os resultados de atividade total (U mL^{-1}) e atividade específica (U mg^{-1}) para o extrato enzimático e o extrato enzimático concentrado. O teor de proteína total aumentou 8 vezes após a liofilização e houve aumento de 19 % na atividade específica. Este cultivo foi caracterizado por uma baixa produção de proteína total, o que contribuiu para bons resultados de atividade específica.

Tabela 1. Atividade enzimática após concentração do extrato.

Extrato	Volume extrato (mL)	Atividade Total (U mL^{-1})	Proteína Total (mg mL^{-1})	Atividade Específica (U mg^{-1})
Extrato enzimático	500,0	0,22 ± 0,031	0,0165 ± 0,001	13,3 ± 0,97
Extrato enzimático concentrado	25,0	2,23 ± 0,19	0,14 ± 0,013	15,9 ± 1,38

3.2.3 Análise do perfil eletroforético das proteínas

As proteínas precipitadas foram submetidas a SDS-PAGE para avaliar o perfil proteico, Fig. 2. No gel corado por prata foi observado proteínas abundantes, indicadas por setas, sendo identificadas 5 bandas em destaque. O perfil apresentado na Fig. 2 já demonstra a complexidade do extrato enzimático integral em relação ao número de bandas e suas intensidades. Esse resultado já era previsto, devido se tratar de um extrato enzimático integral, ou seja, somente a separação da biomassa foi realizada.

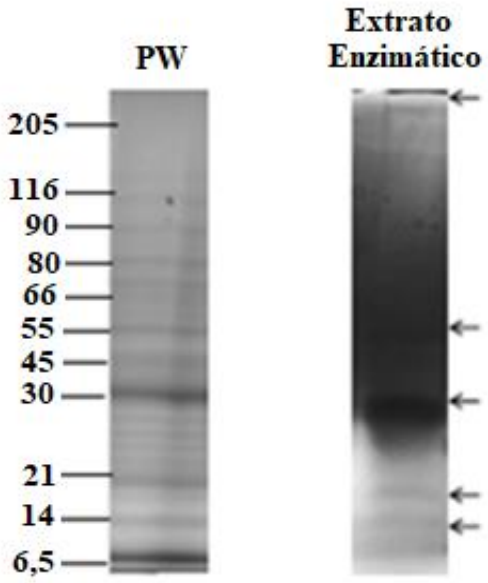


Figura 2. Perfil eletroforético das proteínas secretadas por *Aspergillus nidulans*, comparado com o marcador molecular proteico em kDa (PW).

3.2.4 Identificação das proteínas presentes no extrato enzimático integral

A análise por LC-MS/MS das proteínas secretadas por *Aspergillus nidulans* permitiram a identificação de várias proteínas, listadas na Tabela 2.

Entre as proteínas identificadas tem-se destaque para as quitinases, (E.C 3.2.1.14) e (E.C 3.2.1.52), responsáveis por catalisar a clivagem da quitina e quitosana (Lin, Lin, & Chen, 2009; Qu et al., 2021). De acordo com o tipo de clivagem podem ser classificadas em enzimas endo e exo. As endo-quitinases (E.C 3.2.1.14) catalisam a hidrólise interna das cadeias em pontos aleatórios ao longo do polissacarídeo, enquanto as exo-quitinases (E.C 3.2.1.52) hidrolisam a extremidades da cadeia polimérica, seja redutora ou não redutora (Kidibule et al., 2020).

Tabela 2. Proteínas secretadas por *Aspergillus nidulans* identificadas por LC-MS/MS.

Número de acesso	Proteína/micro-organismo	Função Molecular (E.C)	Peso Molecular (kDa)
Q873X9	Endochitinase B1 OS=*Neosartorya fumigata*	Glicosidase, Hidrolase (3.2.1.14)	47,62
E9QRF2	Endochitinase B1 OS=*Neosartorya fumigata*	Glicosidase, Hidrolase (3.2.1.14)	47,62
Q8J2T0	Beta-hexosaminidase OS=*Aspergillus oryzae*	Glicosidase, Hidrolase (3.2.1.52)	67,52
Q9HGI3	Beta-hexosaminidase OS=*Emericella nidulans*	Glicosidase, Hidrolase (3.2.1.52)	67,98
Q4WM08	Probable 1,4-beta-D-glucan cellobiohydrolase B OS=*Neosartorya fumigata*	Glicosidase, Hidrolase (3.2.1.91)	56,46
A1CU44	Probable 1,4-beta-D-glucan cellobiohydrolase B OS=*Aspergillus clavatus*	Glicosidase, Hidrolase (3.2.1.91)	57,04
O59843	1,4-beta-D-glucan cellobiohydrolase B OS=*Aspergillus aculeatus*	Glicosidase, Hidrolase (3.2.1.91)	57,10
A1DNL0	Probable 1,4-beta-D-glucan cellobiohydrolase B OS=*Neosartorya fischeri*	Glicosidase, Hidrolase (3.2.1.91)	56,05
A1CE97	Probable 1,4-beta-D-glucan cellobiohydrolase A OS=*Aspergillus clavatus*	Glicosidase, Hidrolase (3.2.1.91)	48,21
Q8NK02	1 4-beta-D-glucan cellobiohydrolase B OS=*Emericella nidulans*	Glicosidase, Hidrolase (3.2.1.91)	56,12
A1DMA5	Probable 1,4-beta-D-glucan cellobiohydrolase A OS=*Neosartorya fischeri*	Glicosidase, Hidrolase (3.2.1.91)	48,19
Q5B2Q4	Probable 1,4-beta-D-glucan cellobiohydrolase A OS=*Emericella nidulans*	Glicosidase, Hidrolase (3.2.1.91)	47,64
A2QAI7	Probable 1,4-beta-D-glucan cellobiohydrolase B OS=*Aspergillus niger*	Glicosidase, Hidrolase (3.2.1.91)	56,20
Q9UVS8	1,4-beta-D-glucan cellobiohydrolase B OS=*Aspergillus niger*	Glicosidase, Hidrolase (3.2.1.91)	56,22
Q2U7D2	Probable alpha-L-arabinofuranosidase axhA OS=*Aspergillus oryzae*	Glicosidase, Hidrolase (3.2.1.55)	35,36
Q9UVX6	Alpha-L-arabinofuranosidase axhA OS=*Aspergillus sojae*	Glicosidase, Hidrolase (3.2.1.55)	35,34
Q5AUX2	Alpha-L-arabinofuranosidase axhA-2 OS=*Emericella nidulans*	Glicosidase, Hidrolase (3.2.1.55)	35,56
P79019	Alpha-L-arabinofuranosidase axhA OS=*Aspergillus niger*	Glicosidase, Hidrolase (3.2.1.55)	35,84
Q5BA61	Pectin lyase B OS=*Emericella nidulans*	Liase (4.2.2.10)	39,35

Além da quitinase, foi identificada a enzima celobiohidrolase (E.C 3.2.1.91), também conhecida como exoglucanase. É a enzima responsável pela liberação de unidades de celobiose das extremidades redutoras e não redutoras da celulose (Beer et al., 2020). Esse tipo de enzima pode hidrolisar a quitosana levando à produção de oligômeros de baixo peso molecular. Além disso, foi verificado que o produto de hidrólise da quitosana através da celobiohidrolase é semelhante em comparação ao uso da quitosanase, enzima específica na hidrólise da quitosana (Tegl et al., 2016; Beer et al., 2020).

Outra enzima identificada foi a alfa-L-arabinofuranosidase (E.C 3.2.1.55). Essa enzima libera a L-arabinose e está envolvida na hidrólise de oligossacarídeos e hemiceluloses (Squillaci et al., 2017).

A pectina liase (E.C. 4.2.2.10), também identificada no extrato enzimático, catalisa a clivagem da ligação glicosídica α1-4 em ácido péctico e pectina (Bibi et al., 2019). Além da hidrólise da pectina, vários estudos verificaram que as pectinases podem ser utilizadas na hidrólise da quitosana (Kittur, Kumar, &Tharanathan, 2003; Tishchenko et al., 2011; Wang et al., 2020).

As proteínas identificadas apresentam, em sua maioria, função molecular de glicosil hidrolase com pesos moleculares variando de 35,34 a 67,98 kDa, segundo o banco de dados (http://www.uniprot.proteomes/) (Tabela 2). Esses pesos moleculares corroboram com os resultados encontrados em outros estudos: endoquitinase, 46 a 49 kDa (Sharma et al., 2016; Yan & Fong, 2018); exo-quitinases, 55 a 80 kDa (Liu et al., 2012; Venugopal et al., 2020); celobiohidrolase, 49 a 54 kDa (Jinzu et al., 2010; Sun et al., 2018); alfa-L-arabinofuranosidase, 35 a 35,5 kDa (Guerfali, Gargouri, & Belghith, 2011; Contesini et al., 2017); e pectina liase, 38 kDa (Yadav et al., 2008; Saharan & Sharma, 2019).

Dessa forma, pode-se concluir que o extrato enzimático é rico em enzimas que podem atuar na hidrólise da quitosana, com destaque para as endo-quitinases (E.C 3.2.1.14), exo-quitinases (E.C 3.2.1.52) e celobiohidrolase (E.C 3.2.1.91), pois vários estudos já utilizaram essas enzimas para a produção de oligômeros de quitosana verificando sua eficácia.

3.2.5 Comparação das proteínas identificadas contra o banco de dados de A. nidulans

Devido as análises taxonômicas, baseadas em peptídeos, indicarem que *A. nidulans* seja o micro-organismo de estudo, as proteínas identificadas que não pertenciam à *A. nidulans/ Emericella nidulans*, foram blastadas contra o banco de dados do *A. nidulans*. Todas as proteínas identificadas apresentaram cobertura de sequência superior a 84 % em relação às proteínas de *A. nidulans* (Tabela 3).

Tabela 3. Análise das proteínas identificadas contra banco de dados de *A. nidulans* (https://blast.ncbi.nlm.nih.gov/).

Dados das proteínas identificadas por LC-MS/MS no presente estudo		**Dados das análises de bioinformática das proteínas identificadas por LC-MS/MS no presente estudo contra banco de dados de *A. nidulans*.**		
Número de acesso	Proteínas identificadas – micro-organismo	Proteínas identificadas contra o banco de dados de *A. nidulans*	Número de acesso	Cobertura (%)
Q873X9	Endochitinase B1 OS=*Neosartorya fumigata*	Endochitinase B; AltName: Full=Chitinase B [*Aspergillus nidulans* FGSC A4]	G5EAZ3	90 %
E9QRF2	Endochitinase B1 OS=*Neosartorya fumigata*	Endochitinase B; AltName: Full=Chitinase B [*Aspergillus nidulans* FGSC A4]	G5EAZ3	90 %
Q8J2T0	Beta-hexosaminidase OS=A*spergillus oryzae*	Probable alpha-L-arabinofuranosidase axhA-1; AltName: Full=Arabinoxylan arabinofuranohydrolase axhA-1; Flags: Precursor [A*spergillus nidulans FGSC A4*]	Q5B9Z8	90 %
Q4WM08	Probable 1,4-beta-D-glucan cellobiohydrolase B OS=*Neosartorya fumigata*	1,4-beta-D-glucan cellobiohydrolase B; AltName: Full=Beta-glucancellobiohydrolase B; AltName: Full=Exocellobiohydrolase B; AltName: Full=Exoglucanase B; Flags: Precursor [*Aspergillus nidulans* FGSC A4]	Q8NK02	98 %
A1CU44	Probable 1,4-beta-D-glucan cellobiohydrolase B OS=*Aspergillus clavatus*	1,4-beta-D-glucan cellobiohydrolase B; AltName: Full=Beta-glucancellobiohydrolase B; AltName: Full=Exocellobiohydrolase B; AltName: Full=Exoglucanase B; Flags: Precursor [*Aspergillus nidulans* FGSC A4]	Q8NK02	98 %
O59843	1,4-beta-D-glucan cellobiohydrolase B OS=*Aspergillus aculeatus*	1,4-beta-D-glucan cellobiohydrolase B; AltName: Full=Beta-glucancellobiohydrolase B; AltName: Full=Exocellobiohydrolase B; AltName: Full=Exoglucanase B; Flags: Precursor [*Aspergillus nidulans* FGSC A4]	Q8NK02	100 %
A1DNL0	Probable 1,4-beta-D-glucan cellobiohydrolase B OS=*Neosartorya fischeri*	Full=1,4-beta-D-glucan cellobiohydrolase B; AltName: Full=Beta-glucancellobiohydrolase B; AltName: Full=Exocellobiohydrolase B; AltName: Full=Exoglucanase B; Flags: Precursor [*Aspergillus nidulans* FGSC A4]	Q8NK02	98 %

Tabela 3. Análise das proteínas identificadas contra banco de dados de *A. nidulans* (https://blast.ncbi.nlm.nih.gov/). Continuação.

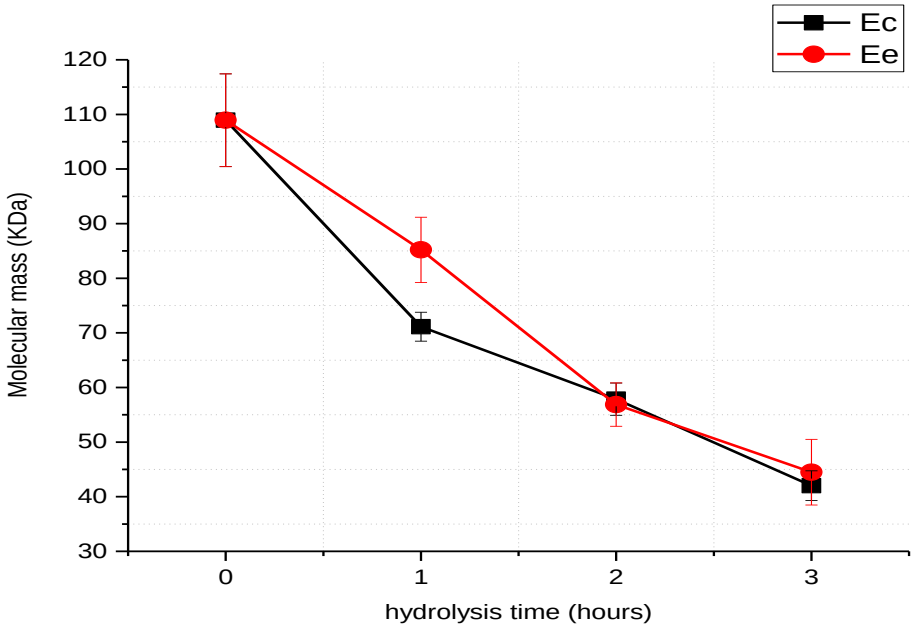
Dados das proteínas identificadas por LC-MS/MS no presente estudo		**Dados das análises de bioinformática das proteínas identificadas por LC-MS/MS no presente estudo contra banco de dados de *A. nidulans*.**		
A1CE97	Probable 1,4-beta-D-glucan cellobiohydrolase A OS=*Aspergillus clavatus*	Probable 1,4-beta-D-glucan cellobiohydrolase A; AltName: Full=Beta-glucancellobiohydrolase A; AltName: Full=Cellobiohydrolase D; AltName: Full=Exocellobiohydrolase A; AltName: Full=Exoglucanase A; Flags: Precursor [*Aspergillus nidulans* FGSC A4]	Q5B2Q4	99 %
A1DMA5	Probable 1,4-beta-D-glucan cellobiohydrolase A OS=*Neosartorya fischeri*	Probable 1,4-beta-D-glucan cellobiohydrolase A; AltName: Full=Beta-glucancellobiohydrolase A; AltName: Full=Cellobiohydrolase D; AltName: Full=Exocellobiohydrolase A; AltName: Full=Exoglucanase A; Flags: Precursor [*Aspergillus nidulans* FGSC A4]	Q5B2Q4	99 %
A2QAI7	Probable 1,4-beta-D-glucan cellobiohydrolase B OS=*Aspergillus niger*	1,4-beta-D-glucan cellobiohydrolase B; AltName: Full=Beta-glucancellobiohydrolase B; AltName: Full=Exocellobiohydrolase B; AltName: Full=Exoglucanase B; Flags: Precursor [*Aspergillus nidulans* FGSC A4]	Q8NK02	84 %
Q9UVS8	1,4-beta-D-glucan cellobiohydrolase B OS=*Aspergillus niger*	1,4-beta-D-glucan cellobiohydrolase B; AltName: Full=Beta-glucancellobiohydrolase B; AltName: Full=Exocellobiohydrolase B; AltName: Full=Exoglucanase B; Flags: Precursor [*Aspergillus nidulans* FGSC A4]	Q8NK02	89 %
Q2U7D2	Probable alpha-L-arabinofuranosidase axhA OS=*Aspergillus oryzae*	Probable alpha-L-arabinofuranosidase axhA-1; AltName: Full=Arabinoxylan arabinofuranohydrolase axhA-1; Flags: Precursor [A*spergillus nidulans* FGSC A4]	Q5B9Z8	90 %
Q9UVX6	Alpha-L-arabinofuranosidase axhA OS=*Aspergillus sojae*	Probable alpha-L-arabinofuranosidase axhA-1; AltName: Full=Arabinoxylan arabinofuranohydrolase axhA-1; Flags: Precursor [*Aspergillus nidulans* FGSC A4]	Q5B9Z8	90 %
P79019	Alpha-L-arabinofuranosidase axhA OS=*Aspergillus niger*	Probable alpha-L-arabinofuranosidase axhA-1; AltName: Full=Arabinoxylan arabinofuranohydrolase axhA-1; Flags: Precursor [*Aspergillus nidulans* FGSC A4]	Q5B9Z8	99 %

3.3 Hidrólise da quitosana

3.3.1 Comparação entre o extrato enzimático integral e celulase comercial

A enzima comercial (*Ec*), Celluclast 1,5 L®, foi utilizada como parâmetro para verificar a eficiência do extrato enzimático integral (*EEI*) produzido nesse estudo, na hidrólise da quitosana, pois essa enzima possui a capacidade de clivar a ligação β-1,4-glicosídica da quitosana (Lin; Lin e Chen, 2009), ou seja, mesma região onde atua a quitosanase (enzima específica para a hidrólise da quitosana).

Na Fig. 4 observa-se diminuição do peso molecular com o aumento do tempo de hidrólise de forma similar, independente do tipo de enzima utilizada. O peso molecular da quitosana hidrolisada por *EEI* e *Ec*, respectivamente, após o tempo de 1 h foi de 85,20 ± 5,97 e 71,14 ± 2,14 kDa e no tempo de 2 h foi de 56,87 ± 3,97 e 57,86 ± 2,95 kDa.



Ee: Extrato enzimático; Ec: Enzima comercial.

Figura 3. Comparação entre diferentes fontes de enzima na hidrólise da quitosana.

Apesar das enzimas presente nos extrato enzimáticos apresentarem atuação catalítica idêntica e a quantidade de proteína presente no extrato comercial, 0,51 mg de proteína/g de quitosana, ser maior do que a quantidade no extrato enzimático, 0,14 mg de proteína/g de quitosana, foi verificado que a partir de 2 h de hidrólise não houve diferença significativa ($p < 0,05$) entre o tipo de extrato utilizado.

Logo, devido a menor quantidade de enzimas presente no extrato enzimático integral, podemos concluir que essas enzimas, com destaque para: endo-quitinases (E.C 3.2.1.14); exo-quitinases (E.C 3.2.1.52); e celobiohidrolase (E.C 3.2.1.91), apresentaram maior eficiência na hidrólise da quitosana em relação a enzima comercial (Celluclast 1,5 L®).

3.3.2 Efeito do tempo de hidrólise através do EEI

A Fig. 5 apresenta o peso molecular da quitosana após diferentes tempos de hidrólise utilizando o *EEI.*

Foi observada rápida diminuição da massa molecular, em um curto período. A quitosana com massa molecular inicial de 108,94 kDa apresentou após o tempo de 2 h de hidrólise redução de 47,80 % (56,87 ± 3,97 kDa); 5 h de hidrólise essa diminuição foi de 75,24 % (26,86 ± 1,10 kDa); e 24 h foi possível reduzir 93,26 % (7,23 ± 2,57 kDa).

A eficiência na hidrólise deve-se a presença de enzimas que apresentam características de hidrólise de polissacarídeos e que já foram utilizadas na literatura para a produção de oligômeros de quitosana (Lin, Lin e Chen, 2009; Tegl et al., 2016; Beer et al., 2020; Qu et al., 2021). Isso mostra que o estudo atingiu seu objetivo em produzir um extrato com enzimas eficientes na hidrólise da quitosana.

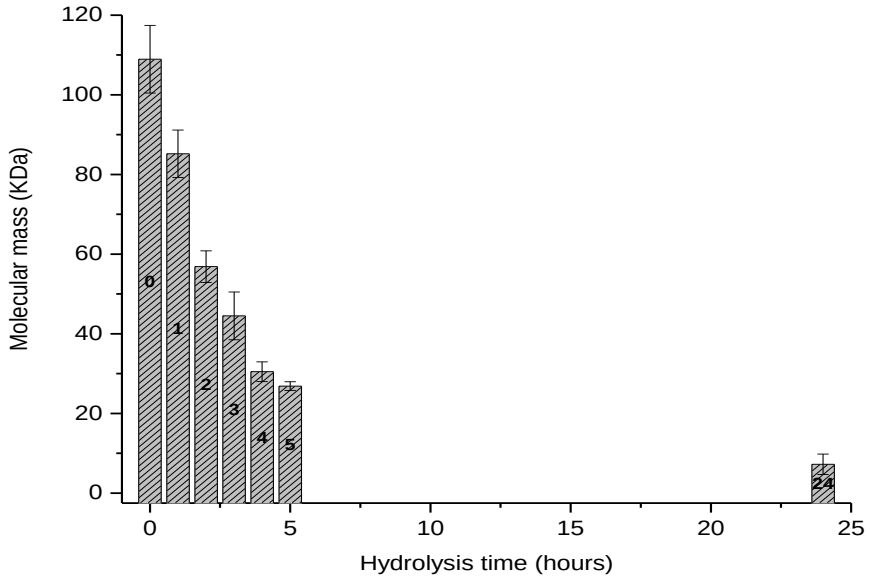


Figura 4. Efeito do tempo de hidrólise no peso molecular da quitosana.

3.3.3 Espectroscopia de Infravermelho com Transformada de Fourier

As estruturas químicas da quitosana e seus oligômeros obtidos pelo *EEI* foram avaliadas por FTIR sendo analisadas as bandas de absorção (Fig. 6).

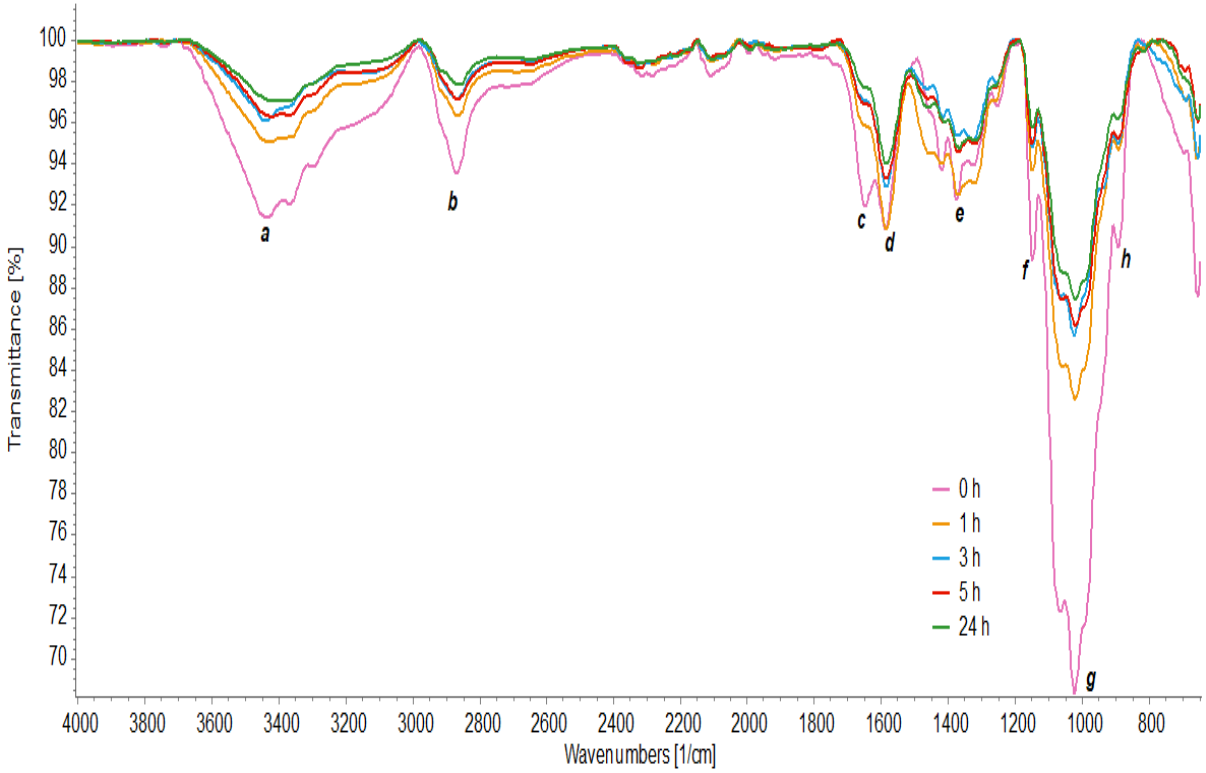


Figura 5. Espectro de FT-IR da quitosana hidrolisada nos tempos de 0 h, 1 h, 3 h, 5 h e 24 h.

Um padrão quase similar entre os espectros foi observado no pico 3435 cm^{-1} (*a*) derivado da vibração de alongamento O–H, mesclada com a banda de alongamento N–H (Ismail, 2019). As bandas 2870 cm^{-1} (*b*) foram atribuídas ao alongamento dos grupos C–H (Águila-Almanza, Salgado-Delgado, Vargas-Galarza, García-Hernández, & Hernández-Cocoletzi, 2019).

Os espectros também apresentaram bandas características dos grupos amida, incluindo: banda característica de alongamento de C=O (amida I) em 1648 cm^{-1} no espectro da quitosana com deslocamento desse pico na quitosana hidrolisada para 1660 cm^{-1} (*c*); em 1589 cm^{-1} (*d*) apresentou vibrações de flexão de N–H acopladas às vibrações de alongamento de C–N (amida II); e em 1330 cm^{-1} (*e*) observa-se característica de amida III (Xu, Mohan, Pitts, Udenigwe, & Mason, 2020).

Além disso, foram obtidas bandas de absorção em 1150 cm^{-1} correspondente ao estiramento assimétrico da ligação C–O–C (*f*), 1067 e 1024 cm^{-1} correspondente à vibração esquelética envolvendo o estiramento C–O (*g*), característica de sua estrutura sacarina (Luo, Han, Zeng, Yu, & Kennedy, 2010), e em 893 cm^{-1} (*h*) tem-se a absorção especial das ligações glicosídicas β-1,4 (Li et al., 2019).

A partir dos resultados observa-se que as características de quitosana foram identificadas nos espectros, o que confirma sua identidade química antes e após a hidrólise,

sendo que em todos os espectros os valores de número de ondas não sofreram deslocamento significativos.

Algumas diferenças foram verificadas entre a quitosana e seus oligômeros. Observam-se os sinais espectrais dos oligômeros de quitosana com menor absorbância devido os grupos funcionais vibrarem com maior liberdade de movimento, pois o impedimento estérico da cadeia polimérica diminuiu. A intensidade do sinal em 3465 cm^{-1} é devido a um maior número de grupos O–H, enquanto a intensidade do sinal em 3364 cm^{-1} está associada ao maior número de unidades N–H (Águila-Almanza, Salgado-Delgado, Vargas-Galarza, García-Hernández, & Hernández-Cocoletzi, 2019).

A banda de absorção a 3435 cm^{-1} deslocou-se para menor número de ondas nos tempos de 5 h e 24 h de hidrólise, indicando que a ordem cristalina natural da quitosana foi destruída (Li et al., 2019). Além disso, a intensidade de absorção relativa à banda de estiramento C–H em 1380 cm^{-1} diminuiu nos tempos de 3 h, 5 h e 24 h, o que indicou que as pontes de hidrogênio intermoleculares e intramoleculares da quitosana foram enfraquecidas e sua cristalinidade foi reduzida (Luo, Han, Zeng, Yu, & Kennedy, 2010).

3.3.4 Grau de desacetilação

Em relação ao grau de desacetilação, foi utilizado os espectros de FTIR e feito sua determinação através da relação entre os valores de integração dos picos em 1350 cm^{-1} e 1465 cm^{-1} segundo a Eq. 8. Os valores observados estão apresentados na Tabela 4. A correlação entre as áreas integradas nos números de ondas descritos e o grau de acetilação ainda não está totalmente esclarecida. Contudo, Brugnerotto et al. (2001) mostraram que esta relação, comparada com outras no espectro do infravermelho, foi a que apresentou maior valor de correlação linear para diferentes biopolímeros de crustáceos.

Tabela 4. Grau de desacetilação (GD) em relação ao tempo de hidrólise utilizando Ee.

Tempo de hidrólise	**Área Integrada (A_{1345})**	**Área Integrada (A_{1460})**	**GD (%)**	**CV (%)**
Sem hidrólise	1.051	1.062	80	0,49
1 hora	1.030	1.154	83	3,23
3 horas	1.175	1.150	79	1,74
5 horas	991,8	1,010	80	0,49
24 horas	1.220	1.991	80	0,49

A_{1345}: Valor da área integrada para o pico de 1345 cm^{-1}; A_{1460}: Valor da área integrada para o pico de 1460 cm^{-1}; GD: grau de desacetilação; e CV: Coeficiente de variação amostral.

Pode ser visto na Tabela 4 que houve uma deslocamento de 5 cm^{-1} dos valores nos picos das áreas de integração, ou seja, foram considerados os valores de 1345 cm^{-1} e 1460 cm^{-1} e não 1350 cm^{-1} e 1465 cm^{-1} como preconiza a Eq. 8. Variações desta magnitude são passíveis de ocorrerem em função do grau de pureza da mostra em comparação com uma amostra padrão. Neste estudo optamos em utilizar uma quitosana com valor comercial não muito elevado, mas este fato não influenciou nas características vibracionais dos grupos funcionais.

Os valores de GD mostram que não houve variação na desacetilção do polímero quando se compara a quitosana antes e após a hidrólise. Este resultado ratifica a idéia da especificidade da hidrólise enzimática; neste caso, em ligações glicosídicas frente à uma hidrólise química convencional com a possibilidade de se obter valores não desejados de GD.

4 Conclusão

O fungo utilizado para a produção do *EEI* foi identificado como *Aspergillus* sp (GenBank ID número de acesso MT135987) e através da análise metaproteômica o micro-organismo foi identificado como *Aspergillus nidulans*.

No extrato produzido por esse fungo foram identificadas as enzimas endo-quitinases, exo-quitinases, celobiohidrolase, alfa-L-arabinofuranosidase e pectina liase. São enzimas com função molecular de glicosil hidrolase e já foram utilizadas em outros estudos na hidrólise da quitosana.

O *EEI* apresentou maior eficiência na hidrólise da quitosana em relação a enzima comercial (Celluclast 1,5 L®). Isso se deve ao fato de que, apesar da celulase comercial possuir característica de hidrólise similar a quitosanase, o extrato enzimático apresenta as enzimas endo e exo-quitinases que são enzimas específicas que catalisam a hidrólise da cadeia interna e externa da quitosana.

Os oligômeros obtidos pela hidrólise utilizando o *EEI* apresentaram menor absorbância dos sinais espectrais devido os grupos funcionais vibrarem com maior liberdade de movimento, além disso, sua cristalinidade foi reduzida e manteve-se seu grau de desacetilação.

Logo, podemos concluir que o *EEI* apresentou eficiência na hidrólise da quitosana com viabilidade para uso industrial pois é um processo com poucas etapas o que reduziria o custo.

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