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CARACTERIZAÇÃO DOS EFEITOS DE QUITOSANAS NA INIBIÇÃO DE FUNGOS FITOPATOGÊNICOS

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Tese de Doutorado apresentada à Faculdade de Engenharia Química como parte dos requisitos exigidos para a obtenção do título de Doutor em Engenharia Química

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Este exemplar corresponde à versão final da Tese de Doutorado em Engenharia Química.

Profª. Drª. Telma Teixeira Franco – Orientadora

Prof.. Dr. Carlos Raimundo Ferreira Grosso – Co-orientador

“A DEUS

A Enio e Maria, meus pais

A Vicentina, Conceição, José Carlos,

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A Anselmo, Leandro, Aline, Mateus e Rafael, meus sobrinhos

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"A teoria sempre acaba, mais cedo ou mais tarde, assassinada pela experiência."

Albert Einstein

RESUMO

Na primeira etapa deste trabalho foi feita uma revisão de literatura referente à atividade antifúngica de quitina, quitosana e seus derivados contra diferentes tipos de microrganismos, como bactérias, fungos e leveduras. Nesta revisão são descritos importantes desenvolvimentos relativos a aplicação de quitosana e seus derivados como substância antimicrobiana contra bactérias, fungos e leveduras, hipóteses envolvidas na suas atividades antimicrobianas e efeitos na qualidade e armazenagem de vegetais frescos tratados com quitosana e seus derivados. A segunda etapa deste trabalho se refere a produção e caracterização de quitosanas com diferentes graus de polimerização (DP) obtidas por tratamento térmico e quitosanas com diferentes graus de acetilação (F_A) obtidas por desacetilação alcalina. A terceira etapa deste trabalho refere-se à avaliação dos efeitos da atividade antifúngica de quinze amostras de quitosana com diferentes graus de polimerização e diferentes graus de acetilação contra quatro fungos fitopatogênicos (*Alternaria alternata, Botrytis cinerea, Penicillium expansum* e *Rhizopus stolonifer*). Os crescimentos fúngicos foram avaliados em micro placas de 96 reservatórios e medidos em leitor de microplacas. A atividade antifúngica de quitosana aumentou com o decréscimo do F_A. O efeito da combinação de quitosana com menor F_A e maior DP teve a mais alta atividade fungistática contra os fungos *A. alternata* e *B. cinerea*. Os resultados tendem a indicar que os grupamentos amino protonados (NH_3^+) foram um importante fator envolvido no efeito antifúngico. Os fungos mais resistentes foram *Penicillium expansum* e *Botrytis cinerea* e os mais sensíveis foram *Alternaria alternata* e *Rhizopus stolonifer.* Na quarta etapa deste trabalho foi investigado o efeito de quitoligômeros no crescimento dos mesmos fungos analisados na terceira etapa. Verificou-se que misturas de quitoligômeros de DP 5 a 8, DP 2 a 12 e de DP 2 a 11, apresentaram baixo ou nenhum efeito no crescimento fúngico na concentração máxima de 1,000 $\mu g \times mL^{-1}$. A última etapa deste trabalho refere-se às mudanças morfológicas nas hifas dos fungos tratados com quitosana. As micrografias obtidas por microscopia eletrônica de varredura com emissão de campo mostraram que os micélios fúngicos tratados com quitosana estavam agregados, excessivamente ramificados, inchados e de comprimentos reduzidos.

Palavras-chave: quitina, quitosana, quitoligômeros, tratamento térmico, desacetilação alcalina, atividade antifúngica, fungos fitopatogênicos, morfologia fúngica.

ABSTRACT

In the first step of this work, a literature review concerning antimicrobial activity of chitin, chitosan and their derivatives against different groups of microorganisms, such as bacteria, yeast and fungi was made. Important developments related to applications of chitosan and their derivatives, as an antimicrobial substance against bacteria, fungi and yeasts, hypotheses involved in the antimicrobial activities and effects on storability and quality of fresh vegetables treated with chitosan and its derivatives are described in this present review. The second step of this work, concerns the production of chitosans with different degrees of polymerization (DP) by thermal treatment and chitosans with different degrees of acetylation (F_A) by alkaline deacetylation. The third step of this work, concerns the evaluation of antifungal effects of fifteen chitosans with different degrees of polymerization and different degrees of acetylation against four phytopathogenic fungi (*Alternaria alternata, Botrytis cinerea, Penicillium expansum* and *Rhyzopus stolonifer*) by using the 96-well microtiter plate and a microplate reader. Antifungal activity of chitosans increased with F_A decreasing. Combination effect of chitosan with low F_A and high DP showed the highest fungistatic activity against *A. alternata* and *B. cinerea*. The results indicated that free amino groups protonated (NH_3^+) were an important factor for antifungal effect. The most resistant fungi were *Penicillium expansum* and *Botrytis cinerea* and the most sensitive fungi were *Alternaria alternata* and *Rhizopus stolonifer*. In the fourth step of this work, it was investigated the effect of chitooligomers in the fungal growth of the same target fungi analyzed in the third step. The results suggested that chitooligomer mixtures of DP 5 to 8, DP 2 to 12 and DP 2 to 11 showed low or no effect on the fungal growth at concentration of 1,000 $\mu g \times mL^{-1}$. The last step of this work, concerns the morphological changes on hyphae of fungi treated with chitosan. Mycelial aggregation and morphological structural changes as excessive branching, swelling of the cell wall and hyphae size reduction were observed in the micrographs obtained by scanning electron microscopy field emission.

Key-words: chitin, chitosan, chitooligomers, thermal treatment, alkaline deacetylation, antifungal activity, phytopathogenic fungi, fungal morphology.

SUMÁRIO

NOMENCLATURA

AV	viscosidade aparente	mPa × s
F_A	fração molar dos grupos N-acetil-D-glicosamina ou grau médio de acetilação	(-)
DR	taxa de despolimerização	% × h^{-1}
KV	viscosidade cinemática	cSt
Mv	massa molar média viscosimétrica	g × mol^{-1}
Mm	Massa molar média	g × mol^{-1}
Mw	massa média da massa molar	g × mol^{-1}
C	concentração da solução de quitosana	g × dm^{-3}
m_0	massa do filtro seco	G
m_1	massa da amostra de quitosana	G
m_2	massa do filtro contendo partículas insolúvies depois da secagem	G
t_1	tempo de fluxo da solução de quitosana	S
t_0	tempo de fluxo do solvente	S
K	constante de Mark-Houwink	dm^3 × g^{-1}
a	constante de Mark-Houwink	(-)
m_{ch}	massa da amostra de quitosana	G
M_{ch}	massa molar da grupo glicosamina	161 g × mol^{-1}
V_{NaOH}	volume da solução de NaOH	cm^3
M_{NaOH}	concentração molar da solução de NaOH	mol × dm^{-3}
DP	grau de polimerização	
GPC-RI	cromatografia de permeação em gel com detector de índice de refração	
MALDI TOF MS	matrix-assisted laser desorption ionization time of flight mass spectrometry	
MC	meio completo	
MEA	ágar extrato de malte	
MIC	concentração inibitória mínima	
PDA	ágar dextrose batata	
RMN ^1H	Espectroscopia de ressonância magnética nuclear de próton	
PT	titulação potenciométrica	
Letras gregas		
$[\eta_i]$	viscosidade intrínseca	dm^3 × g^{-1}
$[\eta_L]$	número da viscosidade limite	dm^3 × g^{-1}

CAPÍTULO I

1 INTRODUÇÃO

A descoberta de compostos antimicrobianos naturais devido a crescente procura pelos consumidores por alimentos sem conservantes químicos tem conduzido a inúmeras pesquisas. Neste contexto, a atividade antimicrobiana de quitina, quitosana e seus derivados contra diferentes tipos de microrganismos, como bactérias, fungos e leveduras têm recebido considerável atenção. Nesta revisão estão descritos importantes desenvolvimentos relativos à aplicação de quitosana e seus derivados como substância antimicrobiana contra bactérias, fungos e leveduras, hipóteses envolvidas na sua atividade antimicrobiana e efeitos na qualidade e armazenagem de vegetais frescos tratados com os mesmos.

1.1 Revisão bibliográfica

Os polímeros quitina e quitosana e quitoligômeros têm sido bastante estudados devido ao elevado potencial de aplicações em indústrias alimentícias, farmacêuticas, cosméticas e na agricultura. As aplicações desses compostos em várias áreas, sobretudo a aplicação de quitosana, justifica-se pelo baixo custo de produção, a qual é produzida a partir dos descartes do processamento de crustáceos, fonte abundante e renovável. Em geral, quitosanas comerciais estão disponíveis nas faixas de massas molares entre 50 e 2.000 kDa e graus de acetilação (F_A) entre 0,1 a 0,4 (Rege & Block, 1999).

Os polímeros quitina e quitosana (Figura 1) são aminoglicopirananas compostas dos monômeros N-acetil-D-glicosamina (GlcNac) e D-glicosamina (GlcN) (Figura 2). Os polímeros podem ser distinguidos por suas solubilidades em solução aquosa de ácido acético 1% (v/v). Quitina, contendo um número ≥ 40% de GlcNac ($F_A \geq 0.4$) é insolúvel, visto que os polímeros solúveis são chamados de quitosana (Peter, 2002a). Quitosana é composta de três grupos funcionais reativos, um grupo amino e dois grupos hidroxilas primário e secundário nas posições dos carbonos C-2, C-3 e C-6 respectivamente (Furusaki

et al., 1996). Processos químicos e biotecnológicos estão sendo atualmente investigados para a produção de quitosana. Industrialmente quitosana é produzida a partir da desacetilação alcalina de quitina (hidrólise alcalina), porém quitosana também pode ser obtida a partir da desacetilação enzimática de quitina, processo este investigado em pesquisas laboratoriais (Figura 1).

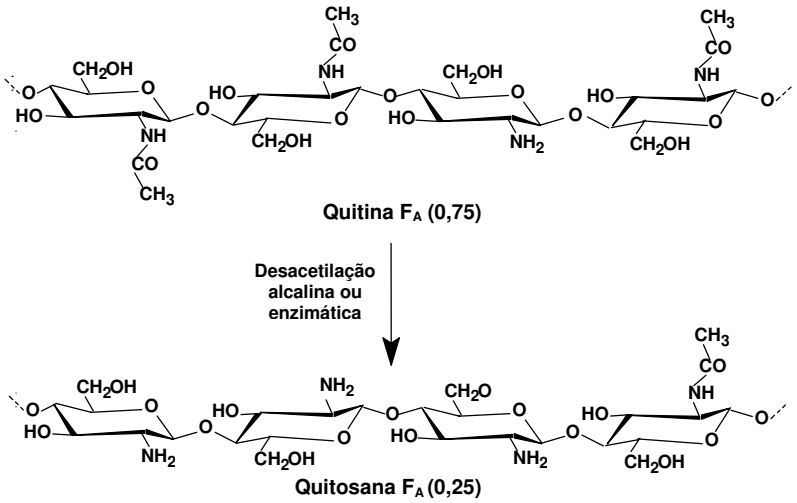

FIGURA 1 Estrutura de quitina e quitosana

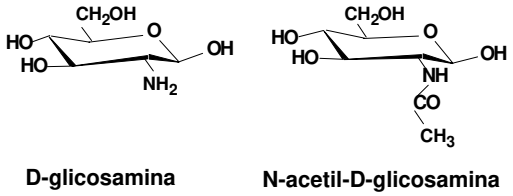

FIGURA 2 Estrutura dos monômeros D-glicosamina e N-acetil-D-glicosamina

O polímero quitina é também largamente encontrado em fungos, ocorrendo em *Basidiomicetos*, *Ascomicetos* e *Ficomicetos* sendo um componente da parede celular e estrutural das membranas micelianas, hastes e esporos (Peter, 2002b). Os teores variam entre traços até 45% da fração orgânica e o restante é composto majoritariamente por proteínas, glucanas e mananas (Roberts, 1992). Porém, nem todos os fungos contêm quitina, a qual pode estar ausente em algumas espécies próximas a outras que contenham quitina como componente da parede celular (Peter, 2002b).

Após a descoberta da atividade antimicrobriana de quitosana e seus derivados por Allan & Hadwiger (1979), Kendra & Hadwiger (1984) e Uchida et al. (1989), muitos pesquisadores têm feito estudos neste campo. Neste contexto a atividade antimicrobiana de quitina, quitosana e seus derivados contra diferentes grupos de microorganismos como bactérias, leveduras e fungos receberam considerável atenção (Yalpani et al., 1992).

1.2 Atividade antibacteriana de quitosanas e quitoligômeros

A procura por alimentos sem preservativos artificiais levou a esforços na descorberta de novos compostos antimicrobianos naturais (Wang, 1992). Diversos estudos têm sido realizados com o intuito de se avaliar a atividade antimicrobiana de quitina, quitosana e seus derivados contra bactérias gram-positivas e gram-negativas (Chang et al., 1989; Uchida et al., 1989; Papineau et al., 1991; Wang, 1992; Darmadji & Izumimoto, 1994; Simpson et al., 1997; No et al. 2002; Xie et al., 2002; Liu et al., 2004; Devlieghere et al., 2004; Qi et al., 2004).

Altas concentrações de quitosana (1-1,5% m/v) são necessárias para se obter completa inativação de *Staphylococcus aureus* depois de dois dias de incubação nos pHs 5,5 e 6,5 (Wang, 1992). Em outro estudo, concentrações de quitosana maiores ou iguais a 0,005% (m/v) foram suficientes para estimular a completa inativação de *Staphylococcus aureus* (Chang et al., 1989). Estes autores também avaliaram o efeito de quitosana na inibição do crescimento de *Escherichia coli*, que foi completamente inibido na concentração de 0,005% (m/v). Entretanto, de acordo com os resultados encontrados por Simpson et al. (1997), as bactérias *E. coli* e *Proteus vulgaris* apresentaram um crescimento mínimo na concentração de 0,005% (m/v) e foram completamente inibidas à concentração de 0,0075% (m/v). O efeito de quitosana na inibição de *E. coli* tem sido analisado em diversos estudos. Wang (1992) observou que a bactéria teve seu crescimento completamente inibido depois de 2 dias de incubação na concentração de 1% (m/v) em pH 5,5. Porém, Darmadji & Izumimoto (1994) encontraram que a concentração de 0,1% (m/v) inibiu o crescimento de *E. coli* e Simpson et al. (1997) observaram que somente 0,0075% (m/v) de quitosana foi necessário para inibir completamente o crescimento da bacteria. As

variações dos resultados observados nos estudos citados anteriormente podem ser explicadas pelos diferentes graus de acetilação das amostras de quitosana usadas; quitosana com F_A 0,075 (Simpson et al., 1997) foi considerada a mais ativa do que quitosana com F_A 0,15 (Darmadji & Izumimoto, 1994).

Seo et al. (1992) testaram o efeito de quitosana no crescimento de onze diferentes bactérias e encontraram que as concentrações inibitórias mínimas (MIC) variaram de 0,001 a 0,1% (m/v). Entre os organismos testados, os crescimentos de *E. coli*, *Pseudomonas fluorescens*, *Bacillus cereus*, e *S. aureus* foram inibidos completamente nas concentrações 0,002; 0,05; 0,1 e 0,002% (m/v), respectivamente. Uchida et al. (1989) relataram que as MIC de quitosana para *E. coli* e *S. aureus* foram de 0,025% (m/v) e 0,05% (m/v), respectivamente. Jeon et al. (2001) observaram que os valores de MIC de quitosana foram menores ou iguais a 0,06% (m/v) contra bactérias gram-positivas e gram-negativas. Segundo Wang (1992), diferentes resultados obtidos em estudos de atividade antibacteriana de quitosana podem estar provavelmente associados aos diferentes métodos experimentais adotados, amostras de quitosana ou pHs dos meios. Acredita-se também que as diferenças de resultados obtidos nos trabalhos citados anteriormente, apesar de microrganismos da mesma espécie terem sido utilizados, possam estar relacionadas às cepas empregadas, o tempo em que as mesmas estiveram armazenadas, o método empregado na preservação (óleo mineral, ultracongelamento, liofilização, etc), assim como o número de repiques realizados até a utilização dos microrganismos.

A relação entre massa molar (Mm) e atividade antimicrobiana de quitoligômeros foi estudada por Jeon et al. (2001), os quais relataram que quitoligômeros de Mm entre 1 a 10 kDa atuaram na inibição de microrganismos e sua eficácia aumentou proporcionalmente com a massa molar. Uchida et al. (1989) relataram que o quitoligômero II (mistura de trímeros a tetrâmeros) não mostrou atividade contra *E. coli* na concentração de 0,5% (m/v), enquanto que o oligômero I (mistura de tetrâmeros a heptâmeros) possuiu atividade antibacteriana na mesma concentração. No et al. (2002) examinaram a atividade antibacteriana de quitosanas e quitoligômeros com diferentes massas molares no crescimento de quatro bactérias gram-negativas (*Escherichia coli*, *P. fluorescens*, *Salmonella typhimurium*, e *Vibrio parahaemolyticus*) e sete bactérias gram-positivas (*Listeria monocytogenes*, *Bacillus megaterium*, *B. cereus*, *Staphylococcus aureus*,

Lactobacillus plantarum, *L. brevis*, e *L. bulgaricus*). Em se tratando de bactérias gram-negativas, a quitosana de Mm 746 kDa mostrou-se mais efetiva contra *E. coli* e *P. fluorescens*, comparado com a quitosana de Mm 470 kDa contra *S. typhimurium* e *V. parahaemolyticus*. Quitosanas cujas Mm eram 1.106 e 224 kDa apresentaram fraca ou nenhuma atividade antibacteriana como observado com a quitosana de Mm 28 kDa contra *S. typhimurium*. Ao contrário da resposta das bactérias gram-negativas, o crescimento das bactérias gram-positivas foi quase ou completamente inibido por todas as amostras de quitosana com diferentes Mm. Com bactérias gram-negativas, a atividade antibacteriana pareceu aumentar com a redução da Mm; porém, esta tendência não foi observada com bactérias gram-positivas. A atividade antibacteriana de quitoligômeros variou dependendo da massa molar e particularmente, da bactéria testada; porém, quitoligômeros de 1 kDa apresentaram relativamente maior contra bactérias gram-negativas, enquanto quitoligômeros de 4 e 2 kDa foram mais ativos contra bactérias gram-positivas comparados com outras massas molares.

A insolubilidade de quitosana em água é desvantajosa para sua aplicação como agente antibacteriano. A atividade antimicrobiana de quitosanas solúveis em água como lactato de quitosana, hidroglutamato de quitosana e quitosana derivada do fungo *Absidia coerulea* foi avaliada em diferentes culturas bacterianas (Sudharshan et al., 1992). Os autores observaram que glutamato e lactato de quitosana apresentaram atividade bactericida, contra bactérias gram-positivas e gram-negativas, e que as reduções dos crescimentos foram de 1 a 5 ciclos logaritmos no período de 1 hora. No mesmo estudo, foi relatado que a quitosana não apresentou atividade bactericida em pH 7 por duas razões principais, a presença de uma porção significativa de grupos amino não carregados e a baixa solubilidade de quitosana. Estes resultados estão de acordo com os encontrados em estudo similar realizado por Papineau et al. (1991), no qual a concentração de 0,2 mg × mL^{-1} de lactato de quitosana reduziu a população de *E. coli* a 2 e 4 ciclos logaritmos durante 2 e 60 minutos de exposição, respectivamente. Foi observado que glutamato de quitosana era efetivo contra cultura de leveduras como *Saccharomyces cerevisiae* e *Rhodotorula glutensis*. A inativação completa das leveduras mencionadas anteriormente foi obtida em 17 minutos expondo-se as culturas a 1 mg × mL^{-1} de lactato de quitosana. Porém, ao contrário

dos resultados encontrados por Sudharshan et al. (1992), Papineau et al. (1991) observaram que hidroglutamato de quitosana foi um antagonista mais efetivo que lactato de quitosana.

Os efeitos antibacterianos de quitosana F_A 0,31 produzida a partir de quitina de camarão, quitosanas com graus de sulfonação de 0,63% (SC1) e 13,03% (SC2), e sulfobenzoila de quitosana na preservação de ostras foram relatados por Chen et al. (1988). Foi observado que com a exceção de *Bacillus cereus*, pelo menos um dos compostos citados inibiu o crescimento bacteriano efetivamente na concentração de 0,02% (m/v). Embora a sulfonação tenha aumentado a solubilidade da quitosana, atividade antibacteriana totalmente diferentes foram observadas para as amostras SC1 e SC2. Para a maioria das culturas bacterianas, a amostra SC1 apresentou pronunciada concentração inibitória mínima a 0,02% (m/v), enquanto SC2 não exibiu efeito antibacteriano para concentrações abaixo de 0,2% (m/v). Os autores sugeriram que a amostra SC2, por possuir maior número de grupos sulfonila, e como conseqüência maior número de cargas negativas, teria maior força repulsiva entre as moléculas carregadas negativemente e as paredes celulares bacterianas do que a amostra SC1.

Xie et al. (2002) prepararam hidroxipropil quitosana, derivado de quitosana solúvel em água, e avaliaram sua atividade contra bactérias. A atividade antibacteriana de quitosana e seus derivados, hidroxipropila de quitosana (HPCTS) e hidroxipropila de quitosana – ácido maleico de sódio (HPCTS-MAS 1, HPCTS-MAS 2, HPCTS-MAS 3) contra *S. aureus* e *E. coli* foi analisada pelos métodos "cut plug" diâmetro das zonas de inibição e contagem de células viáveis. Os autores observaram que a amostra HPCTS não era eficiente contra as bactérias testadas e o derivado HPCTS-MAS 1 apresentou pequenos diâmetros de zonas de inibições. Já os derivados HPCTS-MAS 2 e HPCTS-MAS 3 tiveram aproximadamente os mesmos diâmetros de inibição, todavia os derivados foram mais efetivos contra *E. coli* do que contra *S. aureus*. A contagem de células viáveis de *S. aureus* e *E. coli* tratados com o copolímero HPCTS-MAS 3 foi determinada em diferentes concentrações. Aproximadamente 10^8 células × mL^{-1} de *S. aureus* e *E. coli* foram tratadas com 10,1μg × mL^{-1} e 100ng × mL^{-1} do copolímero em água estéril. Os autores observaram que houve redução do número de células viáveis com o aumento do tempo de contato do copolímero nas três diferentes concentrações testadas. Mais de 99,9% das células de *S.*

aureus e *E. coli* estavam mortas após 30 minutos de contato com o copolímero na mais baixa concentração (100 ng × mL^{-1}).

Qi et al. (2004) avaliaram a atividade antibacteriana *in vitro* de quitosana, nanopartículas de quitosana (CNP) e nanopartículas de quitosana contendo cobre (CNP-Cu) contra *E. coli* K88, *E. coli* ATCC 25922, *S. choleraesuis* ATCC 50020, *S. typhimurium* ATCC 50013 e *S. aureus* ATCC 25923. As CNP e CNP-Cu exibiram maior atividade antibacteriana que quitosana e segundo os autores, a superfície negativamente carregada da célula bacteriana é o sítio alvo do policátion. A atividade antibacteriana de quitosana, CNP e CNP-Cu, contra *E. coli*, *S. choleraesuis*, *S. typhimurium* e *S. aureus* foi avaliada pelo cálculo da concentração inibitória mínima (MIC) e da concentração bactericida mínima (MBC). Os valores de MIC em pH 5.0 variaram dependendo do microrganismo de 8 a 16 μg × mL^{-1} para quitosana, 0,03 a 0,125 μg × mL^{-1} para CNP e de 0,03 a 0,06 μg × mL^{-1} para CNP-Cu contra as bactérias *E. coli* e *S. typhimurium*, respectivamente. Os valores de MBC não serão aqui descritos, para detalhes consultar Qi et al. (2004).

1.3 Atividade antifúngica *in vitro* e *in vivo* de quitosanas e quitoligômeros

A deterioração pós-colheita devido à ação de fungos limita o valor econômico de vegetais armazenados. Embora os fungicidas sejam extensivamente usados no controle de doenças pós-colheita, há interesse público na redução destes resíduos em alimentos bem como aos patógenos resistentes a fungicidas.

Métodos não convencionais de controle de fitopatógenos pós-colheita têm sido relatados na literatura. Além dos estudos com controles de fungos fitopatogênicos por fungicidas (Griffin, 1994), outros métodos têm sido empregados, como por exemplo: controle biológico (Melo, 2002), associação de controle biológico a $CaCl_2$ (Wisniewski et al., 1995; Tian et al., 2001; Tian et al., 2002), associação de controle biológico à atmosfera modificada (Qin et al., 2004), tratamento térmico pós-colheita (Couey, 1989; Fallik et al, 1995), associação de tratamento térmico e etanol (Karabulut et al., 2004) e de quitosana (Shahidi et al., 1999; Bautista-Baños et al., 2006).

Há uma forte evidência que o crescimento miceliano fúngico pode ser inibido completamente ou retardado quando quitosana é adicionada ao meio de cultura de fungos.

Ao se aumentar à concentração de quitosana de 0,75 a 6,0 mg × mL^{-1}, El Ghaouth et al., (1992a) observaram diminuição do crescimento radial dos fungos *Alternaria alternata, Botrytis cinerea, Colletrotichum gloeosporioides* e *Rhizopus stolonifer*. O mesmo efeito foi observado contra *Sclerotinia sclerotiorum* ao se aumentar a concentração de quitosana de 1 a 4% (m/v) (Cheah & Page, 1997). Outros estudos mostraram um decréscimo linear do crescimento de *Rhizoctonia solani* com o gradual aumento da concentração de 0,5 a 6,0 mg × mL^{-1} de quitosana (Wade & Lamondia, 1994). Os crescimentos micelianos de *Fusarium solani* f. sp. *phaseoli* e *F. solani* f. sp *pisi* foram inibidos a concentrações mínimas de 12 e 18 mg × mL^{-1} (Hadwiger & Beckman, 1980; Kendra & Hadwiger, 1984). Inibições completas dos crescimentos dos fungos *F. oxysporum*, *R. stolonifer*, *Penicillium digitatum* e *C. gloeosporioides* foram obtidas na concentração de 3% (m/v) (Bautista-Baños et al., 2003, 2004). Na Tabela 1 são listados alguns estudos nos quais foram avaliados os efeitos de quitosanas *in vitro* no crescimento de fungos fitopatogênicos.

TABELA 1 Efeitos de quitosana *in vitro* na inibição do crescimento de fungos

Fungo	Concentração de quitosana % (m/v)	Efeito	Autor
A. alternata, B. cinerea e *R. stolonifer*	0,075 a 0,6	Diminuição do crescimento radial	El Ghaouth et al. (1992a)
Rhizoctonia solani	0,05 a 0,6	Redução do crescimento	Wade & Lamondia (1994)
F. solani f. sp. *phaseoli*	1,2	Inibição completa	Hadwiger & Beckman (1980)
F. solani f. sp *pisi*	1,8	Inibição completa	Kendra & Hadwiger (1984)
F. oxysporum, *R. stolonifer*, *Penicillium digitatum* e *C. gloeosporioides*	3	Inibição completa	Bautista-Baños et al. (2003, 2004)
Mucor racemosus	0,2	Redução de 73% do crescimento	Roller & Covill (1999)

Allan & Hadwiger (1979) observaram que a quitosana possui forte atividade antifúngica contra um grande número de fitopatógenos, com exceção de Zigomicetos, os quais contêm quitosana como o maior componente da parede celular. Porém, os resultados de Roller & Covill (1999) mostraram que o fungo *Mucor racemosus*, cujas paredes celulares são constituídas de quitosana, teve inibição do seu crescimento a uma concentração de quitosana de 1 g × L^{-1}, contrariando a proposição de Allan & Hadwiger (1979) que incluía cepas de *Mucor* spp. El Ghaouth et al. (1991b) investigaram o efeito do

revestimento de quitosana (1,0 e 1,5% m/v) no controle da deterioração de morangos a 13°C em comparação com o efeito do fungicida ipridione (Rovral®), e concluíram que o revestimento com quitosana era mais efetivo do que o tratamento com o fungicida Rovral® no controle da deterioração pós-colheita. O efeito antifúngico de quitosana *in vitro* no crescimento de patógenos pós-colheita de morangos foi também estudado por El Ghaouth et al. (1992a). De acordo com este estudo, quitosana F_A 0,83 reduziu marcadamente o crescimento miceliano dos fungos *Botrytis cinerea* e *Rhizopus stolonifer*, com um grande efeito a altas concentrações. Estes autores também confirmaram a importância do grande número de grupos carregados positivamente ao longo da cadeia do polímero devido ao fato de se ter observado que N,O-carboximetilquitosana apresentou menor atividade antifúngica do que quitosana (El Ghaouth et al., 1992a). Em um estudo *in vivo*, El Ghaouth et al. (1992b) relataram que sinais de infecção foram observados em frutos de morango depois de cinco dias de armazenamento a 13°C, enquanto que os frutos controle já haviam apresentado sinais de infecção com apenas um dia de armazenamento. Depois de 14 dias de armazenamento, o revestimento de quitosana, cuja concentração era de 15 mg × mL^{-1}, reduziu a deterioração de morangos a 60%, causado pelo mesmo fungo e também foi observado que os frutos revestidos amadureceram normalmente sem apresentarem sinais aparentes de fitotoxicidade. Na Tabela 2 são listados alguns estudos nos quais foram avaliados os efeitos dos revestimentos de quitosanas *in vivo* na superfície de frutos, hortaliças e plantas no controle de fungos fitopatogênicos.

TABELA 2 Efeito do revestimento de quitosana formado na superfície de frutos e hortaliças imersas pós-colheita[a] em soluções ácidas de quitosana ou de frutos e plantas pulverizadas pré-colheita[b] com quitosana. Os frutos, plantas e hortaliças foram inoculados com seus respectivos fungos fitopatogênicos.

Fruto, hortaliça ou planta	**Fungo**	**Concentração de quitosana % (m/v)**	**Efeito (redução da doença%)**	**Autor**
Morango[a]	*B. cinerea*	1,0 e 1,5	77	El Ghaouth et al. (1991b)
Morango[a]	*B. cinerea* e *R. stolonifer*	1,5	60	El Ghaouth et al. (1992b)
Cenoura[a]	*Sclerotinia sclerotiorum*	2,0 e 4,0	68	Cheah & Page (1997)
Planta de pepino[b]	*B. cinerea*	0,1	65	Ben-Shalom et al. (2003)
Morango[b]	*B. cinerea*	0,2 a 0,6	45 a 62	Bhaskara Reddy et al. (2000)
Mamão[b]	*C. gloeosporioides*	1,5	60	Bautista-Baños et al. (2003)

Cuero et al. (1991) observaram que N-carboximetilquitosana reduziu em 90% a produção de aflatoxina por *Aspergillus flavus* e *Aspergillus parasiticus*, enquanto o crescimento fúngico foi reduzido a menos de 50%. Revestimento de quitosana mostrou-se eficaz na inibição de *Sclerotinia sclerotiorum* em cenouras (*Daucus carota* L.) cuja incidência foi significativamente reduzida de 88 para 28% nas raízes revestidas com 2 e 4% (m/v) de quitosana (Cheah & Page,1997). Estudos microscópicos revelaram que o micélio fúngico de *Sclerotinia sclerotiorum* exposto a quitosana, pareceu estar deformado e morto, visto que o micélio controle estava normal.

O controle do mofo cinzento causado por *B. cinerea* em plantas de pepino por meio de oligômeros de quitosana foi estudado por Ben-Shalom et al. (2003), que sugeriram que o principal efeito dos oligômeros no controle da doença é devido ao efeito fungistático na germinação do conídio de *Botrytis*, pois a quitosana é um polímero carregado positivamente que pode prevenir a ligação do conídio a algum sítio. Bhaskara Reddy et al. (2000) analisaram o efeito da pulverização pré-colheita com quitosana na qualidade pós-colheita de morango e incidência do patógeno *B. cinerea* e observaram que as pulverizações preventivas de quitosana foram efetivas no controle da infecção de *B. cinerea* em morangos.

A relação entre massa molar de quitosanas e quitoligosacarídeos e atividade antifúngica tem sido analisada em alguns estudos. Kendra e Hadwiger (1984) observaram que monômeros e dímeros de quitosana não apresentaram atividade antifúngica contra *Fusarium solani,* enquanto heptâmeros tiveram atividade antifúngica equivalente à quitosana. Uchida et al. (1989) relataram que uma mistura de quitoligômeros com DP 2 a 8 (DP médio de 5) foram inativos contra três espécies do gênero *Fusarium* à concentração de 1% (m/v). Zhang et al. (2003) relataram que quitoligômeros com DP médio de 20 inibiram o crescimento de 16 fungos patógenos de plantas. Torr et al. (2005) sugeriram que maiores atividades antifúngicas contra certos fungos podem ser obtidas com quitoligosacarídeos (DP=5, DP=9 e DP=14), quando comparadas às obtidas com quitosana (Mm de 310 kDa a maiores que 375 kDa; DP≈1.925 a 2.329). Acetato de quitosana e as misturas de quitoligômeros, citadas anteriormente, foram testadas contra *Leptographium procerum*, *Sphaeropsis sapinea* e *Trichoderma harzianum*. A taxa de crescimento médio de *T. harzianum* foi dimuída com o aumento da concentração de acetato de quitosana e

quitoligômeros de 0.1 a 0.4% (m/v) o que causou um período inicial de fungistase que foi eventualmente superado pelo fungo. *Sphaeropsis sapinea* e *Leptographium procerum* foram mais susceptíveis às atividades de quitosana e quitoligômeros que *T. harzianum*, cujos crescimentos foram inibidos à concentração de 0,4% (m/v) no período de 35 dias. As atividades antifúngicas das três misturas de quitoligômeros foram maiores em pH 4.0 do que em pH 6.0, cujos DP 9 e 14 foram mais efetivos contra *S. sapinea* e *L. procerum* que a mistura de DP 5.

1.4 Ação de quitosanas na produção de esporos fúngicos

O efeito de quitosanas na produção de esporos pelos fungos *F. oxyporum, R. stolonifer, C. gloeosporioides, A. alternata* f. sp. *lycopersici* e *A. niger* foi analisado pelos autores Bhaskara Reddy et al. (1998), Bautista-Baños et al. (2003, 2004) e Plascencia-Jatomea et al., (2003). A esporulação de fungos tratados com quitosanas é geralmente menor do que a de fungos não tratados. Além disso, em alguns estudos a esporulação foi completamente inibida nos casos de tratamento com quitosana. Entretanto, foi observado em alguns casos que a quitosana estimula a esporulação. A formação de esporos de *A. alternata* na presença de quitosana às concentrações de 100 e 500 $\mu g \times mL^{-1}$ (doses sub letais) foi significativamente maior que o controle sem quitosana (Bhaskara Reddy et al., 1998, Bautista-Baños et al., 2004). Estes autores indicaram que alta esporulação pode ter sido devido a uma resposta ao estresse induzido por este polímero.

1.5 Ação de quitosanas na viabilidade de esporos fúngicos

A viabilidade de esporos fúngicos tem sido analisada após tratamento com quitosana. Concentrações de 0,75 $mg \times mL^{-1}$ reduziram a viabilidade de esporos e o crescimento do tubo de germinação dos fungos *B. cinerea* e *R. stolonifer* (El Ghaouth et al., 1992b). Em outro estudo, baixas concentrações de quitosanas (20-30 $\mu g \times mL^{-1}$) causaram 50% de inibição da germinação e 50 $\mu g \times mL^{-1}$ promoveu a quase total inibição da germinação dos esporos (Ben-Shalom et al., 2003). Estes autores também observaram que quitosana promoveu a redução do tamanho dos tubos de germinação, os quais apresentaram um tamanho médio de aproximadamente 15 μm na presença de água e 2 μm na presença de

10 μg × mL^{-1} de quitosana, ambos tratamentos incubados por 24 horas. Sathiyabama e Balasubramanian (1998) avaliaram o efeito da concentração de quitosana na viabilidade de esporos fúngicos de *Puccinia arachidis* incubados por 4 horas e observaram que com o aumento das concentrações de quitosana de 100 a 1.000 μg × mL^{-1}, houve diminuição da porcentagem do número de esporos germinados de 24±3 para 6±0%, respectivamente, enquanto os esporos não tratados com quitosana tiveram uma germinação de 96±1%.

1.6 Mudanças na morfologia dos fungos devido ao efeito de quitosanas

Observações microscópicas de fungos tratados com quitosana revelaram que o polímero pode afetar a morfologia das hifas. Os fungos *F. oxysporum* f. sp. *radicis-lycopersici*, *R. stolonifer* e *S. sclerotiorum* tratados com quitosana mostraram excessivas ramificações micelianas, formas anormais, inchamento e redução do tamanho das hifas (Benhamou, 1992; El Ghaouth et al., 1992a, b; Cheah & Page, 1997). Similarmente, quitosanas causaram mudanças morfológicas (Figura 3) como grandes vesículas ou células vazias isentas de citoplasma nas hifas de *B. cinerea* e *F. oxyporum* f. sp. *albedinis* (Ait Barka et al., 2004; El Hassni et al., 2004). Em outros estudos, análises de imagens foram usadas para medir o efeito de quitosana nos parâmetros morfológicos individuais dos esporos de *C. gloeosporioides, R. stolonifer, P. digitatum* e *F. oxysporum*. Área, tamanho e forma dos conídios de cada um dos fungos testados foram afetadas de acordo com a espécie de fungo e tempo de incubação nas soluções de quitosana (Bautista-Baños et al., 2003, 2004). Em outro estudo foi relatado que a morfologia do esporo de *A. niger* foi também afetada quando tratado com quitosana (Plascencia-Jatomea et al., 2003).

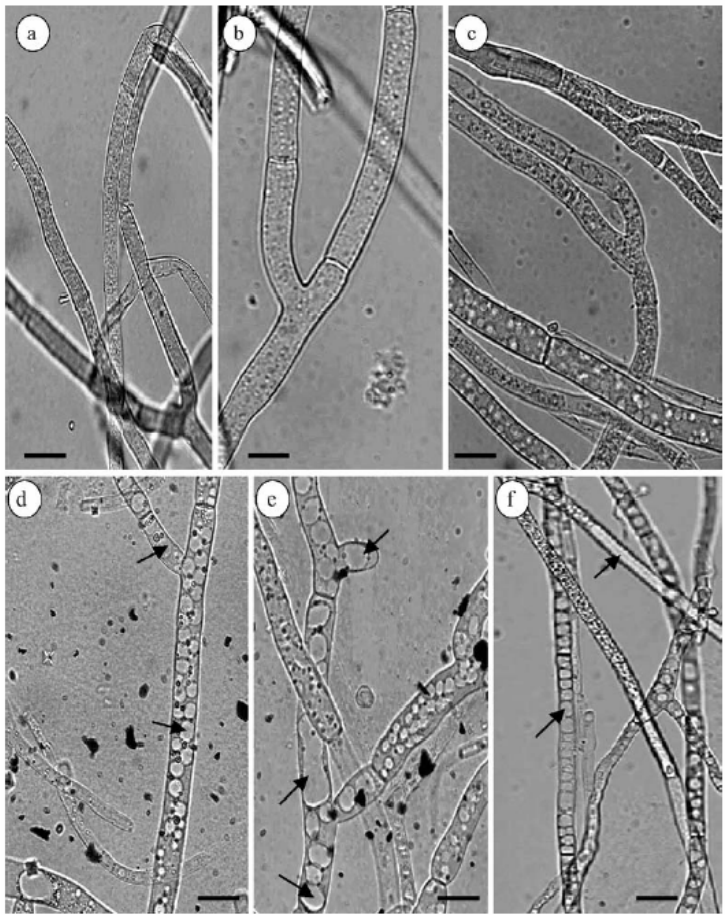

FIGURA 3 Mudanças estruturais microscópicas nos fragmentos de hifas de *B. cinerea* em resposta a presença de quitosana. (a) e (b) Micélio controle, (c) a (f) Micélios das culturas fúngicas crescidas em PDA contendo 1,75% (v/v) de quitosana (Chitogel®). *Barras*: 40μm. Pequenas e grandes vesículas apareceram nas amostras tratadas com quitosana e em alguns casos o citoplasma estava isento de alguma organela (*setas*). Reproduzido de Ait Barka et al. (2004).

1.7 Quitosana como indutor dos mecanismos de respostas em plantas

Estimuladores são substâncias (oligossacarídeos, glicoproteínas, peptídeos e lipídeos) que podem induzir respostas de defesa quando aplicadas aos tecidos de plantas ou a cultura de células vegetais. Os oligossacarídeos indutores mais estudados são os oligômeros de glucano, quitina, quitosana e de ácidos galacturônicos. Quando uma planta é atacada por um patógeno, rapidamente mecanismos de defesa são ativados no sítio infectado e uma variedade de respostas de defesas bioquímicas ocorrem ao redor das células mortas. Dentre as respostas de defesas bioquímicas incluem-se, a produção de

oxigênio reativo, mudanças estruturais na parede celular, acúmulo de proteínas relacionadas à defesa e biossíntese de fitoalexinas (Rabea et al., 2003).

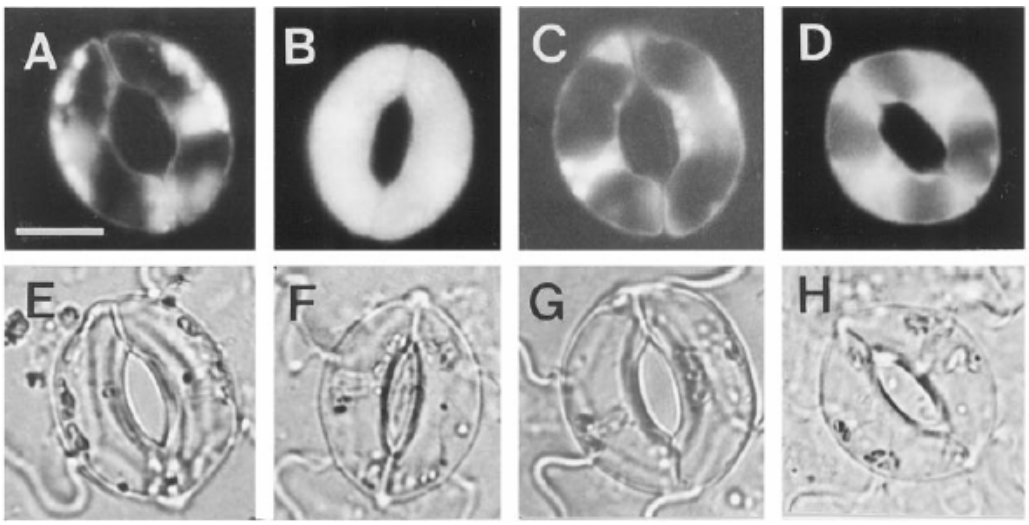

FIGURA 4 Indução da produção de H_2O_2 por quitosana nas células guarda em folhas de tomate. Pedaços epidermais de folhas de tomate sem quitosana (controles A e E) ou com tratamentos de 30 minutos somente com quitosana (B e F), com quitosana e catalase (C e G), ou com quitosana e ácido ascórbico (D e H). Microscopia de fluorescência são mostradas nas Figuras de A a D e microscopia ótica são mostradas nas Figuras de E a H. A barra em A é de 10μm, e se aplica a todas as Figuras. Reproduzido de Lee et al. (1999).

As habilidades estimuladoras de quitosanas nas respostas naturais de defesa de plantas foram extensivamente estudadas. Mudanças fisiológicas e bioquímicas que ocorrem em plantas devido ao estímulo por quitosana foram descritas em inúmeros estudos (Bohland et al., 1997; Vander et al., 1998; Pearce e Ride, 1982; Benhamou e Thériault, 1992; Lafontaine e Benhamou, 1996; Benhamou et al., 1994; El Ghaouth et al., 1994, 1997; Benhamou et al., 1998). Mudança fisiológica primária foi observada em plantas tratadas com quitosana, cujas aberturas dos estômatos foram diminuídas o que dificulta o acesso fúngico no interior dos tecidos das folhas. Lee et al. (1999) observaram que células guarda em folhas de plantas, produzem H_2O_2, que é um composto mediador do estímulo promovido por quitosana que induz a diminuição das aberturas estomatais (Figura 4).

Oligossacarídios de quitosana estimularam o acúmulo de lignina, calose, fitoalexinas, e/ou inibidores de proteases em vários tecidos de plantas. O mecanismo de

ação pelo qual quitosana induz esta lignificação tem sido estudado em diferentes tipos de plantas (Lesney, 1990 e Vander et al., 1998).

Indução a várias enzimas relacionadas ao processo de defesa de plantas tem sido estudada (Bohland et al., 1997, Vander et al., 1998). Estas enzimas participam dos mecanismos de defesa iniciais e previnem a infecção por patógenos. Oligômeros de quitina e quitosana foram associados ao estímulo de outros sistemas envolvidos na resistência, como as atividades de lipoxigenase e fenilalanina amônia-liase e a formação de lignina em folhas de trigo (Bohland et al., 1997, Vander et al., 1998).

A indução de barreiras estruturais à região atacada por fungos é o processo mais comum de resposta à invasão de patógenos. A suberização celular e a lignificação entre outros processos de defesa são estimulados durante o processo de infecção em alguns órgãos das plantas. Relatos descrevem que a quitosana restringe, em alguns casos, a penetração fúngica e induz a formação de diferentes barreiras estruturais. Uma lignificação moderada como um resultado do tratamento de quitosana assim como o inóculo de paredes celulares de *B. cinerea* foram relatados em folhas de trigo depois de 48 e 72 horas (Pearce e Ride, 1982). Microscopia eletrônica de transmissão mostrou a formação de estruturas particulares e novos materiais, por exemplo, as principais reações observadas nas células hospedeiras de raízes e folhas de tomate tratadas com quitosana e infectadas por *F. oxyporum* f. sp. *radicis-lycopersici* foram: (1) obstrução dos vasos do xilema por um material opaco ou fibroso granular ou estrutura em forma de bolha, (2) revestimento da membrana secundária tornando-a mais espessa e marcada por lesões, (3) formação de papilas (aposição da parede) dentro do córtex e dos tecidos endotérmicos (Benhamou e Thériault, 1992; Lafontaine e Benhamou, 1996). Outras reações da planta hospedeira especificamente raízes de plantas de tomate tratadas com quitosana apresentaram células epidermais deformadas (Benhamou et al., 1994). Em frutos de pimenta do tipo sino "bell pepper", respostas de defesa estruturais foram observadas somente nas primeiras camadas de tecidos próximas às células rompidas, como aumento da espessura das paredes celulares, formação de protuberâncias esféricas e hemisféricas ao longo das paredes celulares, e obstrução de espaços celulares devido a formação de materiais fibrilares (El Ghaouth et al., 1994, 1997). Outros estudos demonstraram que a combinação de dois métodos de controle (aplicação de quitosana e controle biológico com *Bacillus pumilus*) aumentaram as reações

de defesa da planta hospedeira (Benhamou et al., 1998). Plantas de pepino cultivadas em soluções de nutriente contendo quitosana, e inoculadas com *P. aphanidermatum*, tiveram reações similares às observadas em raízes de tomate tratadas com quitosana como obstrução dos espaços celulares com materiais opacos e fibrilares e formação de papilas ao longo da parede celular hospedeira (El Ghaouth et al., 1994).

1.8 Efeito de quitosana na qualidade pós-colheita de produtos vegetais

Produtos vegetais têm o tempo de armazenamento extendido quando revestidos com quitosana. Quitosana forma um filme semipermeável que regula as trocas gasosas e reduz as perdas por transpiração, conseqüentemente o amadurecimento do fruto é retardado. Diferentes frutos revestidos com quitosana, geralmente têm suas taxas de respiração e perdas de água reduzidas, dentre os quais, tomates, morangos, "longan", maçãs, mangas, bananas e pimentas tipo sino "bell peppers" (El Ghaouth et al., 1991a,1992c; Du et al., 1997, 1998; Jiang e Li, 2001; Kittur et al., 2001). A eficácia de quitosana na redução da produção de CO_2 interno é descrito em tomates e pêras (El Ghaouth et al., 1992c; Du et al., 1997). Revestimentos de quitosana associado à temperatura de armazenamento podem estar associados com a redução da produção de CO_2. Pepinos e pimentas tiveram menores taxas de respiração a 13°C do que a 20°C (El Ghaouth et al., 1991a). Além da inibição de CO_2 resultante do revestimento de quitosana, a produção de etileno em frutos é também reduzida. Ambos efeitos inibitórios foram observados em pêssegos e tomates revestidos com quitosana (Li & Yu, 2000; El Ghaouth et al., 1992c). Frutos como morangos, framboesas, tomates, pêssegos, mamões e outros frutos tiveram suas perdas de firmeza retardadas durante o armazenamento quando tratados com quitosana (El Ghaouth et al., 1991b, 1992c; Li e Yu, 2000; Bautista-Baños et al., 2003). Pulverizações de quitosana pré-colheita nas concentrações 2, 4 e 6 $g \times L^{-1}$, em plantas de morangos não causaram fitotoxicidade e os frutos tratados com o tempo e temperatura de armazenamento se tornaram mais firmes que os não tratados (Bhaskara Reddy et al., 2000). Em geral, a degradação de antocianinas em frutos tratados com quitosanas é retardada, a qual foi demonstrada em lichia, morango e framboesa (Li e Chung, 1986; Zhang e Quantick, 1997, 1998). Porém outro estudo relatou um efeito oposto ao descrito anteriormente, no qual é mencionado haver síntese de antocianinas em morangos tratados

com quitosana (El Ghaouth et al., 1991b). Morangos, tomates e pêssegos tratados com quitosana após armazenamento, apresentaram maior acidez titulável comparada à dos frutos controle, enquanto outros frutos como mangas e "longan" tiveram acidez titulável reduzida lentamente (El Ghaouth et al.,1992c; Li e Yu, 2000; Jiang e Li, 2001; Srinivasa et al., 2002). Sólidos solúveis totais de frutos armazenados e tratados com quitosana diferiram de acordo com o tipo de fruto. Mangas e bananas revestidas com quitosana apresentaram menores teores de sólidos solúveis totais que os frutos não tratados, todavia maiores teores foram relatados em pêssegos tratados com quitosana. Em outro estudo foi observado não haver diferença nos teores de sólidos solúveis totais de mamões tratados com quitosana e não tratados (Du et al., 1997; Kittur et al., 2001; Srinivasa et al., 2002; Bautista-Baños et al., 2003). Os teores de açúcar redutor de frutos é também afetado por revestimento de quitosana: menores teores de açúcar redutor em bananas tratadas com quitosana foram observados do que em frutos não tratados no final do período de armazenamento (Kittur et al., 2001). Porém, relatos contraditórios a respeito dos teores de açúcar redutor de mangas tratadas com quitosana foram descritos na literatura. Uma provável explicação para isto poderia estar relacionado ao modo de aplicação de quitosana na superfície do fruto. No primeiro estudo os frutos de manga foram embalados em caixas de papelão e cobertos com filme de quitosana, neste caso os teores de açúcares redutores foram maiores que os dos frutos controle, enquanto que no segundo estudo, os frutos de manga foram imersos em uma solução de quitosana, e estes frutos tiveram menores teores de açúcares redutores que os frutos controle (Kittur et al., 2001; Srinivasa et al., 2002) indicando que os frutos imersos tiveram redução do metabolismo comparado aos frutos não tratados com quitosana. O teor de ácido ascórbico em mangas e pêssegos tratados com quitosana também foram avaliados (Li e Yu, 2000; Srinivasa et al., 2002). Nestes estudos, o teor desta vitamina em mangas tratadas decresceu gradualmente durante o período de armazenamento e foi menor que em frutos não tratados. Porém, em pêssegos, os teores de ácido ascórbico foram maiores nos frutos tratados com quitosana que os dos frutos controle e também tratados com fungicida Prochloraz depois de doze dias de armazenamento. Embora poucos estudos relatem o efeito de quitosana nos atributos sensoriais dos produtos vegetais tratados, geralmente, o sabor e gosto permanecem inalterados. Mangas e morangos tratados com quitosana obtiveram maiores pontuações nos atributos sensoriais comparados com os frutos não tratados armazenados por 21 e 15 dias, respectivamente (Li e Chung, 1986; Kittur et

al., 2001). Em outros estudos morangos revestidos com quitosana e armazenados por 12 dias a 7°C tiveram um gosto levemente amargo somente no dia zero (Devlieghere et al., 2004).

1.9 Modo de ação da quitosana

O mecanismo de ação da atividade antimicrobiana de quitina, quitosana e seus derivados não é ainda bem esclarecido, entretanto diferentes mecanismos têm sido propostos. Acredita-se que a quitosana interfira nos grupos carregados negativamente das macromoléculas expostas na superfície da parede celular fúngica e, desta maneira, há modificação da permeabilidade da membrana plasmática (Benhamou, 1996) o que conduz a liberação de proteínas e outros constituintes intracelulares (Seo et al., 1992; Chen et al., 1998; Fang et al., 1994; Hadwiger et al., 1986; Jung et al., 1999). A interação eletrostática entre as moléculas de quitosana e os grupos negativamente carregados encontrados na membrana celular, ocorre devido a carga positiva no carbono dois (C-2) dos monômeros de glicosamina a pH abaixo de 6 que torna o polímero quitosana mais solúvel e com melhor atividade antimicrobiana comparado a quitina (Chen et al., 1998). Esta importante propriedade do polímero quitosana, a capacidade de se protonar em soluções ácidas, é devido à presença de aminas na molécula que se ligam a prótons como mostrado na equação (1) abaixo:

$$\textbf{Quitosana - } NH_2 \textbf{ + } H_3O^+ \rightleftharpoons \textbf{Quitosana - } NH_3^+ \textbf{ + } H_2O \qquad \textbf{(1)}$$

O valor de pKa de quitosana é de aproximadamente 6,3. A quitosana se solubiliza quando mais que 50% dos grupos amino estejam protonados (Rinaudo et al., 1999), desta maneira a solubilidade de quitosana diminui agudamente quando o pH aumenta acima de 6,0 a 6,5 (Vårum et al., 1994).

Sudarshan et al. (1992) e Papineau et al. (1991) observaram aglutinação bacteriana ao se usar baixas concentrações de quitosana menores que 0,2 mg × mL^{-1} devido provavelmente à ligação do polímero policatiônico à superfície bacteriana carregada

negativamente; todavia a altas concentrações a aglutinação não foi observada, o que segundo os autores pode estar relacionado ao número elevado de cargas positivas que podem ter formado uma rede de carga positiva na superfície bacteriana mantendo-as em suspensão.

A interação entre quitosana e a célula também pode alterar a permeabilidade da membrana celular. Por exemplo, a fermentação de leveduras usadas em panificação é inibida por certos cátions que agem na superfície da célula e previnem a entrada de glicose (Rabea et al., 2003). A interação entre quitosana e células de *Pythium oaroecandrum* foi estudada por Leuba & Stossel (1985), os quais usaram a técnica de UV e observaram que houve considerável liberação de material protéico das células a pH 5,8.

Quitosana age também como agente quelante que seletivamente se liga a traços de metais e desta forma inibe a produção de toxinas e crescimento microbiano (Cuero et al, 1991).

Liu et al. (2004) avaliaram a atividade antibacteriana de solução de acetato de quitosana contra *Escherichia coli* e *Staphylococcus aureus*. A integridade da membrana celular de ambas espécies foi investigada por determinação da liberação de materiais intercelulares que absorvem na faixa de 260 nm. Foi observado aumento gradativo de material intercelular das suspensões de bactérias tratadas com acetato de quitosana (0,5 e 0,25% m/v) durante duas horas de monitoramento. Os autores também observaram que de acordo com os resultados dos espectros de infravermelho e dos perfis de termogravimetria e termogravimetria diferencial, houve formação de ligação iônica entre o grupo NH_3^+ do acetato de quitosana e o grupo fosforila da fosfatidilcolina. Este resultado parece confirmar a interação eletrostática entre acetato de quitosana e fosfatidilcolina que é um dos componentes da membrana celular bacteriana.

1.10 Conclusões

Quitina, quitosana, derivados e quitoligômeros têm sido amplamente estudados e a existência de inúmeras patentes ou aplicações de patentes registradas, aproximadamente 9.772 foram encontradas na base de dados "SciFinder Scholar" usando-se o termo quitosana, assim como o grande número de artigos científicos que têm sido publicados na

literatura, refletem o grande potencial de aplicações destes polímeros, derivados e oligômeros. Levando em consideração a tendência mundial de preferência dos consumidores por alimentos sem preservativos químicos, a quitosana entre outros compostos naturais, mostra-se uma substância alternativa no combate de fungos e bactérias, embora os preservativos químicos sejam ainda usados extensivamente no controle destes microrganismos, sobretudo os fungicidas, empregados no controle de doenças pós-colheita de frutos. Quitosana comercial "ChitoClear®" produzido pela Primex ehf. é indicada pelo fabricante como produto antimicrobiano comestível usado como revestimento de frutos, cujos os tempos de armazenamento são prolongados. O produto "ChitoClear®" também é indicado no tratamento pré-colheita de plantas atuando na indução de respostas de defesa das plantas e no tratamento de águas como floculante. No Brasil, quitosana comercial "Fybersan" produzida pela Polymar Ciência e Nutrição S/A é indicada para perda de peso e diminuição de colesterol em humanos. Estudos pré e pós-colheita de vegetais têm mostrado que o versátil polímero quitosana apresenta triplo efeito no tratamento destes: controle de microrganismos patogênicos, ativações de várias respostas de defesa induzindo e/ou inibindo diferentes atividades bioquímicas durante a interação planta-patógeno e aumento do tempo de armazenamento de vegetais frescos devido às propriedades filmogênicas da quitosana. Estudos de campo necessitam ser realizados no sentido de se avaliar a viabilidade de aplicação comercial de quitosana no controle de microrganismos patogênicos de produtos vegetais, assim como a qualidade sensorial dos produtos tratados.

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CAPÍTULO II

Este capítulo corresponde ao artigo submetido:

Oliveira Junior, E. N., Franco, T. T. Physicochemical characterization of thermally treated chitosans and chitosans obtained by alkaline deacetylation. ***Brazilian Journal of Chemical Engineering.***

Neste artigo é relatada à produção de quitosanas de diferentes massas molares obtidas por tratamento térmico, assim como a produção de duas amostras de quitosana de diferentes graus de acetilação obtidas por desacetilação alcalina. As quitosanas termicamente tratadas e obtidas por desacetilação alcalina foram produzidas e caracterizadas por viscosimetria, cromatografia de permeação em gel e titulação potenciométrica no nosso Laboratório de Engenharia Bioquímica da FEQ/UNICAMP. As análises de RMN H^1 e de viscosidade intrínseca foram realizadas na Universidade de Potsdam com a colaboração do Prof. Dr. Martin G. Peter. O processo de degradação térmica foi avaliado por meio de viscosimetria e cromatografia de permeação em gel com detector índice de refração e os graus de acetilação das amostras foram determinados através dos métodos de titulação potenciométrica e ressonância magnética nuclear de próton.

As técnicas viscosimétricas e de cromatografia de permeação em gel utilizadas para caracterizar o processo de despolimerização de quitosana mostraram boa correlação de resultados. Seis horas de tratamento térmico à temperatura de 100°C foram suficientes para se obter quitosanas com massa molar 90% menor comparada a massa molar da quitosana sem tratamento. Os métodos de titulação potenciométrica e de RMN H^1 também mostraram resultados de graus de acetilação similares para as amostras de quitosana caracterizadas neste estudo.

Physicochemical characterization of thermally treated chitosans and chitosans obtained by alkaline deacetylation

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Abstract - The thermal depolymerization of chitosan and alkaline deacetylation of chitin were characterized by measurement of viscosity, gel permeation chromatography (GPC-RI), potentiometric titration (PT) and proton nuclear magnetic resonance spectroscopy (^{1}H NMR). The depolymerization rates (DR) measured by kinematic viscosity (KV), apparent viscosity (AV) and GPC (Mw) after 4 hours of treatment were DR_{KV}=21.9, DR_{AV}=25.5 and DR_{Mw}=23.3 % h^{-1} and for 5 to 10 hours of treatment they decreased slowly to produce of DR_{KV}=0.545, DR_{AV}=0.248 and DR_{Mw}=1.11 %h^{-1}. The mole fraction of N-acetylglucosamine residues (F_A) of chitosans was not modified after 10 hours of thermal treatment at 100°C. The initial F_A values of chitosan without any treatment were $F_{A\ PT}$=0.21 and $F_A{}^1{}_{H\ NMR}$=0.22 and of chitosan treated for 10 hours were F_{APT}=0.27 and $F_A{}^1{}_{H\ NMR}$=0.22. The work compares the capability of the analytical methods to evaluate the thermal degradation process and alkaline deacetylation of chitosan.

Keywords: chitosan; chitin; thermal treatment; alkaline deacetylation.

1.0 INTRODUCTION

Chitin, a natural biopolymer, is a structural polysaccharide found in the exoskeleton of marine crustaceans (crab and shrimp shells) and insects. It is also widely found in fungi, such as *Basidiomycetes*, *Ascomycetes*, and *Phycomycetes,* where it is a component of cell walls and structural membranes of mycelia, stalks and spores (Peter, 2002). The chemical structure of chitin is similar to that of cellulose with 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) monomers attached via β(1→4) linkages (Shahidi et al., 1999). Chitosan is a linear binary copolymer consisting of GlcNAc and 2-amino-2-deoxy-β-D-glucopyranose (GlcN) monomers distributed along the chitosan chain (Vårum et al., 1991).

Chitosan and chitin are polydisperse polymers and the number of their subunits varies. They are distinguished by their solubility in 1% aqueous acetic acid. Chitin, containing ca. >40% GlcNAc residues (F_A >0.4) is insoluble, whereas soluble polymers are named chitosan (Peter, 2002).

Several characteristics of chitosan are fundamental in describing the particular molar batch and predicting its chemical and physical properties: the average molar mass of the sample, its average degree of acetylation (DA, given as a percentage) or the fraction of

acetylation (F_A, given as the mole fraction) and the local and global distribution of the acetylated amide moieties along the chain as well as the polydispersity index, the viscosity and the ash content (Peter, 2002). Presently, a substantial amount of research is devoted to the application of chitosan for antimicrobial purposes against a wide range of phytopathogenic fungi (El Ghaouth et al., 1992) and pathogenic bacteria (No et al., 2002).

Chitosan, like other polysaccharides, is susceptible to a variety of degradation mechanisms, including oxidative-reductive free radical depolymerization and acid-, alkaline- and enzyme-catalyzed hydrolysis. Degradation of polysaccharides occurs via cleavage of the glycosidic bonds (Holme et al., 2001). Several studies have been done to evaluate the acid hydrolysis of chitosan. The acid hydrolysis of chitin was studied for the first time in 1937 by Meyer and Wehrli, who used HNO_3 (Roberts, 1992). Different acids have been used in the hydrolysis of chitosan: hydrochloric acid (Horowitz et al., 1957; Rupley, 1964; Rege & Block, 1999; Lee et al., 1999; Vårum et al., 2000), phosphoric acid (Hasegawa et al., 1993; Jia & Shen, 2002), sulfuric acid and acetic anhydride (Schanzenbach et al., 1997), nitrous acid (Allan & Peyron, 1995) and hydrogen fluoride (Bosso et al., 1986).

Unlike acid hydrolysis, enzymatic hydrolysis of chitin and chitosan by chitinases (EC 3.2.1.14) and chitosanases (EC 3.2.1.132) permits the production of different oligomers. Hydrolysis of chitin and chitosan catalyzed by specific chitinase and chitosanase enzymes has been employed in several studies (Dixon & Webb, 1979; Aiba, 1994; Stoyachenko et al., 1994; Izume & Ohtakara, 1987; Fenton & Eveleigh, 1981). A few studies have been done to evaluate the degradation of chitosan by thermal treatment (Alonso et al., 1983; Köll et al., 1991; Peniche-Covas et al., 1993; Holme et al., 2001; Mucha & Pawlak, 2005).

The aims of this work were to investigate chitosan degradation by thermal treatment and alkaline deacetylation of chitin. In order to evaluate the extension of these processes, the capability of some analytical methods was further investigated. For molar mass determination two methods were used: viscometry and gel permeation chromatography (with a refractive index detector, GPC-RI). For a determination of the degree of acetylation, potentiometric titration (PT) and high-field ^{1}H NMR spectroscopy were used.

2.0 MATERIALS AND METHODS

2.1 Raw materials

Chitosan was supplied by *Polymar (Fortaleza, Brazil)*. Chitin (*Sigma Chemical Co. St. Louis, USA*) was used in the deacetylation reaction to obtain chitosans with different degrees of acetylation. Dextran standards (*American Polymers, Ohio, USA*) were used for calibration of GPC columns (Mw: 11, 38, 72, 260 and 530 KDa). Sodium azide was from *Sigma* (*St. Louis,USA*), lactic acid was from *Synth* (*Diadema, Brazil*), acetic acid, sodium chloride, urea, hydrochloric acid and deuterium chloride were from *E. Merck* (*Darmstadt, Germany*).

2.2 Thermal depolymerization of chitosan

Thermal depolymerization of chitosan samples was achieved in accordance with a modified procedure developed by Holme et al. (2001). Solid chitosan (8 g) was transferred to glass Petri dishes (Ø = 9cm) and treated at 100°C in a drying oven for ten hours with the addition of 1.5 mL distilled water at one-hour intervals.

2.3 Kinematic and apparent viscosities

The thermal depolymerization of all chitosans was analyzed by kinematic and apparent viscosities. Thermally treated chitosans were transferred to lactic acid solution (0.15 mol/dm^3), stirred in an orbital shaker for 3 hours and filtered through a glass sintered filter n° 4. Kinematic viscosity (cSt) and apparent viscosity (Pa.s) of the filtrate were determined using a Cannon-Fenske n°200 viscometer and a Brookfield Programmable DV-II rheometer at 25±0.1ºC, respectively, in accordance with the manufacturer's instructions.

2.4 Determination of average molar mass by GPC-RI

For the GPC analysis, all chitosan samples (1.0 mg × cm^{-3}) were diluted in sodium acetate buffer (0.33 mol × dm^{-3} acetic acid, 0.1 mol ×dm^{-3} NaOH, pH=3.9±0.2) and filtered in sintered glass filter n° 4. A calibration curve was obtained with 2.5 mg × cm^{-3} dextran

standard dissolved in water containing 0.05% (m/v) sodium azide (Sigma Chemical Co., St. Louis, USA). The GPC-RI system consisted of a pump, model 515; an automatic injector, model 717 and a refractive index detector, model 410, and the software used was *Millenium*® GPC (all from Waters). Two polymethyl methacrylate hydroxylate columns (Ultrahydrogel 1000 and Ultrahydrogel 500) with exclusion volumes of 1.0×10^6 and 8.0×10^4, respectively, were connected in series. The volume of the injected sample was 200 μL and the mobile phase was sodium acetate buffer (0.33 mol × dm^{-3} acetic acid, 0.1 mol × dm^{-3} NaOH, pH=3.9±0.2) at a flow rate of 0.8 mL × $minute^{-1}$ and a temperature of 40ºC.

2.5 Determination of viscosity average molar mass (Mv) of chitosan

The viscosity average molar mass of the five chitosans described in Table 1 was also determined by intrinsic viscosity (Roberts, 1992).

Table 1: Chitosan samples used to determine the viscosity average molar masses (Mv)

Treatment	**Temperature (ºC)**	**Time (hours)**	**Code**
Thermally treated chitosan	100	0 (control)	A
		3	B
		10	C
Chitosan obtained by alkaline deacetylation of chitin	100-110	1	D
	110-122	1.5	E

Three different solvents were tested in order to find the most suitable (Table 2). *K* and *a* are constants that are independent of molar mass over a considerable range of molar masses and depend on the polymer, solvent, temperature and, in the case of polyelectrolytes, the nature and concentration of the low-molar-mass electrolyte added. The Ubbelohde viscometer was kept at a temperature at a temperature of 25.0 ± 0.1°C by means of a water bath. The solvent flow times were preferably longer than 100s. Chitosan average molar mass was determined using a Ubbelohde viscometer, type 53110/I from Schott GmbH.

Table 2: Viscosity parameters of chitosans (for references and discussion, see Roberts, 1992)

Code	Mv (g mol^{-1})	Solvent	K (dm^3/g)	a
1	113,000-492,000	0.2 mol dm^{-3} HOAc, 0.1 mol dm^{-3} NaCl, 4 mol dm^{-3}	0.893×10^{-4}	0.71
2	90,000-1,140,000	0.1 mol dm^{-3} HOAc, 0.2 mol dm^{-3} NaCl	0.181×10^{-5}	0.93
3	13,000-135,000	0.33 mol dm^{-3} HOAc, 0.3 mol dm^{-3} NaCl	0.341×10^{-5}	1.02

In order to select the best solvent, Mv of sample A (raw material) were determined using solvents 1, 2 and 3, and for samples B, C, D and E solvent 3 was used. A 0.5% stock solution (w/v) was prepared for each chitosan and the range of concentrations used was 1.0 to 5.0 g × dm^{-3}. When the dissolution was complete, the solution was filtered through a Schott glass sintered filter n°4. The correct concentration of dissolved polysaccharide was calculated as the difference between the initial amount of polymer and the insoluble part, using equation 1.

$$C = \frac{m_1 - (m_2 - m_0)}{V} \qquad (1)$$

where C is the concentration of chitosan solution, m_0 is the mass of dry filter, m_1 is the mass of chitosan sample and m_2 is the mass of filter containing insoluble particles after drying.

The intrinsic viscosity was determined according to equation 2.

$$[\eta_i] = \frac{(t_1 - t_0)/t_0}{C} \qquad (2)$$

where t_1 is the flow time for the chitosan solution, t_0 is the flow time for the solvent system and η_i is the intrinsic viscosity.

The limiting viscosity number was found by the extrapolation of Mark-Houwink´s relationship between the intrinsic viscosity and the concentration of chitosan in the investigated solution to concentration of chitosan of zero.

The average molar mass was obtained according to the equation 3.

$$Mv = (\eta_L / K)^{1/a} \qquad (3)$$

where *Mv* is the viscosity average molar mass, η_L is limiting viscosity number and *K* and *a* are Mark-Houwink constants.

2.6 Alkaline deacetylation of chitin

Deacetylation of chitin was achieved in accordance with a modified procedure developed by Canella and Garcia (2001). Sixteen grams of chitin (*Sigma Chemical, St. Louis, USA*) were suspended in 200mL of 50% NaOH solution (m/v) and stirred at 900 r.p.m in a batch reactor under reflux. In Table 3 the temperatures and times used to obtain samples D and E are described and a summary of the purification process is given.

Table 3: Conditions of alkaline deacetylation of chitin and purification of chitosan samples.

Batch	Temperature (°C)	Time (minutes)	Cooling	Centrifugation 10,000g for 5 minutes	Washing of pellet until low conductivity is achieved	Solubilization in 0.15 dm^3/L lactic acid and orbital shaker at 200 o.p.m	Centrifugation 10,000g for 5 minutes	Obtention of supernatant	Neutralization of supernatant with NaOH 1dm^3/L until pH=8.5 is reached	Washing of suspension until low conductivity is achieved	Freezing at -80°C and lyophilization	Sample
1	100-110	60										D
2	110-122	80										E

2.7 Determination of the degree of N-acetylation by potentiometric tritation

The mole fraction of N-acetylglucosamine residues (F_A) was determined using potentiometric tritation, as described by Raymond *et al.* (1993). The 1% chitosan sample (w/v) was added to HCl 0.1 mol dm^{-3} and titrated with a solution of NaOH 0.1 mol dm^{-3}. The neutralization point was determined potentiometrically.

The values of F_A were calculated according to equation (4),

$$F_A = 1 - \frac{V_{NaOH} \, x \, M_{NaOH}}{(m_{ch}/M_{ch})} \qquad (4)$$

where F_A is the mole fraction of N-acetylglucosamine residues, m_{ch} is the chitosan sample mass, M_{ch} is the molar mass of glucosamine unit, V_{NaOH} is the volume of NaOH 0.1 mol dm^{-3} solution used to neutralize the protonated free amino groups and M_{NaOH} is the molar concentration of NaOH solution.

2.8 Determination of the degree of N-acetylation by high-field ^{1}H NMR spectroscopy

In accordance with a procedure adapted from a publication by Vårum et al. (1991), a sample of roughly 100mg of chitosan was suspended in 10 mL of 0.07 mol dm^{-3} HCl at room temperature with stirring overnight. A small mass of $NaNO_2$ (9-10mg) was added to the stirring solution and left to react for 4 hours. The solution was lyophilized and then ion-exchanged with D_2O three times. The samples were dissolved in roughly 1.5 mL of D_2O and filtered through cotton to remove any insoluble mass. ^{1}H NMR spectra of the samples were obtained in a Bruker 300 MHz NMR spectrometer at room temperature after 32 scans with a delay time of 3 to 4 seconds. The degree of acetylation is given by equation 5 according to Vårum et al. (1991),

$$F_A = 7\,(I_B + I_E)/[4(I_A + I_C + I_D) + I_B + I_E] \qquad (5)$$

where **I** is the peak intensity, represented as an integral, and the subscripts A to E identify particular peaks indicated in Figures 10 and 11. Peaks **A** and **B** correspond to the anomeric protons GlcN and GlcNAc, respectively; **C** and **D** correspond to ring protons; **E** corresponds to the methyl protons.

2.9 Statistical analysis

The result of each treatment was the average of two or three repetitions depending of analyze realized. The results were analyzed by ANOVA and regression at $p \leq 0.05$. The models were selected analyzing the determination coefficients (R^2) and significance of regression coefficients tested by Student t test. Statistical analyses were carried out using the Sisvar software 4.3.

3.0 RESULTS AND DISCUSSION

3.1 Evaluation of thermal depolymerization

The most commonly used methods for determination of Mv, Mw and Mn are viscosity, light scattering (SLS – static light scattering and MALLS – multiple-angle laser light-scattering) and GPC (gel-permeation chromatography) or SEC (size exclusion chromatography) (Terbojevich & Cosani, 1997).

Insoluble materials were observed in the raw material from Polymar. Approximately, 14% of insoluble material was retained on sintered glass filter n° 4 from 1% chitosan solution (m/v) in lactic acid (0.15 mol × dm^{-3}). The formation of insoluble material increased with increase in treatment time. Approximately, 27% and 67% of isoluble materials were retained on sinterized glass filter n° 4 from 1% chitosan solutions of samples thermally treated for 3 and 10 hours, respectively. Some insoluble materials were also observed by Holme et al. (1999). The formation of insoluble material can be explained by interchain cross-link formation involving free amino groups and reducing ends (Roberts & Taylor, 1989).

The thermal degradation of chitosan samples in solid state treated at 100°C during 10 hours was analyzed by viscosity (kinematic, intrinsic and apparent viscosities) and gel permeation chromatography with a refractive index detector (GPC-RI). Values of kinematic and apparent viscosities and molar mass (Mw) are shown in Figure 1a,b. The empirical models adjusted to the experimental data of kinematic viscosities (Equation 6), apparent viscosities (Equation 7) and average molar masses (Equation 8) were statistically significant at $p \leq 0.05$.

$$Y_{KV} = -0.03373X^3 + 0.85342X^2 - 7.05007X + 20.19318 \qquad R^2 = 0.99193 \qquad (6)$$

$$Y_{AV} = -0.05609X^3 + 1.62181X^2 - 1.10938X + 4.17165 \qquad R^2 = 0.96158 \qquad (7)$$

$$Y_{MW} = 4.29172X^2 - 74.75343X + 323.88758 \qquad R^2 = 0.95506 \qquad (8)$$

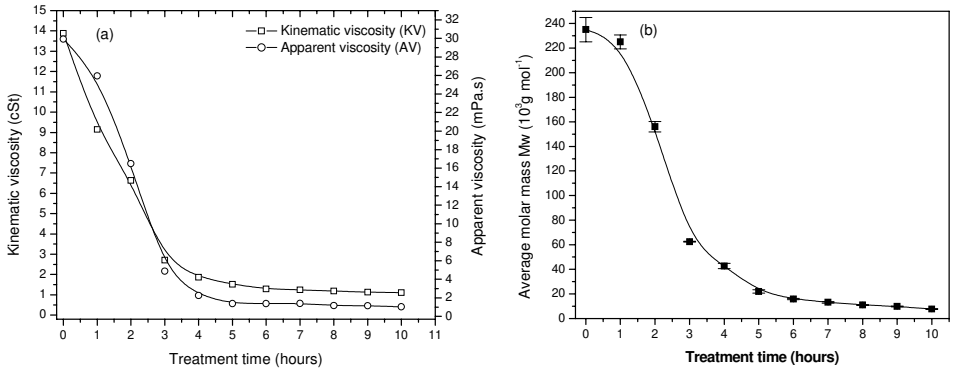


Figure 1: Kinematic and apparent viscosities (a) and average molar mass (b) of chitosans in solid state thermally degraded at 100°C for up to10 hours.

Values of Mw determined by GPC-RI were calculated using chromatograms and calibration curve for the dextran standard shown in Figure 2a,b. Both chromatograms (Figure 2a) and calculation of the calibration curve (Figure 2b) for the dextran standard were obtained with the software Millenium.

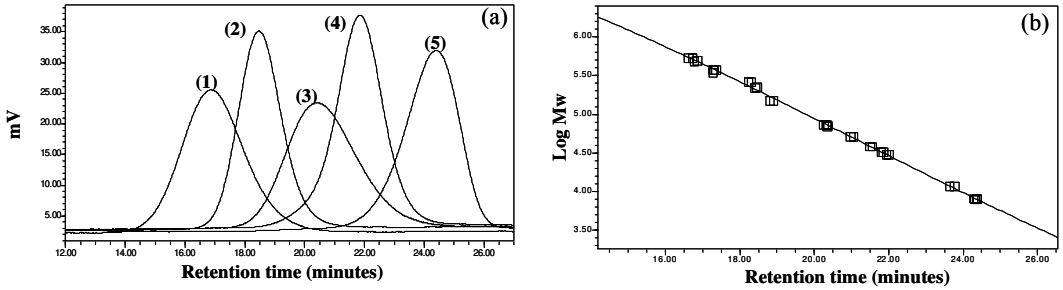


Figure 2: Chromatograms (a) and calibration curve (b) of GPC-RI for the dextran standards (1) DXT530K M_w=5.34x10^5 g mol^{-1}; (2) DXT260K M_w=2.61x10^5 g mol^{-1}; (3) DXT72K M_w=7.27x10^4 g mol^{-1}; (4) DXT38K M_w=3.82x10^4 g mol^{-1}; (5) DXT11K M_w=1.17x10^4 g mol^{-1}.

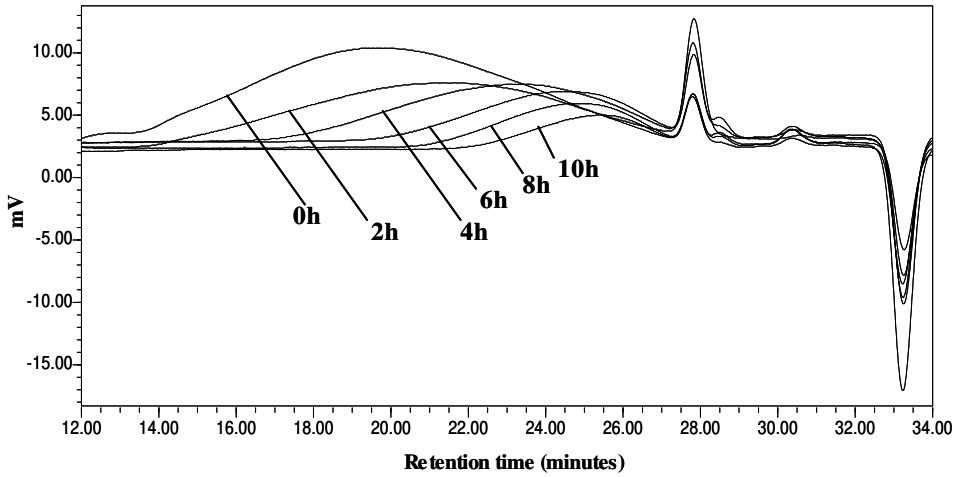


Figure 3: Chromatografic profiles obtained by GPC-RI for control chitosan without thermal treatment (0 hour) and chitosans treated for up to 10 hours.

The kinematic and apparent viscosities and molar masses of chitosan samples treated for six hours at 100ºC decreased more than 90% (Figures 3 and 4 and Table 4). After 10 hours of treatment, decreases in kinematic and apparent viscosities and Mw of the chitosans of 92.0%, 96.5% and 96.7%, respectively, were observed.

Thermal depolymerization of chitosan chloride with different F_A was studied by Holme et al. (2001) at different temperatures. The decrease in apparent viscosity obtained by Holme et al. (2001) for chitosan F_A=0.35 treated at 105ºC for 10 hours was about 90%. Similar result was obtained in our study for chitosan F_A=0.22% treated at 100ºC for 10 hours, where a decrease of 96.5% in apparent viscosity was observed (Figure 4 and Table 4).

The thermal depolymerization of chitosan occurs in in two phases. In the first stage (0-4 hours of treatment) an increase in chitosan depolymerization, predicted by the linear model was observed and in the second stage (5-10 hours of treatment) a tendency towards process stabilization, also predicted by the linear model (Figures 5 and 6), was observed.

Similar results were observed by viscosity and GPC-RI measurements. Figure 5 depicts viscosity and molar mass reductions plotted against time for 4 hours of chitosan degradation at 100ºC. In Figure 5 it can be seen that the depolymerization rate increased with time as expected. The depolymerization rates measured by kinematic viscosity, apparent viscosity and molar mass for 4 hours were 21.9, 25.5 and 23.3 % h^{-1}, respectively.

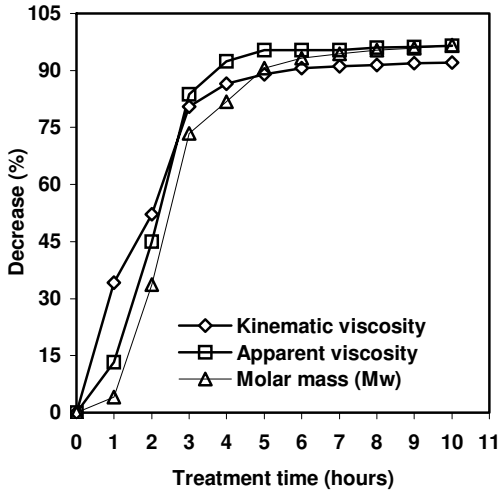


Figure 4: Decrease in kinematic and apparent viscosities and molar masses of chitosans in solid state thermally degraded at 100°C for 10 hours.

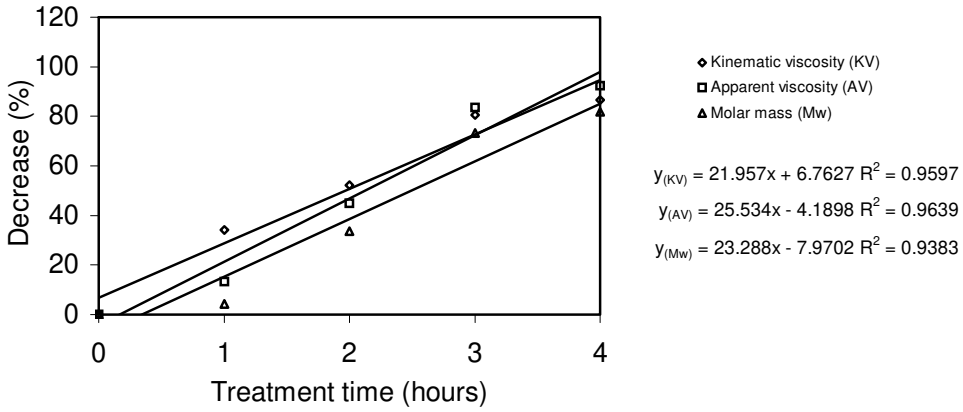


Figure 5: Decrease in kinematic and apparent viscosities and molar masses of chitosans in solid state thermally degraded at 100°C for 4hours ($p \leq 0.05$).

It was observed that the depolymerization rates in the period of 5 to 10 hours of treatment decreased slowly, and the values observed were DR_{KV}=0.545, DR_{AV}=0.248 and DR_{MW}=1.11 % h^{-1} (Figure 6).

Viscometry and GPC-RI gave similar results in the analysis of chitosans with a range of molar masses from 235,000 to 43,000 g × mol^{-1} and polydispersity indices from 7

to 3.5, however, in the analysis of chitosans with a range of molar masses from 22,000 to 8,000 g × mol^{-1} and polydispersity indices from 2.3 to 1.3 the methods gave different results. This difference may be related to the hydrodynamic volume of the macromolecules, which is a function of molar mass, conformational properties and polymer-solvent interactions, as described by Terbojevich and Cosani (1997).

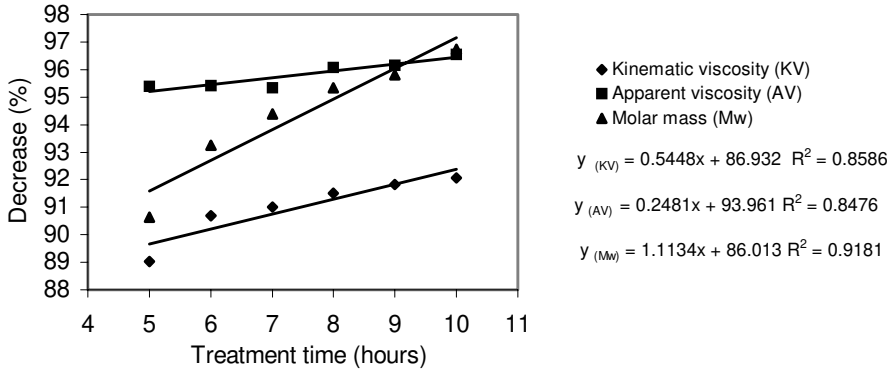


Figure 6: Decrease in kinematic and apparent viscosities and molar masses of chitosans in solid state thermally degraded at 100°C for a period of 5 to 10 hours ($p \leq 0.05$).

Table 4 shows the decrease in the values of kinematic and apparent viscosities and molar mass as measured by viscometry and GPC-RI. Each parameter had significantly different values for the decrease up to t_{KV}=7 hours, t_{AV}=4 hours and t_{Mw}=6 hours. Chitosans thermally treated at 100ºC for 8, 9 and 10 hours did not show a significant decrease in AV and Mw. The degradation of chitosan in the first 4 hours of thermal treatment, measured by KV, AV and GPC-RI was significant ($p \leq 0.05$).

Table 4: Decrease in kinematic and apparent viscosities and molar mass of chitosans in solid state thermally degraded at 100°C for 10 hours ($p \leq 0.05$).

	Decrease (%)		
Time (hours)	**Kinematic viscosity (KV)**	**Apparent viscosity (AV)**	**Molar mass (Mw)**
0	0 [a]	0 [a]	0 [a]
1	34.1 [b]	13.3 [b]	4.1 [b]
2	52.2 [c]	44.9 [c]	33.7 [c]
3	80.5 [d]	83.7 [d]	73.4 [d]
4	86.6 [e]	92.5 [e]	81.8 [e]
5	89.0 [f]	95.4 [e]	90.6 [f]
6	90.7 [g]	95.4 [e]	93.3 [g]
7	91.0 [h]	95.3 [e]	94.4 [g]
8	91.5 [i]	96.1 [e]	95.3 [h]
9	91.8 [j]	96.2 [e]	95.8 [h]
10	92.0 [j]	96.5 [e]	96.7 [h]

Different letters in the same column indicate significant differences between the means obtained with the Tukey test ($p \leq 0.05$).

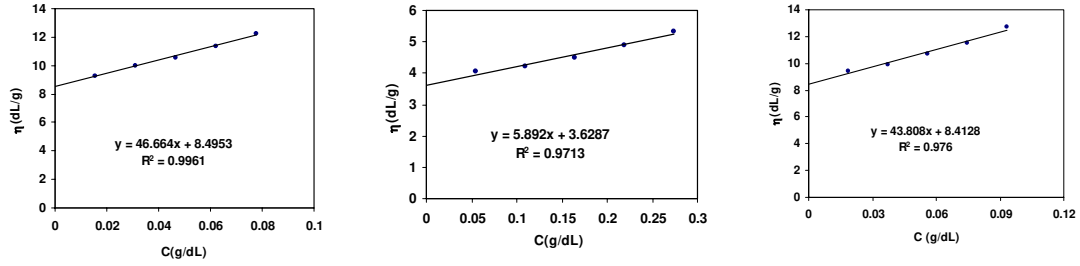
In our work, two depolymerization rates were found, the first one for up to 4 hours of heating (DR_{KV}=21.9, DR_{AV}=25.5 and DR_{Mw}=23.3 % × h^{-1}) and the lower rate (DR_{KV}=0.545, DR_{AV}=0.248 and DR_{Mw}=1.11 % × h^{-1}) for the treatment time interval from 5 to 10 hours, when a decrease in molar mass of more than 90% had already been achieved. Three techniques used to evaluate the thermal depolymerization process had linear relationships ($p \leq 0.05$) in the treatment time interval up to 4 hours, i.e., the slope of the linear regression line predicts the depolymerization rate (DR) of chitosan treated thermally in a specific time interval. Analysis of thermal depolymerization kinetics of chitosans based on the KV, AV and Mw data showed good agreement with DR values that did not vary statistically at $p \leq 0.05$. The viscosities and the average molar masses had a statistical tendency to become stable from 5 hours on, and further heating did not seem to cause further chitosan depolymerization.

The mechanism of thermal degradation of chitosan was studied by Holme et al. (2001). The oxidative-reductive degradation mechanism was discarded after it was confirmed that thermal degradation with and without oxygen (nitrogen atmosphere) did not affect the degradation rate. Chitosan chloride in solid state F_A=0.16 with pH 4, 5 and 6 was

thermally treated at 105°C and an increase in thermal degradation with the increase in H^+ concentration was observed. It was confirmed that acid hydrolysis is the primary mechanism of the thermal degradation of chitosan chloride. Acid hydrolysis of the glycosidic linkages involves both protonation of the glycosidic oxygen and addition of water to yield the reducing sugar end group (BeMiller, 1967). In our study chitosan in solid state was used with the addition of water at time intervals of 1 hour to maintain the H^+ concentration during the heating treatment and to hydrolyze the glycosidic linkages.

3.2 Determination of viscosity average molar mass of chitosan

The Mv of control chitosan (A) was determined using solvent 1 (0.2mol dm^{-3} of acetic acid and 0.1mol dm^{-3} of sodium chloride and 4mol dm^{-3} of urea), solvent 2 (0.1mol dm^{-3} of acetic acid and 0.2mol dm^{-3} of sodium chloride) and solvent 3 (0.33mol dm^{-3} of acetic acid and 0.3mol dm^{-3} of sodium chloride) as described by Roberts (1992). In Figure 7 the linear regressions, intrinsic viscosities and Mv of control chitosan (A) using solvents (1), (2) and (3) are shown; Mv values were 1,254,259g mol^{-1}, 121,048 g mol^{-1} and 193,400g mol^{-1}, respectively.



(1)

η_i(dL/g)= 8.4953
Mv=1,254,259 g mol^{-1}

(2)

Chitosan sample A
η_i(dL/g)= 3.6287
Mv=121,048 g mol^{-1}

(3)

η_i(dL/g)= 8.4128
Mv=193,400 g mol^{-1}

Figure 7: Linear regressions to obtain the limiting viscosity number by extrapolation of Mark-Houwink's relationship between intrinsic viscosity and chitosan concentration of control sample (A) and Mv values using solvents 1, 2 and 3.

The strong effects of the three different solvents were shown to be significant ($p \leq 0.05$) when used for determination of viscosity average molar mass. The Mv values of chitosan (A) determined in solvent 1 (0.2mol dm^{-3} of acetic acid, 0.1mol dm^{-3} of sodium chloride and 4mol dm^{-3} of urea) was 1,254,259 g mol^{-1}, that in solvent 2 (0.1mol dm^{-3} of acetic acid and 0.2mol dm^{-3} of sodium chloride) was 121,048 g mol^{-1} and that in solvent 3 (0.33mol dm^{-3} of acetic acid and 0.3mol dm^{-3} of sodium chloride) was 193,400 g mol^{-1}. Several set values for *K* and *a* of chitosan have been proposed in the literature (Roberts, 1992). The *K*-values depend on the average molar mass used and on the molar mass distribution of the samples (Ottøy et al., 1996). The polydispersity index of sample A determined by GPC-RI was 6.97, and for this reason different values of Mv were found for different *K*-values and solvents. By GPC-RI analysis the average molar mass of chitosan A was 235,000 g mol^{-1}, of the closest value to the Mv determined by viscosity (value 193,400 g mol^{-1}) in solvent 3. The same was observed for samples B, C, D and E, indicating that 0.33 mol dm^{-3} of acetic acid and 0.3 mol dm^{-3} of sodium chloride was the most suitable solvent for this determination.

The relationship between Mv and intrinsic viscosity η_i is expressed by the Mark-Houwink-Kuhn-Sakurada equation, $\eta_i = kM^a$, where the viscosity parameters *K* and *a* depend on the polymer, the temperature, the solvent, and the salt concentrations (Peter, 2002). Different values of Mv were obtained by intrinsic viscosity η_i for chitosan sample A using three different solvents by the high polydispersity index of raw material (6.97) calculated by GPC-RI. Roberts (1992) reports different ranges of Mv for different solvents (Table 2). Chitosan samples with a high polydispersity index (6.97), whose average molar masses were determined by viscometry, showed very large discrepancies; however for chitosans with a low polydispersity index there was no discrepancy of average molar masses obtained by viscometry.

It was observed that methods used to characterize thermally treated chitosan samples and chitosan samples obtained by alkaline deacetylation showed a good correlation between parameter values analyzed to determine molar masses and viscosities. Of these three methods, GPC-RI and apparent and kinematic viscosities cited and discussed as well as intrinsic viscosity used in the determination of Mv, intrinsic viscosity was shown to be

reliable and efficient, since it can be closely correlated to the average Mw obtained by GPC-RI (Figure 1b).

Figure 8 shows chromatographic profiles for chitosans obtained by thermal treatment and alkaline deacetylation. Chromatographic profiles for chitosans without thermal treatment (A) and thermally treated for 3 hours (B) and 10 hours (C) are shown in Figure 8a and chromatografic profiles for chitosans D and E, obtained by alkaline deacetylation, are shown in Figure 8b.

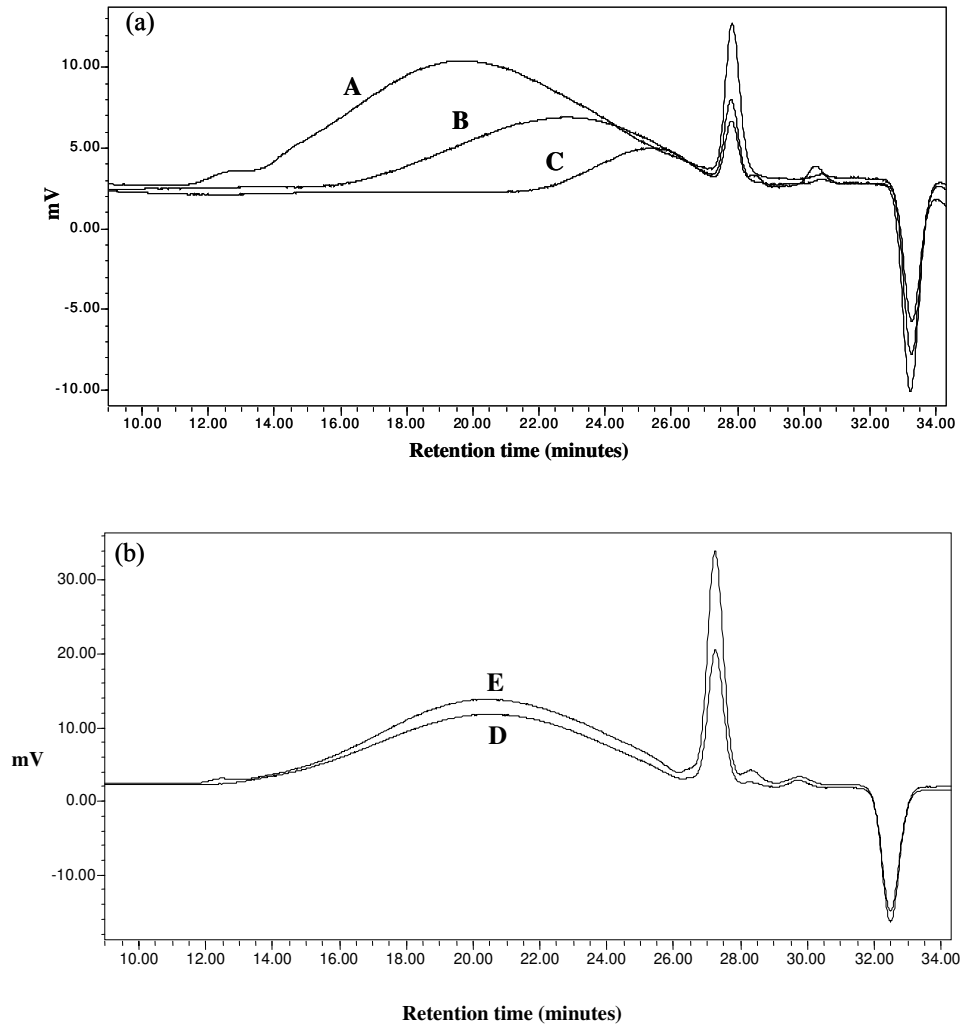


Figure 8: Chromatografic profiles obtained by GPC-RI for chitosans: (a) without thermal treatment A, and thermally treated for 3 hours B and 10 hours C at 100ºC and (b) chitosans D and E, obtained by alkaline deacetylation (Table 2).

In Table 5 the average molar masses, degrees of polymerization, apparent and kinematic viscosities and viscosity average molar masses of chitosan obtained by thermal treatment and alkaline deacetylation are described. In Table 6 the observed decrease in Mw (GPC-RI), Mv (intrinsic viscosity) and apparent and kinematic viscosities of chitosans thermally degraded at 100°C for 3 and 10 hours are described.

Table 5: Average molar mass (Mw), degree of polymerization, kinematic and apparent viscosties and viscosity average molar mass (Mv) of chitosans obtained by thermal treatment and alkaline deacetylation

Code	GPC[1] Mw (g mol^{-1})	DP[2]	Apparent viscosity[3] (mPa.s)	Kinematic viscosity[4] (cSt)	Intrinsic viscosity[5] Mv (g mol^{-1})
A	235,000	1,383	29.9	13.9	193,400
B	62,500	366	2.3	1.9	93,594
C	7,700	45	1.4	1.2	10,978
D	193,000	1,171	4.9	5.1	30,219
E	184,000	1,089	4.7	5.2	91,203

[1]Gel permeation chromatography. [2]Degree of polymerization [$DP=Mw/(203\times F_A+161\times(1-F_A))$]. [3]Brookfield rheometer. [4]Cannon-Fenske N° 200 viscometer. [5]Ubbelohde type 53110/I Schott GmbH viscometer.

Table 6: Decrease (%) in average molar mass (Mw), viscosity average molar mass (Mv) and apparent and kinematic viscosities of chitosan in solid state thermally treated at 100°C

Code	Treatment time (hours)	Decrease (%)			
		Mw	Mv	Apparent viscosity	Kinematic viscosity
A	0	0	0	0	0
B	3	73.4	52.9	92.3	86.3
C	10	96.7	94.5	95.3	91.4

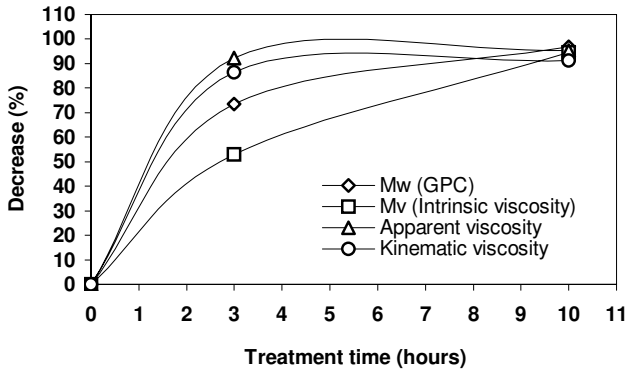


Figure 9: Decrease in Mw (GPC), Mv (intrinsic viscosity) and apparent and kinematic viscosities of chitosans thermally degraded at 100°C for 3 and 10 hours.

Determination of the molar mass by viscometry is useful to compare modifications of chitosan average molar mass but not to determine the absolute molar mass. In the present study, it was possible to verify the average molar masses calculated by more than one technique and to compare the viscometry method with gel permeation chromatography. GPC-RI offers the possibility to obtain the molar mass distribution and its polydispersity. Comparing the decrease (%) in the values of parameters Mw, Mv, apparent viscosity and kinematic viscosity after 3 hours of thermal treatment, it can be observed that the techniques used to evaluate thermal degradation gave different values. But in comparing the same parameters after 10 hours of thermal treatment, it was observed that the techniques gave similar values (Table 6 and Figure 9). Differences observed by techniques used in this study to evaluate the process of depolymerization of chitosan can be explained by the different polydispersity indices determined by GPC-RI after 3 hours (4.24) and after 10 hours (1.33) of thermal treatment. The polydispersity index of chitosans treated for 3 hours was shown to be about three times larger than that of chitosans treated for 10 hours. The polydispersity index and molar mass distribution can affect the results of the methods used to determine the molar mass of chitosan samples.

3.3 Determination of mole fraction of N-acetylglucosamine residues in chitosan

Initially, it was also our goal, to compare two analytical methods to determine mole fraction of N-acetylglucosamine residues in chitosans. The most commonly applied methods for determination of the F_A in chitin and chitosan are infrared (IR), cross-polarization/magic angle spinning nuclear magnetic resonance (CP/MAS ^{13}C-NMR), first-derivative UV and CD spectroscopy, potentiometric and dye adsorption titration, pyrolysis-gas chromatography, quantification of acetic acid in hydrolysates by liquid chromatography and thermal and elemental analysis (Peter, 2002). The potentiometric titration method and the ^{1}H NMR were chosen for study of their ability to determine the acetyl group fraction of our group of chitosans (Table 7). Titration was chosen because it is a very simple and inexpensive analytical procedure and ^{1}H NMR because it is one of the most cited tools currently used for this task.

Table 7: Degree of acetylation of chitosan samples determined by potentiometric titration and ^{1}H NMR.

Code	F_A	
	PT[1]	NMR[2]
A	0.21	0.22
B	0.23	nd
C	0.27	0.22
D	0.09	0.08
E	0.19	0.16

[1]Potenciometric titration. [2]Nuclear magnetic ressonance. [nd]Not determined.

Another goal of this present work was to verify the effect of the heating on the degree of acetylation of chitosans. The same group of chitosan samples which had been previously analyzed for average Mw was also analyzed for F_A.

It was observed that the thermal treatment used to depolymerize chitosans did not seem to significantly affect the F_A of the chitosan samples, when determined either by titration or by ^{1}H NMR (Table 7). Figures 10 and 11 show ^{1}H NMR spectra of chitosan samples A and C. F_A of control chitosan (A) and chitosan thermally treated for 10 hours (C) was 0.22 and 0.22, respectively, as determined by ^{1}H NMR, and 0.21 and 0.27,

respectively, as determined by PT. Thus, thermal treatment of chitosan at 100°C for 10 hours has very little or even no ability to modify the glucosamine bonds.

The F_A value obtained by the titration method is precise when some steps are taken to standardize the HCl and NaOH solutions and filtrate the chitosan hydrochloride solution to avoid mistakes when chitosan samples are partially soluble. Raymond et al. (1993) observed that titration of chitosan is useful for analysis of low F_A samples. In our study the range of F_A samples was 0.08 to 0.22 and the accuracy of the ^{1}H NMR and PT methods in determining F_A did not differ.

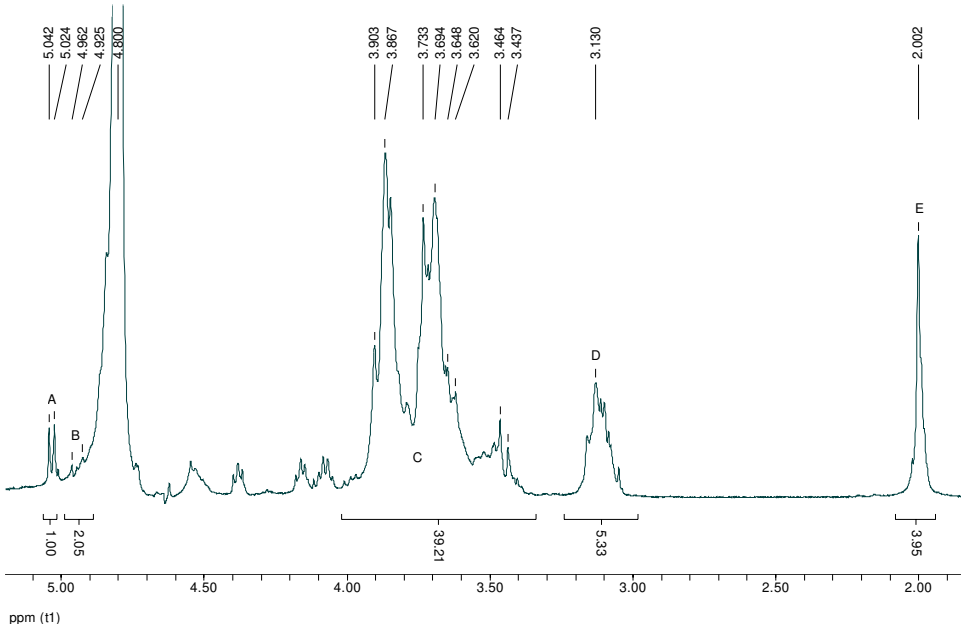


Figure 10: ^{1}H NMR spectrum of control chitosan sample (A).

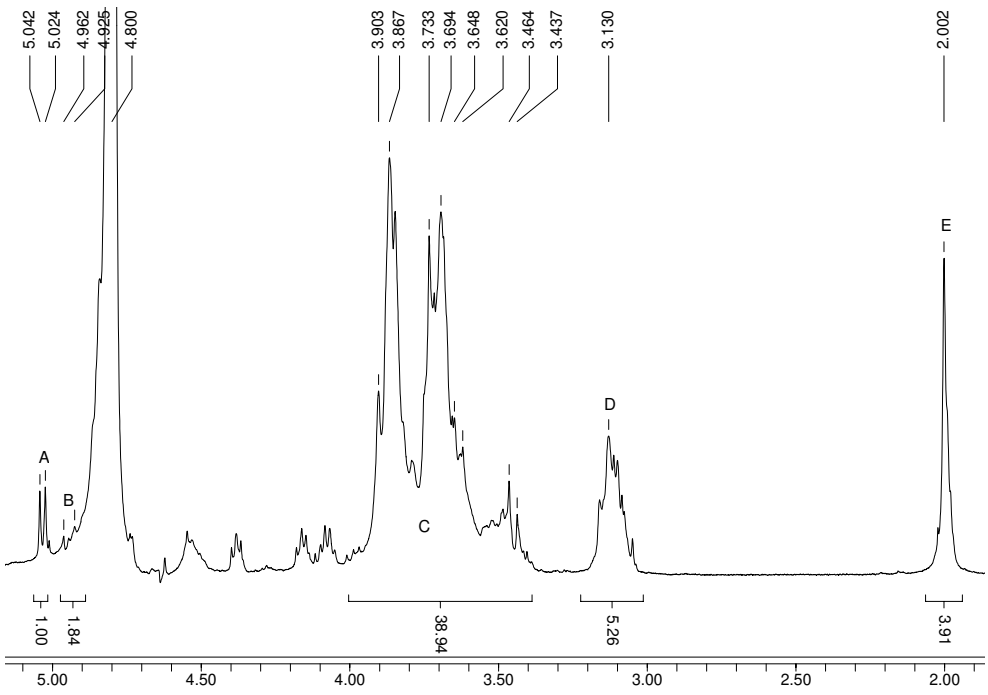


Figure 11. ^{1}H NMR spectrum of chitosan sample (C), thermally treated at 100°C for 10 hours.

4.0 Conclusions

This study showed that thermal treatment results in chitosans with different molar masses but the same degree of acetylation, as determined by ^{1}H NMR spectroscopy and potentiometric titration. The process of thermal depolymerization under the conditions used in this study was shown to be appropriate for obtaining chitosans with smaller molar masses, given that in this process strong hydrolytic reagents, such as hydrocloric, sulfuric and phosphoric acids, are not used. The variables used to characterize the depolymerization process showed a good correlation. Six hours of thermal treatment were sufficient to obtain chitosans with a molar mass 90% smaller than that of the control chitosan without treatment.

ACKNOWLEDGEMENTS

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NOMENCLATURE

AV	apparent viscosity	mPa s
F_A	mole fraction of N-acetylglucosamine residues	(-)
DR	depolymerization rate	% h^{-1}
KV	kinematic viscosity	cSt
Mv	viscosity average molar mass	g mol^{-1}
Mw	Mass average of molar mass (determined by GPC-RI)	g mol^{-1}
C	concentration of chitosan solution	g dm^{-3}
m_0	mass of dry filter	g
m_1	mass of chitosan sample	g
m_2	mass of filter containing insoluble particles after drying	g
t_1	flow time for the chitosan solution	s
t_0	flow time for the solvent system	s
K	Mark-Houwink constant	dm^3 g^{-1}
a	Mark-Houwink constant	(-)
m_{ch}	chitosan sample mass	g
M_{ch}	molar mass of glucosamine unit	161g mol^{-1}
V_{NaOH}	volume of NaOH solution	cm^3
M_{NaOH}	molar concentration of NaOH solution	mol dm^{-3}
PT	Potentiometric titration	(-)

Greek Letters

η_i	intrinsic viscosity	dm^3 g^{-1}
η_L	limiting viscosity number	dm^3 g^{-1}

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CAPÍTULO III

Este capítulo corresponde ao artigo submetido:

E. N. Oliveira Junior, N. E. Gueddari, B. M. Moerschbacher, and T. T. Franco. Characterization of the effects of chitosan on the inhibition of phytopathogenic fungi. *Brazilian Journal of Microbiology*.

Este capítulo corresponde à avaliação da atividade antifúngica de quatro grupos de quitosanas de diferentes graus de acetilação e de polimerização contra quatro fungos fitopatogênicos: *Alternaria alternata*, *Botrytis cinerea*, *Penicillium expansum* e *Rhizopus stolonifer*. Esta etapa do trabalho foi realizada no Instituto de Bioquímica e Biotecnologia de Plantas da Universidade de Münster (Alemanha) com a colaboração do Prof. Dr. Bruno M. Moerschbacher. O grupo de quitosana III foi gentilmente fornecido pelo Prof. Dr. Kjell M. Vårum da Universidade de Trondheim, Noruega, e o grupo de quitosana IV foi fornecido pelo Prof. Dr. Alain Domard da Universidade Claude Bernard, Lyon, França. Os bioensaios foram realizados em microplacas de 96 reservatórios e os crescimentos fúngicos foram medidos por meio dos valores de absorbância no comprimento de onda de 405 nm. Microplacas plásticas com 96 reservatórios foram bastante eficientes para o crescimento dos fungos filamentosos testados e inibições completas do crescimento de *A. alternata*, *B. cinerea*, *R. stolonifer* e redução do crescimento de *P. expansum* foram obtidas com quitosanas de DP 45 a 1.460 e de F_A 0.08 a 0.22. Os grupos de quitosana III e IV por apresentarem faixas similares de F_A e diferentes DP médios de 175 e 3.780, respectivamente, tiveram sua atividades antifúngicas comparadas e foi observado que a combinação de menor F_A e maior DP de uma mesma quitosana resultou em maior atividade antifúngica contra os fungos *A. alternata* e *B. cinerea*. Mudanças morfológicas e formação de agregados micelianos fúngicos foram observados por microscopia ótica nos fungos tratados com quitosana. F_A de quitosana mostrou ser um importante fator envolvido na atividade antifúngica, cuja atividade máxima foi observada para os menores valores de F_A, destacando a importância dos grupos amino protonados (NH_3^+) na atividade antifúngica.

Characterization of the effects of chitosan on the inhibition of phytopathogenic fungi

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Abstract - The antifungal effects of fifteen chitosans with different degrees of polymerization (DP) and different degrees of acetylation (F_A) on four phytopathogenic fungi (*Alternaria alternata, Botrytis cinerea, Penicillium expansum* and *Rhyzopus stolonifer*) were examined using a 96-well microtiter plate and a microplate reader. The antifungal activity of the chitosans increased with the decrease in F_A. Chitosans with low F_A and high DP showed the highest fungistatic activity against *A. alternata*, *B. cinerea, Penicillium expansum* and *Rhizopus stolonifer* fungi. The most resistant fungi were *Penicillium expansum* and *Botrytis cinerea* and the most sensitive fungi were *Alternaria alternata* and *Rhizopus stolonifer*. The minimum inhibitory concentrations (MICs) of the chitosans ranged from 100 μg/mL to 1,000 μg/mL depending on the fungi tested and the DP and F_A of the chitosan. Microscopic observations of fungi treated with chitosans revealed that chitosan affects the morphology of the hyphae.

Keywords: chitosan; antifungal activity; fungal morphology; phytopathogenic fungi.

1.0 Introduction

Chitin and chitosan are aminoglucopyranans composed of N-acetylglucosamine (GlcNAc) and glucosamine (GlcN) residues. These polysaccharides are renewable resources currently being studied by an increasing number of academic and industrial research groups (Peter, 2002). Indeed, numerous applications have been suggested in the literature: preservation of foods from microbial deterioration, formation of biodegradable films, purification of water and clarification and deacidification of fruit juices (Shahidi et al., 1999).

The biological activity of chitosan and chitooligomers has attracted considerable attention and there are reports on some antimicrobial (Kendra and Hadwiger, 1984; Sudarshan et al., 1992), antitumor (Suzuki et al., 1986; Tokoro et al., 1988) and hypocholesterolemic functions (Muzzarelli, 1996). After discovery of the antimicrobial activity of chitosan and its salts by Allan and Hadwiger (1979), Kendra and Hadwiger (1984) and Uchida et al. (1989), many researchers have continued studies in this field. In this context, the antimicrobial activity of chitin, chitosan and their derivatives against different groups of microorganisms, such as bacteria, yeast and fungi, has received

considerable attention (Shahidi et al., 1999). Chitosan has been used as a coating for fruits (El Ghaouth et al., 1991a,b; Jiang and Li, 2000) and it has shown activity against a wide range of fungi (El Ghaouth et al., 1992a,b; El Ghaouth, 1994; Bhaskara Reddy et al., 2000). Most studies involve only one or a few different molar masses and F_A of chitosans. Thus, important information is lacking on the antifungal activity of chitosans with widely different molar masses and F_A.

Growth of filamentous fungi in liquid culture is usually measured as an increase in dry mass, using either stationary or shake cultures in Erlenmeyer flasks. Large-scale physiological experiments, for instance when testing the effect of various compounds on the growth of a fungi, can become very space-demanding and laborious, limiting the scale of studies (Langvad, 1999). In order to overcome the difficulties related to the conventional methods to measure biomass content, this study used a 96-well microtiter plate and a microplate reader to examine the *in vitro* antifungal effect of fifteen chitosans with widely different molar masses and F_A against the phytopathogenic fungi *B. cinerea, P. expansum, R. stolonifer* and *A. alternata*.

2.0 Materials and methods

2.1 Chitosan samples

The chitosan samples used in this study were classified into four groups, according to their source and treatment. Group I contained chitosans with different DP and the same F_A raw material coded as A (Polymar, Fortaleza, Brazil) was thermally treated to produce samples B and C. Group II included chitosans with different F_A obtained from alkaline deacetylation of chitin (Sigma Chemical Co., St. Louis, USA), in accordance with the method described by Canella and Garcia (2001). More details about the preparation and physicochemical characterization of these chitosan samples (Table 1) will be published elsewhere.

Group III included chitosans with of rather constant DP_n the ca. 190 (obtained from number average molar mass), but F_A varying from 0.01 to 0.69 (Table 2), which had been previously prepared by partial de-N-acetylation of highly acetylated chitosan polymers. The acetyl groups were distributed randomly along the linear polymer chains, and the polymers were all fully water soluble. Chitosans in group III were prepared as described previously

by Vander et al. (1998) and were generously provided by Dr. Kjell M. Vårum, Dept. of Biotechnology, Univ. of Trondheim, Norway.

TABLE 1 Average degree of polymerization (DP_w) and degree of acetylation (F_A) of chitosans (group I) without treatment (A) and thermally treated for 3 and 10 hours (B and C) and chitosans (group II) obtained by alkaline deacetylation of chitin (D and E).

	Group I			**Group II**	
Code	A	B	C	D	E
DP_w	1,383	366	45	1,171	1,089
F_A	0.22	0.23[a]	0.22	0.08	0.16

[a] F_A determined by potentiometric titration. F_A of other samples were determined by high-field ^1H NMR (proton nuclear magnetic resonance spectroscopy).

Chitosans (group IV) with a constant DP_w (obtained from mass average molar mass) of around 3,800 were prepared by re-N-acetylation of a fully de-N-acetylated chitosan polymer (Table 2) as described by Lamarque et al. (2005) and were provided by Dr. Alain Domard.

TABLE 2 Chitosan samples (groups III and IV)

	Group III[a]						Group IV[b]			
Code	X_1	X_2	X_3	X_4	X_5	X_6	Y_1	Y_2	Y_3	Y_4
DP	190	320	68	121	210	224	3,726	3,726	3,820	3,850
F_A	0.01	0.15	0.35	0.49	0.60	0.69	0.10	0.30	0.40	0.50

[a] DPn (number average molar mass). [b] DPw (mass average molar mass)

2.2 Microorganisms and cultivation

The filamentous fungi used in this study included *Alternaria alternata* (CCT 2816), *Penicillium expansum* (CCT 4680) and *Rhizopus stolonifer* (CCT 2002); all of these strains were purchased from André Tosello Foundation Research and Technology (Campinas, Brazil). *Botrytis cinerea,* an isolate from spoiled grape, was provided by the Institute of Botany of the University of Muenster. The *Botrytis cinerea* and *Penicillium expansum* were cultured on potato dextrose agar (PDA) and in malt extract agar (MEA) supplemented with 2% (m/v) of glucose and peptone, respectively, while the *Rhizopus stolonifer* and *Alternaria alternata* were both cultured on MEA. In order to achieve sporulation, the *A. alternata*, *B. cinerea* and *P. expansum* were incubated in slants (25×800 mm) for eight days and the *R. stolonifer* for four days at 25°C 100 cm beneath Hg lamps (32 watts) for a twelve-hour photoperiod. Spores were harvested by pouring sterile water into the slant and

stirring with a vortex for 20 seconds. Suspensions of spores and mycelia were filtered through cotton. The concentration of spores was assessed using a hemocytometer (Fuchs-Rosenthal Hell Linie) under optic microscopy (magnification 400×). The concentration of *Rhizopus stolonifer* spores was adjusted to 10,000 spores × mL^{-1} and of *B. cinerea*, *A. alternata* and *P. expansum* spores, to 20,000 spores × mL^{-1}.

2.3 Antifungal activity

Inhibition of fungal growth by chitosan was assessed using complete medium (CM) with pH 4.3 and monitoring absorbance at λ=405 nm. Complete medium was prepared as described by Pontecorvo (1953). Aliquots (150 μL) of sterile CM containing the required volume of chitosan stock solution (2 mg ×mL^{-1}) and sterile water were dispensed into the wells containing either 10 μL of a spore suspension of a test fungus or 10μL of sterile water alone (blanks). The sterile 96-well microtiter plates of polystyrene (Roth®, Karlsruhe, Germany) were incubated at 25°C under agitation at 200 o.p.m (orbits per minute) for three days for the *R. stolonifer*, six days for the *B. cinerea* and *A. alternata* and five days for the *P. expansum*. The absorbance of the wells was measured at one-day intervals for the *A. alternata, B. cinerea* and *P. expansum* and twelve-hour intervals for the *R. stolonifer*. The experiments were carried out in triplicate, and the absorbances correspond to the mean of the three well readings.

2.4 Optical microscopy

Material for optical microscopy examination of the fungi studied was obtained from cultures that were grown on 2% liquid malt extract (ME) for five days at 25ºC under cool white fluorescent lamp with an alternating cycle of 12 h of light and 12 h of darkness. The fungal spores were inoculated in 1 mL of medium (ME) dispensed into 2 mL Eppendorf vials. Growth media of the *Alternaria alternata* and *Rhizopus stolonifer* were amended with 500 μg × mL^{-1} and growth media of the *Botrytis cinerea* and *Penicillium expansum*, with 1,000 μg × mL^{-1} of chitosan D (Table 1). The fungal mycelia and a drop of sterile water were placed on a microscope slide. A cover slip was placed on top of the suspension, and

observations were made using the microscope (Nikon, China). For each treatment, three Eppendorf vials were used.

3.0 Results

3.1 Growth curve

The growth curves of the filamentous fungi *R. stolonifer*, *B. cinerea*, *P. expansum* and *A. alternata* (Figure 1) showed that CM (pH 4.3) was suitable for developing the *in vitro* assays in the microtiter plates. It was observed that 20% (v/v) 40 mmol L^{-1} acetic acid in CM (pH 3.9-4.2) did not affect the fungal growth rate. According to visual observation the *R. stolonifer* was the fastest growing of the test fungi, since after thirty six hours the fungus started to sporulate and after seventy two hours growth stabilized. Growth of the *B. cinerea*, *P. expansum* and *A. alternata* was slower than that of the *R. stolonifer,* which stabilized after four days of incubation. The absorbances varied with regard to the fungi studied due to the different colors of the mycelia and their agglomerations.

The growth of five species of filamentous fungi (*Saprolegnia parasitica*, *Mucor mucedo*, *Aspergillus niger*, *Coniophora puteana* and *Dacrymyces stillatus*) in a 96-well microtiter plate was studied by Langvad (1999), who observed a linear relationship between absorbance at λ=630 nm and fungal dry mass and validated this technique. The author suggested that the mechanism behind the photometric measurement of fungal growth is probably a mixture of light absorbance and light scattering, and therefore a variation in percentage of cell solids did not influence the readings as much as would otherwise be expected. We observed that the microtiter plate is an efficient support for the filamentous fungi, since errors varied from 0.01 to 0.18 (Figure 1).

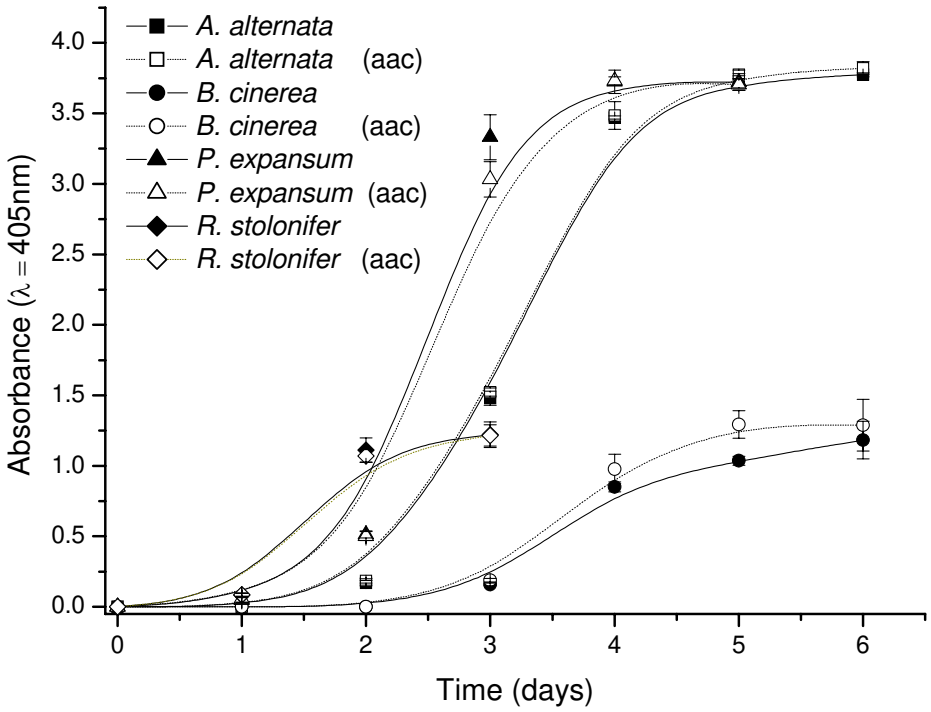

FIGURE 1 *In vitro* growth curves of *A. alternata*, *B. cinerea*, *P. expansum* and *R. stolonifer* in the absence and presence of acetic acid control (aac). Each value is the mean for three replicates and vertical bars represent standard error values.

3.2 Antifungal effects of chitosans

Chitosans markedly inhibited or completely prevented the growth of all four fungi tested. A dose-response relationship was generally observed for each fungus, with average fungal growth rates decreasing with increasing concentration of chitosan (Figure 2). The *Alternaria alternata* and *Rhizopus stolonifer* were more susceptible to chitosan than the *Botrytis cinerea* (Figure 3) and *Penicillium expansum* (Figure 2 (3)). Concentrations of chitosans from 20 to 1,000 μg × mL^{-1} were investigated for the four different fungi. Fifteen chitosan samples were tested: thirteen, whose MICs were from 100 to 800 μg × mL^{-1}, completely inhibited the *Alternaria alternata*, and ten samples with a range of MICs from 400 to 1,000 μg × mL^{-1} completely inhibited the *Botrytis cinerea* Group I (A, B and C) and group II (D and E) chitosans were tested against all fungi studied. Of these four phytopathogenic fungi tested, after preliminary results, the *A. alternata* and *B. cinerea* were selected for further experimentation as fungi that were more sensitive and more resistant, respectively. Therefore the antifungal activities of all chitosans were tested against these two fungi. However, only five chitosans were tested against the *P. expansum* and *R.*

stolonifer (Table 1). Chitosans (group I), A (DP=1,383), B (DP=366) and C (DP=45) showed different antifungal activities against the *Alternaria alternata* with MICs of 300, 200 and 100 μg × mL^{-1}, respectively. However against the *B. cinerea* (MIC=1,000 μg × mL^{-1}) and *R. stolonifer* (MIC=200 μg × mL^{-1}), the chitosans mentioned had the same antifungal effects (Figure 3). *P. expansum* growth was not completely inhibited by chitosans A (Figure 2(3)), B and C (data not shown); however with an increase in concentration of chitosans from 200 to 800 μg × mL^{-1}, there was a decrease in fungal growth rates.

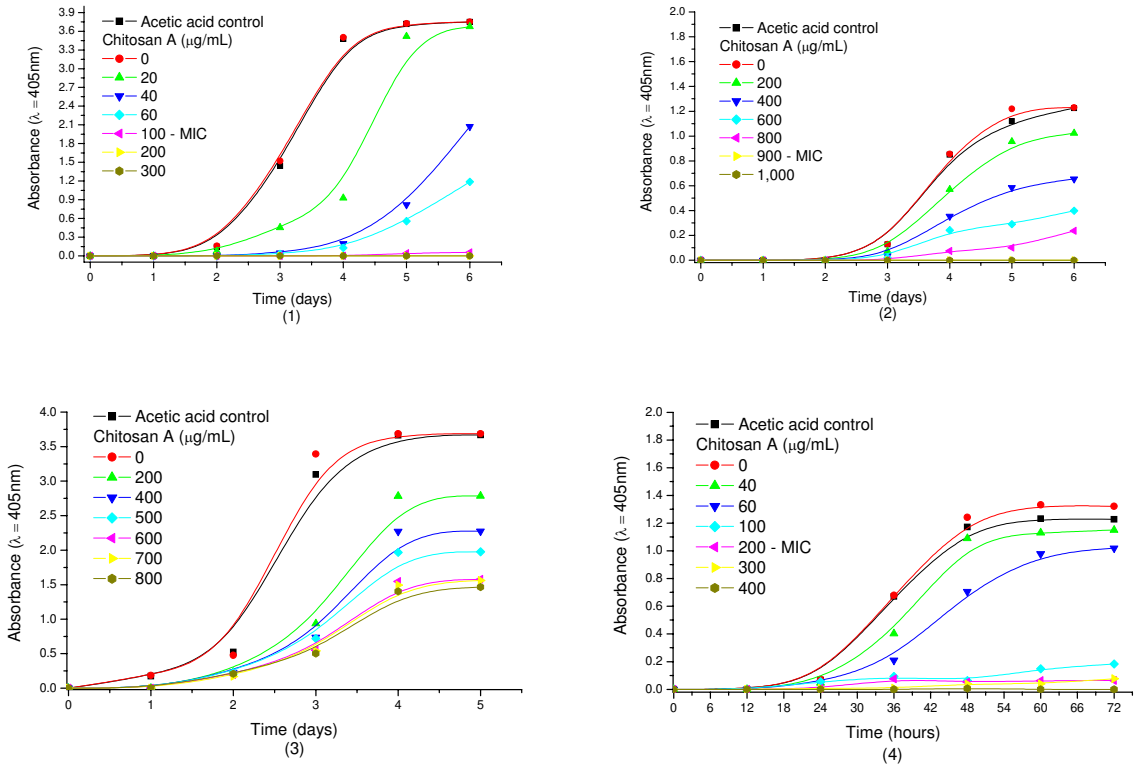

FIGURE 2 *In vitro* growth curves of *A. alternata* (1), *B. cinerea* (2), *Penicillium expansum* (3) and *Rhizopus stolonifer* (4) in CM analyzed in absence and presence of acetic acid control and the presence of chitosan A. Each value is the mean of three replicates.

Group II chitosans D (F_A=0.08) and E (F_A=0.16), whose MICs were 100 μg × mL^{-1} for *A. alternata*, 900 μg × mL^{-1} for *B. cinerea* and 100 μg × mL^{-1} for *Rhizopus stolonifer*, had the same antifungal activities. Chitosans D and E did not completely inhibit the *P. expansum*, as seen in Figure 4; however their antifungal effects were stronger than those of chitosan A (Figure 2).

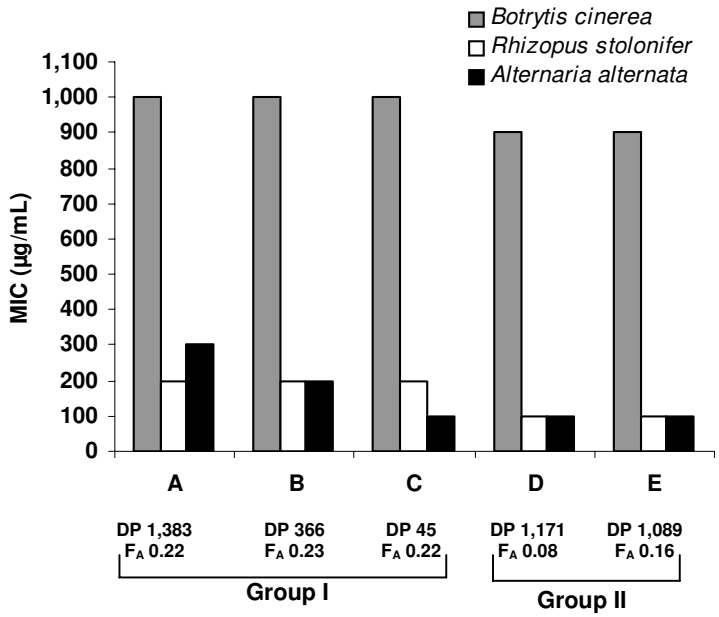

FIGURE 3 Minimum inhibitory concentrations of chitosans (group I) A, B and C with different DP and chitosans (group II) D and E with different F_A against *A. alternata, B. cinerea* and *R. stolonifer*.

The increase in antifungal activities of the chitosans tested against the pathogens *B. cinerea* and *R. stolonifer* may follow the trend (A=B=C<D=E) and against the pathogen *A. alternata*, the trend (A<B<C=D=E). In Figure 3 it can be seen that of these five chitosans tested against the phytopathogens, A, B, C, D and E, chitosans D and E showed the highest antifungal activities against the *A. alternata* (100 µg × mL^{-1}), *B. cinerea* (900 µg × mL^{-1}), *P. expansum* (higher than 800 µg × mL^{-1}) and *R. stolonifer* (100 µg × mL^{-1}). The highest antifungal activities of samples D and E were probably due to the low F_A values (0.08 and 0.16, respectively), analyzed by ^{1}H NMR. No differences between the antifungal activities of chitosans D and E against *A. alternata, B. cinerea* and *R. stolonifer* were noted, although the polymers have different F_A and the same DP values. This result suggests that the difference between F_A values described above was not substantial enough to promote different antifungal activities.

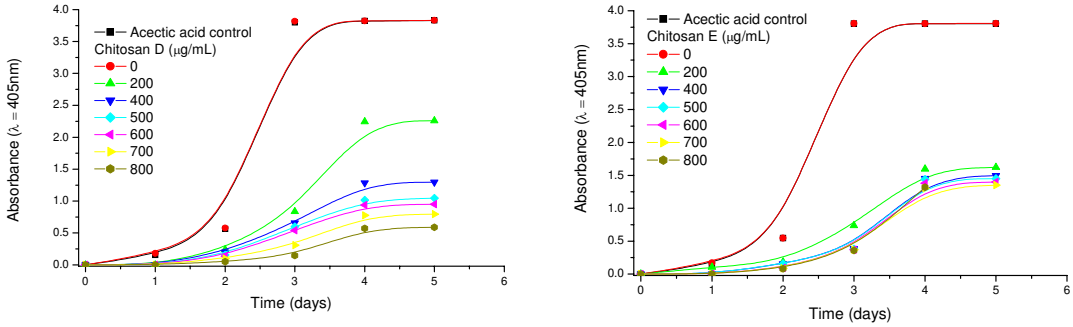

FIGURE 4 *In vitro* growth curves of *P. expansum* in CM analyzed in the absence and presence of acetic acid control (aac) and the presence of chitosans (D and E). Each value is the mean of three replicates.

The antifungal effects of group III chitosans were tested against the pathogens *B. cinerea* and *A. alternata*. Of these samples, only chitosan sample X_1, whose MIC was 800 μg × mL^{-1}, completely inhibited *B. cinerea* growth. Complete inhibition of the *B. cinerea* pathogen was not obtained with samples X_2, X_3, X_4, X_5 and X_6 using the maximum concentration of 800 μg × mL^{-1}. In contrast to the response of the *B. cinerea,* growth of the *A. alternata* was completely suppressed by the chitosan samples X_1, X_2, X_3 and X_4, whose MICs were 200, 400, 800 and 800 μg × mL^{-1}, respectively; however, complete inhibition of the *A. alternata* was not obtained with samples X_5 and X_6 using maximum concentration of 800 μg × mL^{-1} (Figure 5). The *A. alternata* was more susceptible to chitosans in group III than the *B. cinerea*. Chitosan sample X_1 prevented the growth of both fungi with MICs of 800 μg × mL^{-1} for *B. cinerea* and 200 μg × mL^{-1} for *A. alternata*.

As seen in Figure 5, the antifungal activities of X_1 to X_4 chitosans, whose MICs were 200, 400, 800 and 800 μg × mL^{-1} and F_A were 0.01, 0.15, 0.35 and 0.49, respectively, against *A. alternata* may follow the trend (X_1>X_2>X_3=X_4). An increase in MIC values for the *A. alternata* was observed with an increase in F_A of the chitosans, with approximately the same DP. These results showed that the fungistatic activities of the chitosans were influenced by the F_A of the polymers.

The antifungal effects of the group IV chitosans (Y_1, Y_2, Y_3 and Y_4), listed in Table 2, were also tested against the fungi *B. cinerea* and *A. alternata*. All chitosan samples completely inhibited the *B. cinerea* and *A. alternata* pathogens, as shown in Figure 6.

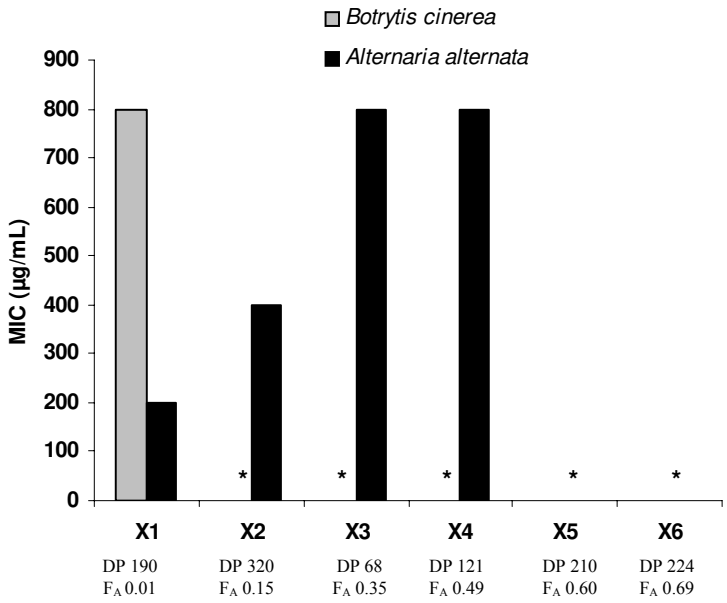

FIGURE 5 Minimum inhibitory concentrations of chitosans (group III) with similar DP and different F_A against *A. alternata* and *B. cinerea.* *MICs were not obtained for the chitosans against the fungi tested.

An increase in the antifungal activity of chitosan against the *A. alternata* ($Y_1=Y_2>Y_3>Y_4$) with a decrease in F_A was observed (Figure 6). Chitosans in group IV have similar DP in a range of 3,726 to 3,850 with a different F_A range of 0.10 to 0.50. Although the same trend in antifungal activities as that obtained with the group III chitosans was also noted with the group IV chitosans, the bioactivity of the group IV chitosans against *A. alternata* and *B. cinerea* was stronger than that of the group III chitosans. For example, the combined effects of DP=3,726 and F_A=0.10 (chitosan Y_1) completely inhibited the *A. alternata* (100 µg × mL^{-1}) and *B. cinerea* (400 µg × mL^{-1}) and DP=190 and F_A=0.01 (chitosan X_1) also completely inhibited the *A. alternata* (200 µg × mL^{-1}) and *B. cinerea* (800 µg × mL^{-1}); however the bioactivity of chitosan Y_1 was higher than that of chitosan X_1.

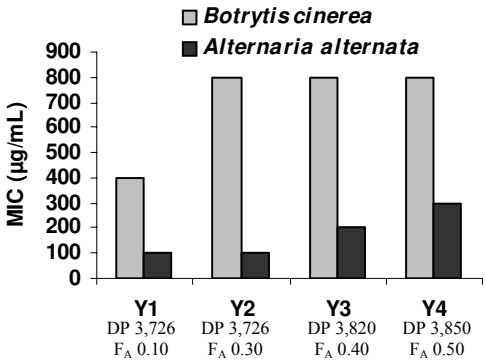

FIGURE 6 Minimum inhibitory concentrations of chitosans (group IV) with similar DP and different F_A against *A. alternata* and *B. cinerea*.

4.0 Discussion

Our results suggest that chitosan bioactivity is highly dependent on F_A and DP. The The combined effects of DP and F_A of groups III and IV chitosans against *A. alternata* is shown in Figure 7. We observed that chitosans with an average DP of 3,780 had stronger antifungal activity than chitosans with an average DP of 175, although both groups have almost the same F_A ranges.

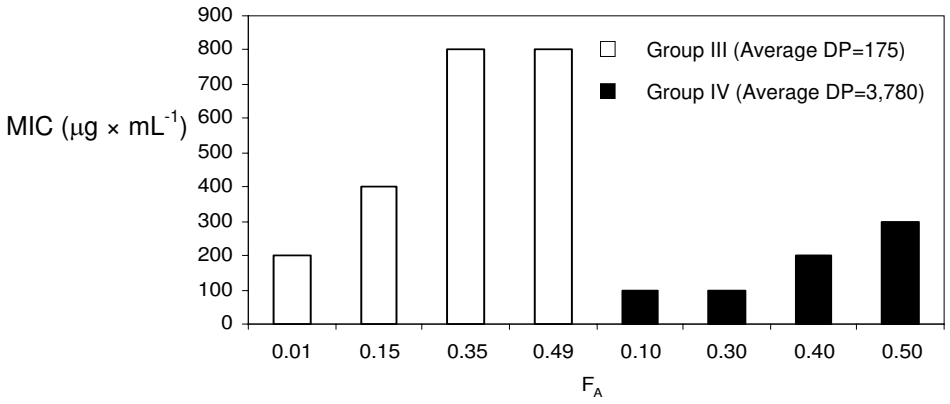

FIGURE 7 Minimum inhibitory concentrations of chitosans (groups III and IV) with similar DP and different F_A against *A. alternata*.

Data from the literature suggest that protonated free amino groups interact with anionic groups on the fungal cell surface, and this could be one of the causes for bioactivity (Shahidi et al., 1999; Roller and Covill, 1999). Roller and Covill (1999) reported that amino

groups in chitosan have the ability to interact with a multitude of anionic groups on the yeast cell surface, thereby forming an impervious layer around the cell. Because of its property to form films, chitosan may also act as a barrier to the outward flux of nutrients, and consequently may reduce the availability of nutrients to a level that will not sustain growth of the pathogen (Bautista-Baños et al., 2006). Our results suggest that this barrier to nutrients may be related to the combined effects of chitosans with high molar mass (average DP=3,780) that were highly deacetylated (F_A=0.10).

In our present study, it was observed that F_A in combination with DP of chitosans had the ability to influence growth inhibition of the filamentous fungi tested. Combined effects of both parameters, DP and F_A, on the bioactivity of chitosan is lacking. Our results point towards enhanced antifungal activities against the pathogens tested, generally with high DP and low F_A chitosans. However, before generalizations can be made, we also observed that depending on fungus sensitivity and source of the chitosan sample, e.g. against the *A. alternata*, chitosans (group I) with DP 45, 366 and 1,383, different MICs of 100, 200 and 300μg × mL^{-1} were obtained.

The relationship between DP of chitosan and chitooligosaccharide and bioactivity against fungi has been investigated in some studies (Kendra and Hadwiger, 1984; Uchida et al., 1989; Zhang et al., 2003; Torr et al., 2005); however the combined effects of DP and F_A of chitosans and antifungal effects is lacking in the literature. Our results suggests that high antifungal activity against *A. alternata, B. cinerea, P. expansum* and *R. stolonifer* pathogens could be obtained using chitosans with low F_A (0.1) and high DP (3,800) under the experimental conditions adopted in this study.

4.1 Changes in fungal morphology due to the effect of chitosan

The present study demonstrates that chitosan is not only effective in restricting the mycelial growth of *A. alternata*, *B. cinerea*, *P. expansum* and *R. stolonifer*, but also induces marked morphological changes in the fungal mycelium (Figure 8).

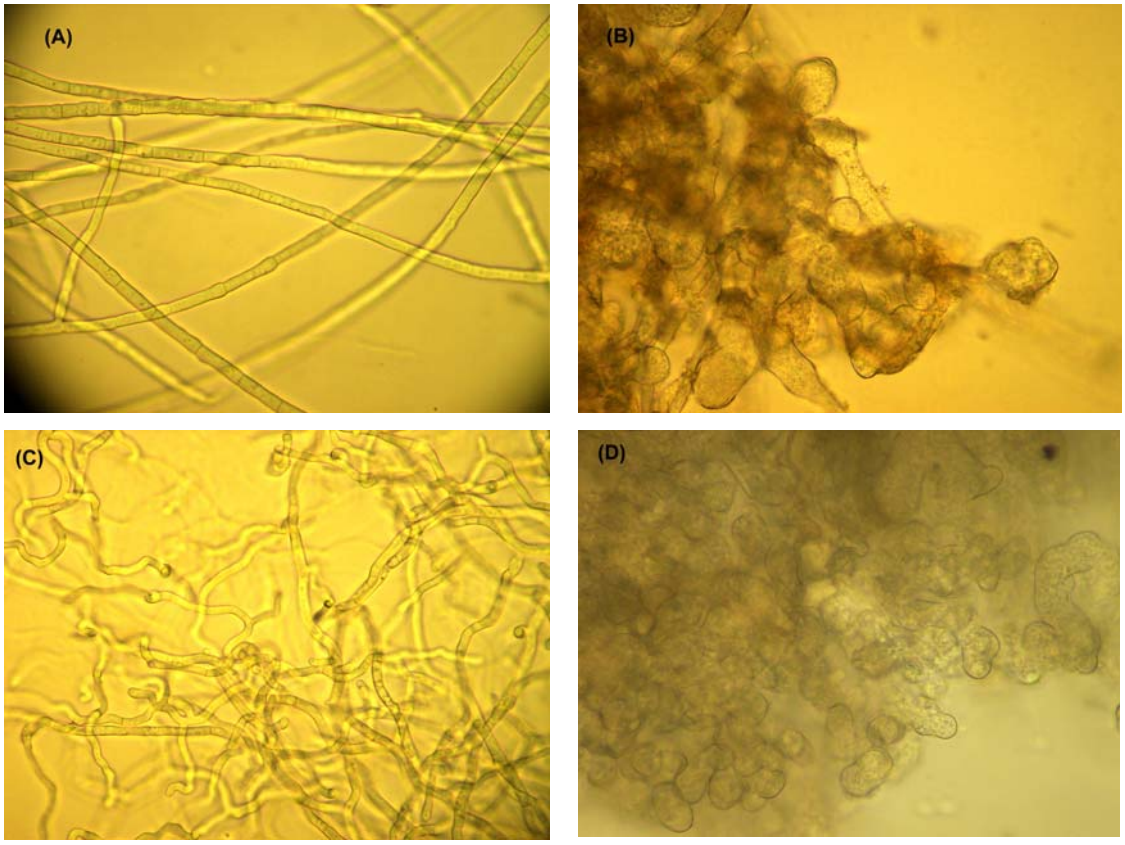

FIGURE 8 Optical microscopy of hyphae of *A. alternata* (A) control and (B) treated with chitosan; *B. cinerea* (C) control and (D) treated with chitosan. Media of *A. alternata* were amended with 500 µg/mL and media of *B. cinerea*, with 1000 µg × mL^{-1} of chitosan D. The *A. alternata* and *B. cinerea* were incubated for five days in liquid malt extract at 25°C. Magnification 400×.

Microscopic observations of four filamentous fungi treated with chitosan D (DP 1,089 and F_A 0.16) revealed that it can affect the morphology of the hyphae and cause their aggregration. For all fungi studied whose media were amended with chitosan, aggregation and excessive mycelial branches and hyphae size reduction were observed. Optical microscopies of the *P. expansum* and *R. stolonifer* are not shown. With optical microscopy of the *A. alternata* (Figures 8A and B) and *B. cinerea* (Figures 8C and D) treated with chitosan, in addition to having the morphological changes mentioned above, abnormal shapes and swelling of their mycelia were observed. El Ghaouth et al. (1992c) reported that chitosan causes severe morphological changes in *Rhizopus stolonifer* that are characterized by excessive branching and swelling of the cell wall. In further studies, image analysis was used to measure the effect of chitosan on the morphology of fungi such as *F. oxysporum* f.

sp. *radicis-lycopersici*, and *S. sclerotiorum* treated with chitosan showed excessive mycelial branching, abnormal shapes, swelling and hyphae size reduction (Cheah et al., 1997). Large vesicles or empty cells devoid of cytoplasm in the mycelium of *B. cinerea* treated with chitosan were observed by Ait Barka et al. (2004).

4.2 Hypothesis for the mode of antimicrobial activity of chitosan

After discovery of the antimicrobial activities of chitosans and its derivatives by Allan and Hadwiger (1979), Kendra and Hadwiger (1984) and Uchida and Ohtakara (1988), many researchers have continued studies in this field. A hypothesis for the mechanism of antimicrobial action of chitosan has been proposed. Protonation of amino groups is believed to be an important factor in the antifungal activity of chitosan and chitooligosaccharides, although the exact mechanism of action is still unknown. A polycationic chitosan or oligomer can potentially interact with negatively charged fungal cell membrane components (i.e., proteins, phospholipids) and/or selectively chelate trace metals, thus interfering with the normal growth and metabolism of the fungal cells (Fang et al. 1994; Roller and Covill, 1999; Shahidi et al., 1999). Liu et al. (2004) evaluated the bactericidal activity of chitosan acetate solution against *Escherichia coli* and *Staphylococcus aureus* by enumeration of viable organisms at different incubation times. The integrity of the cell membranes of both species was investigated by determining the release from cells of materials that absorb at 260 nm. A gradual increase in intercellular material from bacterial suspensions treated with chitosan acetate (0.5 and 0.25% m/v) monitored during two hours was observed. The authors also observed that, according to the results from Fourier-transform infrared spectra, thermogravimetry and differential thermogravimetry, the endothermic peak at 176°C for complexes is most likely due to the ionic bond of $NH3^{+}$ in chitosan acetate with the phosphoryl group in phosphatidylcholine. This result appears to confirm the electrostatic interaction between chitosan acetate and phosphatidylcholine.

In the present study, F_A, DP and the combined effects of the two were shown to be important variables in the bioactivity of chitosans. The chitosan coating observed on the surface of the mycelia (details will be published elsewhere) suggests that fungal growth inhibition could be explained by the direct interaction of chitosan on the fungal cell wall as

a consequence of the polycationic nature of chitosan. This was supported by scanning electron microscopy for all fungi treated with chitosan (data not shown).

5.0 Conclusions

The 96-well microtite plates were shown to be an efficient support to evaluate fungal growth of the filamentous fungi *Alternaria alternata, Botrytis cinerea, Penicillium expansum* and *Rhizopus stolonifer*. Results of this study indicates that chitosan samples with low F_A were most effective against the phytopathogenic fungi tested, but chitosan with high F_A did not have the ability to inhibit the fungal *in vitro* growth. Complete inhibition was obtained for the fungi *B. cinerea*, *R. stolonifer* and *A. alternata* and growth reduction, for *P. expansum*, using chitosan samples with different F_A and DP. The combined effects of low F_A and high DP had good fungistatic activity against the fungi *A. alternata, B. cinerea, Penicillium expansum* and *Rhizopus stolonifer*. Optical microscopy showed that chitosan induced marked morphological changes and severe structural alterations in the fungal mycelia. Different hypotheses have been proposed to explain the antifungal activity of chitosans. F_A of chitosan was shown to be an important factor in antifungal activity, with higher activity observed for lower F_A values. These results highlight the importance of free amino group protonated (NH_3^+) for antifungal activity.

Acknowledgments

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CAPÍTULO IV

Este capítulo é referente à artigo a ser submetido:

E. N. Oliveira Junior, N. E. Gueddari, B. M. Moerschbacher, M. G. Peter and T. T. Franco. Effects of chitooligosaccharides on the growth of phytopathogenic fungi. *Biomacromolecule*.

O presente trabalho teve por objetivo avaliar o efeito de três amostras de quitoligômeros no crescimento dos fungos: *Alternaria alternata*, *Botrytis cinerea*, *Penicillium expansum* e *Rhizopus stolonifer*. Os bioensaios foram realizados na cidade de Münster, Alemanha, no Instituto de Bioquímica e Biotecnologia de Plantas da Universidade de Münster com a colaboração do Prof. Dr. Bruno M. Moerschbacher. As amostras de quitoligômeros foram gentilmente fornecidas pela empresa Genis ehf., Reykjavik, Islândia, e degradadas enzimaticamente por Bahrke et al. (2002). As misturas de quitoligômeros tiveram suas massas molares determinadas por MALDI TOF MS, cuja análise foi executada pela Dr. Sophie Haebel no Centro de Pesquisas Interdisciplinares para Biopolímeros da Universidade de Potsdam com a colaboração do Prof. Dr. Martin G. Peter.

As misturas de quitoligômeros promoveram menor efeito no crescimento fúngico na concentração de 1,000 $\mu g \times mL^{-1}$ do que as quitosanas em geral. O crescimento do fungo *A. alternata* foi levemente induzido na presença de 1,000 $\mu g \times mL^{-1}$ das misturas de quitoligômeros DP 5 a 8 e DP 2 a 12, enquanto a mistura de quitoligômeros de DP 2 a 11 não afetou significativamente o crescimento. Os fungos mais resistentes tiveram seus crescimentos fracamente inibidos (*B. cinerea*) ou não afetados (*P. expansum*) pela presença de 1,000 $\mu g \times mL^{-1}$ dos quitoligômeros testados.

Effects of chitooligosaccharides on the growth of phytopathogenic fungi

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Abstract - Three mixtures of chitooligomers, whose degrees of polymerization (DP) were Q1(DP=5-8), Q2(DP≈2-12) and Q3(DP≈2-11), were analyzed by MALDI TOF MS (Matrix-assisted laser desorption ionization time-of-flight mass spectrometry). Chitooligomer mixtures were assayed against four phytopathogenic fungi (*Alternaria alternata, Botrytis cinerea, Penicillium expansum* and *Rhyzopus stolonifer*). Growth of the fungi was slightly inhibited or induced by the chitooligomers. *A. alternata* growth was slightly induced in the presence of chitooligomers Q1 and Q2 for up to six days, while in the presence of Q3 it was not significantly affected. The growth of *B. cinerea*, one of the most resistant fungi, was unaffected by the presence of chitooligomers Q1 and Q2 and was slightly retarded in the presence of Q3. *P. expansum* growth was largely unaffected by the presence of the three samples of chitooligomers. The Q2 and Q3 chitooligomers affected the growth of *Rhizopus stolonifer* by causing an initial period of fungistasis that was overcome by the fungus. In the polyacrylamide gels containing glycol chitin and chitosan as substrates, chitinolytic and chitosanolytic activity were detected in the fungal spent media of *Alternaria alternata* and *Penicillium expansum*, while only slight chitosanolytic activity was found in the spent medium of *Rhizopus stolonifer*. For *Botrytis cinerea* no activity was detected on the spent medium using both substrates. MALDI TOF MS of the fungal spent media of the four fungi showed a mixture of chitooligomers with a smaller DP, than the mixture of chitooligomers. In the case of *Penicillium expansum*, more resistant fungus, spectra of the fungal spent media revealed that disaccharides (A_2 and D_1A_1) and trisaccharides (A_3 and D_2A_1) were present in the fungal medium after six days of growth.

Keywords: chitooligomer; antifungal activity; phytopathogenic fungi.

1.0 Introduction

Chitin and chitosan are aminoglucopyranans composed of N-acetylglucosamine (GlcNAc) and glucosamine (GlcN) residues (Peter, 2002a). The chemical structure of chitin is similar to that of cellulose with 2-acetamide-2-deoxy-β-D-glucopyranose (GlcNAc) monomers attached via β(1→4) linkages (Shahidi et al., 1999). Chitosan is a linear binary copolymer consisting of GlcNAc and 2-amino-2-deoxy-β-D-glucopyranose (GlcN) monomers randomly distributed along the chitosan chain (Vårum et al., 1991).

Chitosan and chitooligomers have attracted considerable attention due to their biological activities, that is, antimicrobial (Kendra and Hadwiger, 1984; Sudharshan et al., 1992), antitumor (Suzuki et al., 1986; Tokoro et al., 1988) and hypocholesterolemic functions (Muzzarelli, 1996).

After discovery of the antimicrobial activities of chitosan and its salts by Allan and Hadwiger (1979), Kendra and Hadwiger (1984) and Uchida et al. (1988), many researchers have continued studies in this field. In this context, the antimicrobial activity of chitin, chitosan and their derivatives against different groups of microorganisms, such as bacteria, yeast and fungi has received considerable attention (Yalpani et al., 1992). Chitosan has been used as a coating for fruits (El Ghaouth et al., 1991a,b; Jiang and Li, 2001) and it has shown antifungal activity against a wide range of fungi (El Ghaouth et al., 1992a,b; El Ghaouth, 1994; Bhaskara Reddy et al., 2000).

Chitooligosaccharides have also been shown to inhibit fungal growth (Zhang et al., 2003). Kendra and Hadwiger (1984) characterized the heptamer as the smallest chitooligosaccharide to show antifungal activity equivalent to that of chitosan against *Fusarium solani* (Mart.) Sacc. Uchida et al. (1988) found that chitosan as well as chitosan that had been slightly (5%) hydrolyzed with chitosanase was effective against three *Fusarium* species, whereas lower molar mass oligomers with degrees of polymerization (DP) from 2 to 8 were not. Torr et al. (2005) examined the bioactivities of chitosan and chitosan oligomers against *Leptographium procerum*, *Sphaeropsis sapinea* and *Trichoderma harzianum* on nutrient media. *Leptographium procerum* and *S. sapinea* growth was prevented by chitosan acetate and chitosan oligomers at concentrations from 0.3 to 0.4% (m/v), whereas *T. harzianum* was able to overcome the fungistatic action of these compounds. Of these three chitooligomer preparations with average degrees of polymerization of 5, 9 and 14 and chitosan acetate, the authors observed that the oligomer with DP=14 had higher specific activity than the both oligomer with DP=5 and chitosan acetate. The exact mechanism by which chitosan exerts antimicrobial activity is unknown; however, factors which can influence bioactivity include the molar mass (Mm) and degree of acetylation (F_A) of the chitosan and the pH of the test medium. The results of Hirano and Nagao (1989) indicate that low-Mm chitosans were more effective than high-Mm chitosans

in inhibiting mycelium growth of a selection of phytopathogenic fungi and wood-inhabiting fungi.

The objective of this study was to examine *in vitro* the antifungal effect of three chitooligomer mixtures against the phytopathogenic fungi *A. alternata, B. cinerea, P. expansum* and *R. stolonifer*, whose sensitivities were correlated with degradation products characterized by MALDI TOF MS.

2.0 Materials and methods

2.1 Chitooligosaccharides

Chitooligosaccharides were supplied by Genis ehf. (Reykjavik, Iceland) in the form of powders, resulting from enzymatic degradation of chitosan (*cf*. Bahrke et al., 2002). The powders were dissolved in 0.05 M ammonium acetate buffer, pH 4.2, to give a concentration of ca. 12 mg × mL^{-1} and fractionated by sequential ultrafiltration through a 0.8 µm and a 0.2 µm cellulose acetate membrane (Schleicher & Schuell) and a 3,000 Da cut-off membrane (Amicon). Finally, the filtrates were lyophilized. Sample Q2 was separated by gel permeation chromatography (GPC) on Biogel P4, fine grade (BioRad, Munich, Germany). Column dimensions were 5 cm i.d. × 200 cm; mobile phase 0.05 M ammonium acetate buffer, adjusted with 0.23 M acetic acid to pH 4.2; flow rate 60 mL × h^{-1}; and detector Shimadzu RID 6A. Fractions of 20 mL were collected, combined, concentrated into small volume and finally lyophilized. A fraction showing a DP of approximately 5-8, labelled Q1, was used for bioassays in this study.

2.2 Mass spectrometry

Samples containing ca. 100 µg of oligosaccharides were mixed with 8 µL of methanol/water (v/v 50/50). An aliquot of the solution (0.5 µL) was mixed in the target with 0.5 µL of a solution of 2,5-dihydroxybenzoic acid (DHB) as a matrix (27 mg × mL^{-1}) in 20% aqueous methanol, and the drop was dried under a gentle stream of air. Crystallization of the matrix usually occurred spontaneously. Mass spectra were recorded on a Bruker Reflex II mass spectrometer (Bruker Daltonik, Bremen, Germany) in the positive ion mode. A nitrogen laser (337nm, 3ns pulse width, 3Hz) was used. All spectra

were measured in the reflector mode using external calibration by means of angiotensin II (Bahrke et al., 2002).

2.3 Microorganisms and cultivation

The filamentous fungi used in this study included *Alternaria alternata* (CCT 2816), *Penicillium expansum* (CCT 4680) and *Rhizopus stolonifer* (CCT 2002); all of these strains were purchased from André Tosello Foundation Research and Technology (Campinas, Brazil). The *Botrytis cinerea,* a grape isolate, was provided by the Institute of Food Technology (Campinas, Brazil). The *Botrytis cinerea* and *Penicillium expansum* were cultured on potato dextrose agar (PDA) and in malt extract agar (MEA) supplemented with 2% (m/v) of glucose and peptone, respectively, while the *Rhizopus stolonifer* and *Alternaria alternata* were both cultured on MEA. In order to achieve sporulation, the *A. alternata*, *B. cinerea* and *P. expansum* were incubated in Petri dishes (Ø=9cm) for 8 days and the *R. stolonifer*, for 4 days at 25°C and 100cm beneath Hg lamps for a twelve-hour photoperiod. Spores were harvested by pouring sterile water into the slant and stirring with a vortex for 20 seconds. Suspensions of spores and mycelia were filtered through cotton. The concentration of spores was assessed by using a hemocytometer (Fuchs-Rosenthal Hell Linie) under optic microscopy (magnification 400×). The concentration of *Rhizopus stolonifer* spores was adjusted to 10,000 spores mL^{-1} and of *B. cinerea*, *A. alternata* and *P. expansum* spores, to 20,000 spores mL^{-1}.

2.4 Antifungal activity

Inhibition of fungal growth by chitooligomers was assessed by using complete medium (CM) with pH 4.3 and monitoring absorbance at λ=405nm. Complete medium was prepared as described by Pontecorvo (1953). Aliquots (150µL) of sterile CM containing the required volume of chitooligomer stock solution (4mg × mL^{-1}) and sterile water were dispensed into the wells containing either 10 µL of a spore suspension of a test fungus or 10 µL of sterile water alone (blanks). The sterile 96-well microtiter plates of polystyrene (Roth®) were incubated at 25°C under agitation of 200 o.p.m (orbits per minute) for up to three days for the *R. stolonifer*, six days for the *B. cinerea* and *A. alternata* and five days

for the *P. expansum*. The absorbances of the wells were measured at one-day intervals for the *A. alternata, B. cinerea* and *P. expansum* and twelve-hour intervals for the *R. stolonifer*. The experiments were carried out in triplicate, and the absorbances correspond to the mean of the three well readings.

2.5 Chitinase and chitosanase activities

Chitinolytic and chitosanolytic activities of the spent fungal media, both desalted and not desalted, were evaluated for the *Alternaria alternata*, *Botrytis cinerea*, *Penicillium expansum* and *Rhizopus stolonifer*. Spent fungal media were obtained from 100 mL of complete medium inoculated with fungal spores dispensed into Erlenmeyers (250 mL) incubated at 26°C under agitation of 120 o.p.m for five days for all fungi tested. Mycelial suspension was filtered through Whatman paper n°1 and the filtrate was frozen at -40°C and lyophilized. Lyophilized spent media (1 g) were dissolved in 4 mL of ultrapure water (MilliQ). Samples were desalted in prepacked Sephadex G-25 columns (PD-10, Amersham Biosciences, Uppsala, Sweden) equilibrated with ultrapure water, lyophilized and resuspended in a minimum volume of water (approximately 50µl). Proteins showing chitinolytic activity were detected in accordance with the method developed by Trudel and Asselin (1989) using gels containing 0.01% (m/v) glycol chitin and chitosan (F_A 0.22; DP 1,383). Aliquots of 20 µL of spent media, both desalted and not desalted, were added on the papers for electrophoresis (Amersham Biosciences, Uppsala, Sweden) on gel surfaces. After incubation in 100 mM sodium acetate buffer (pH 5.0) for 90 min at 37°C, gels were incubated for 5 min in solution containing 0.01% (m/v) calcofluor M2R (Sigma-Aldrich, Steinheim, Germany) in 500 mM Tris–HCl (pH 8.9). After 5 min, the calcofluor solution was removed and the gel was rinsed overnight in distilled water. Lytic zones were visualized and recorded under UV light.

3.0 Results

3.1 MALDI TOF MS of Chitooligosaccharide Mixtures

The MALDI TOF MS spectra of the three samples of chitooligosaccharide mixtures (Q1, Q2 and Q3) are shown in the Figures 1, 2 and 3, respectively. Q1 was

composed of low-Mm (molar mass) oligomer mixtures (DP 5 to 8), as observed in the sodiated pseudomolecular ions from D_1A_4 at m/z 1,014.48 to D_4A_4 at *m/z* 1,497.58 (Figure 1). A high-intensity peak appears at *m/z* 1,174.53 (D_2A_4) as the main component. Figure 2 depicts the spectrum of sample Q2, whose sodiated main components are between DP 2 (*m/z* 447.22) and DP 7 (*m/z* 1,336.55) and sodiated minor components are between DP 8 (*m/z* 1,455.61) and DP 12 (*m/z* 2,225.76). The spectrum of Q3 shows the sodiated ions from A_2 (*m/z* 447.20) to D_3A_3 (*m/z* 1,133.47) as the main and minor components from D_2A_4 (*m/z* 1,175.48) to D_6A_5 (*m/z* 2,023.75).

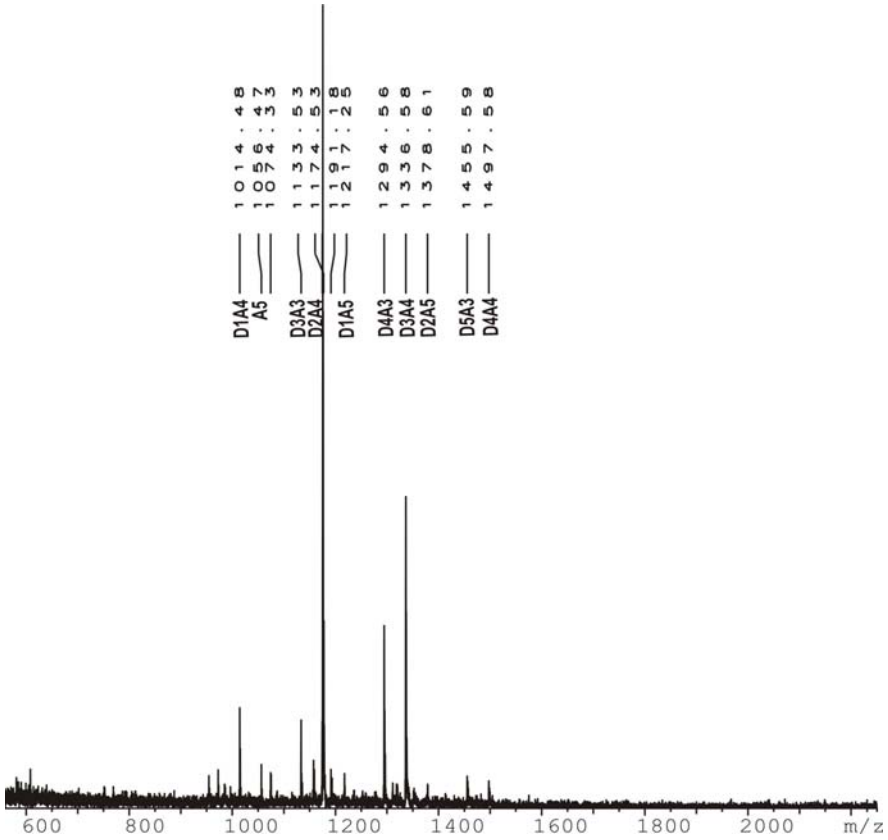

FIGURE 1 MALDI TOF MS of chitooligomer sample Q1.

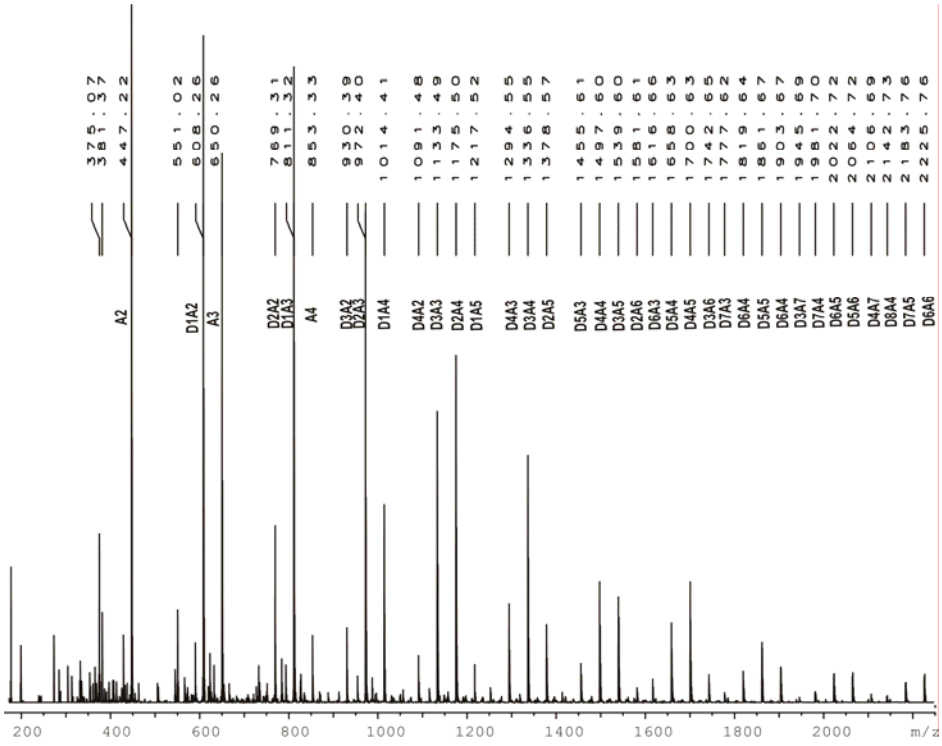

FIGURE 2 MALDI TOF MS of chitooligomer sample Q2.

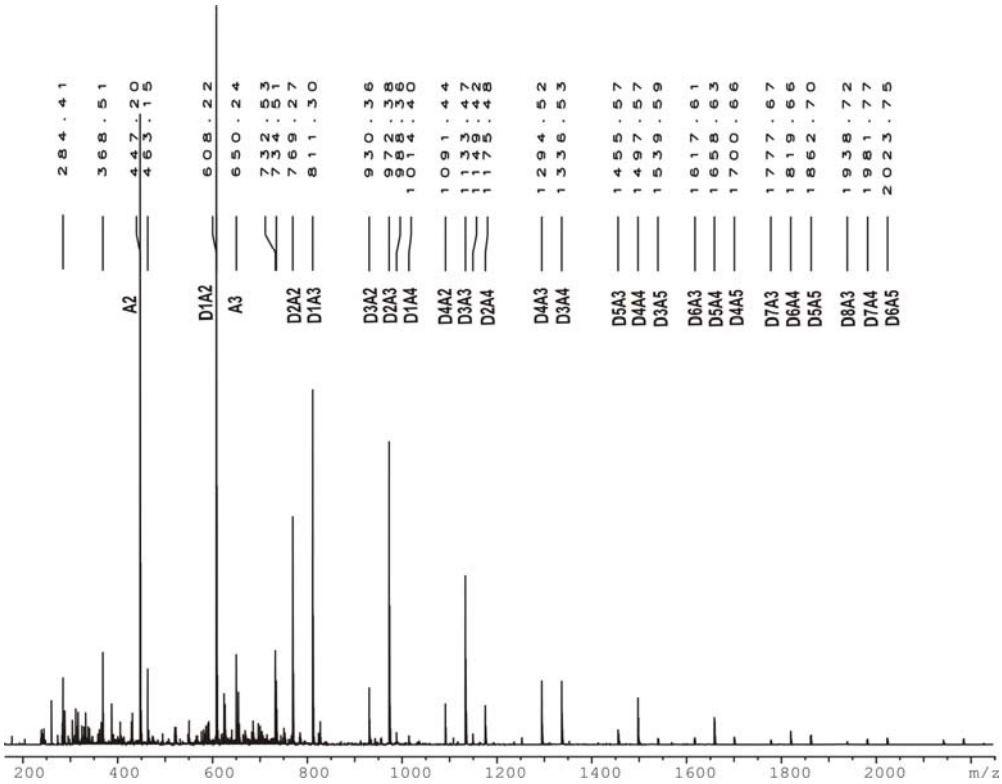

FIGURE 3 MALDI TOF MS of chitooligomer sample Q3.

3.2 Bioassays of chitooligosaccharides

The antimicrobial activity of chitooligosaccharides in complete medium (pH 4.3) was assessed using the following four target organisms: *Alternaria alternata*, *Botrytis cinerea*, *Penicillium expansum* and *Rhizopus stolonifer*. According to visual observation the *R. stolonifer* was the fastest growing of the fungi, since after thirty six hours the fungus started to sporulate and after seventy two hours there was growth stabilization. Growth of the *B. cinerea*, *P. expansum* and *A. alternata* was slower than that of the *R. stolonifer,* which stabilized after four days of incubation. The absorbance values varied with regard to the fungi studied due the different colors of mycelia and their agglomerations (Table 1). Growth of the fungi tested was slightly inhibited or induced by chitooligomers. Figure 4 (A and B) shows that growth of the *A. alternata* was slightly induced in the presence of 1,000 $\mu g \times mL^{-1}$ of chitooligomers Q1 and Q2 for up to six days, while growth in the presence of Q3 was not significantly affected. Growth of the *B. cinerea*, one of the most resistant fungi, was unaffected by the presence of chitooligomers Q1 and Q2 at a concentration of 1,000 $\mu g \times mL^{-1}$, while it was slightly retarded in the presence of Q3. *P. expansum* growth was largely unaffected by the presence of 1,000 $\mu g \times mL^{-1}$ of the three chitooligomers used.

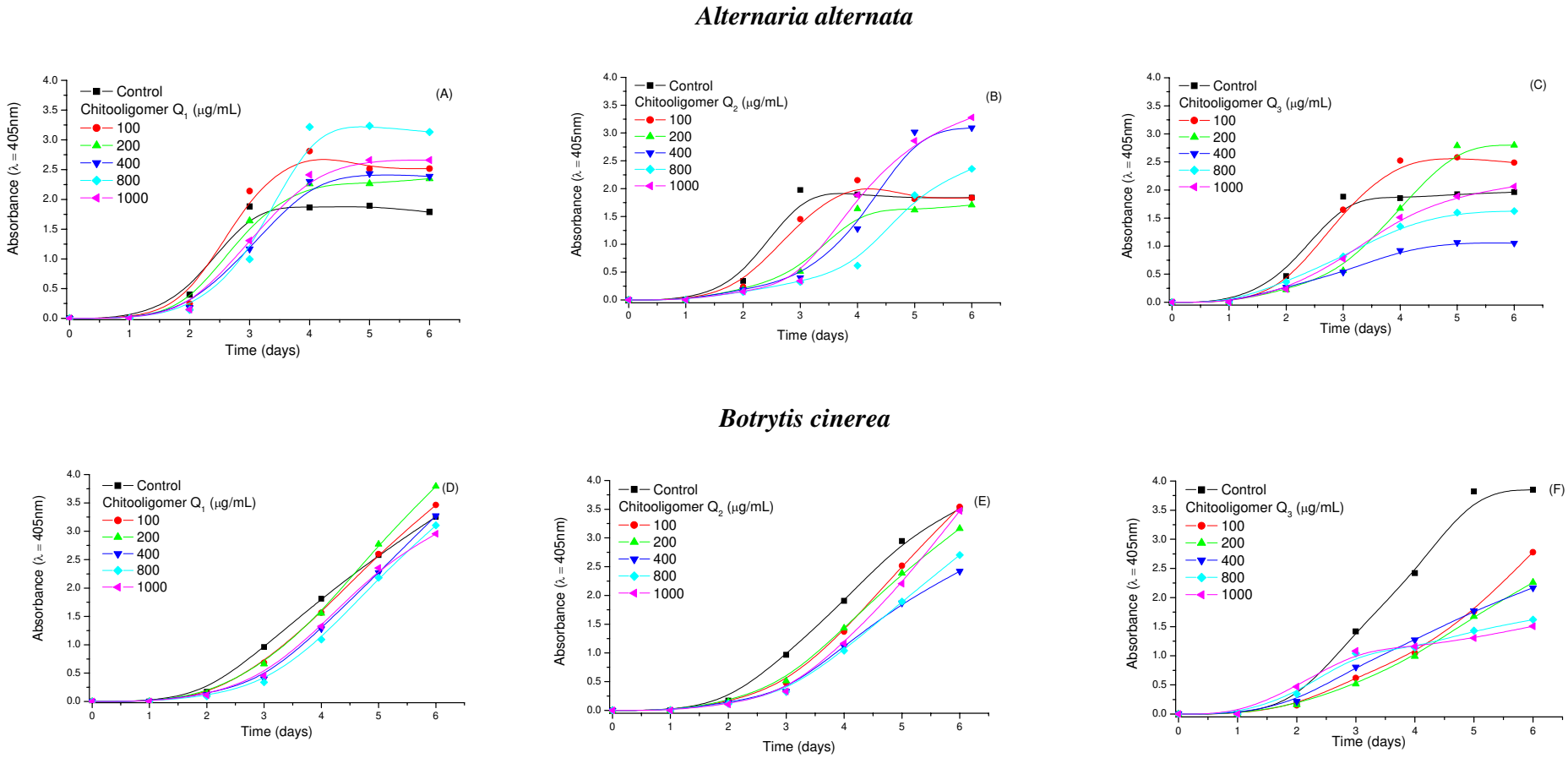

FIGURE 4 Activities of chitooligomers Q1, Q2 and Q3 against *Alternaria alternata* (A, B and C) and *Botrytis cinerea* (D, E and F).

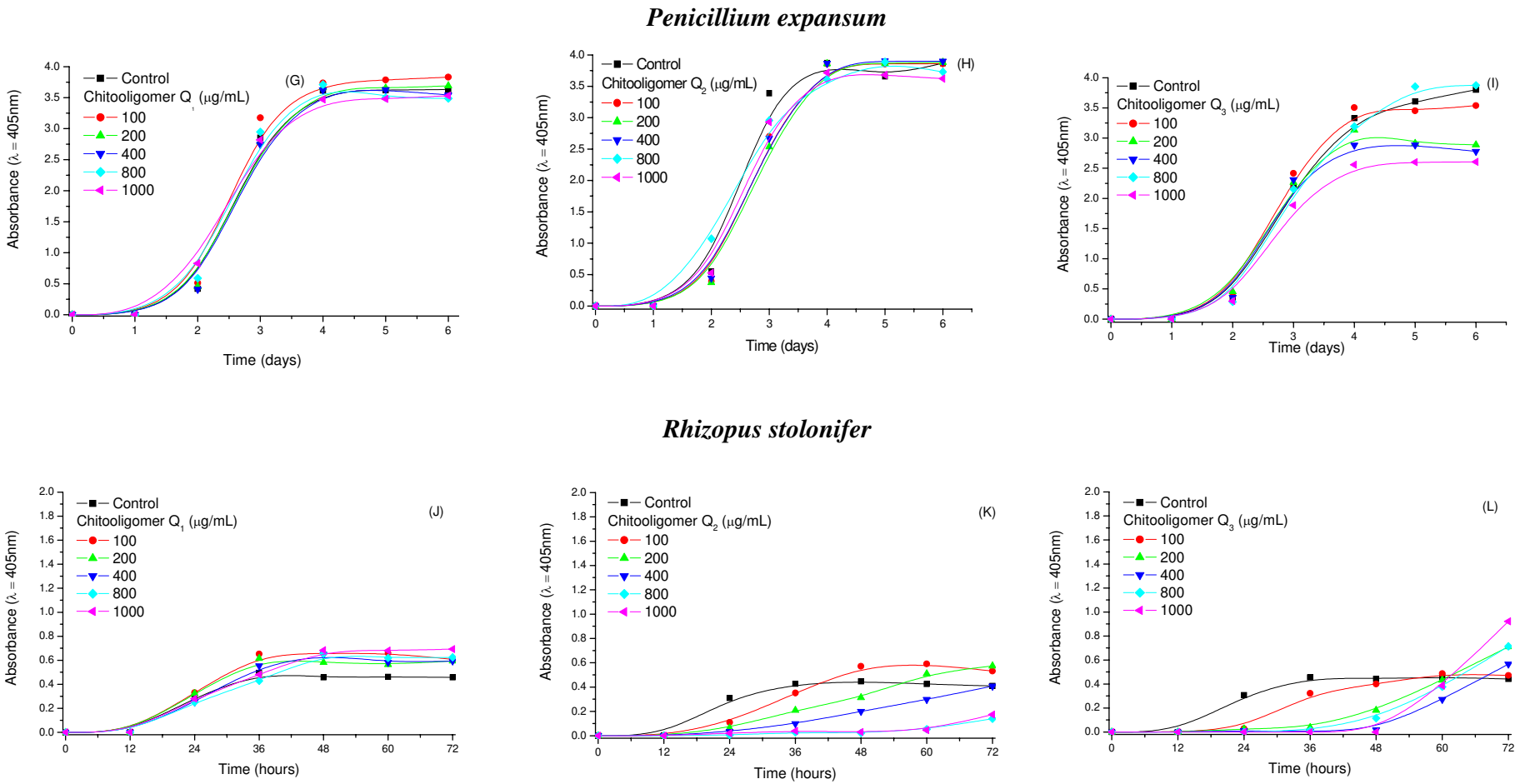

FIGURE 5 Activities of chitooligomers Q1, Q2 and Q3 against *Penicillium expansum* (G, H and I) and *Rhizopus stolonifer* (J, K and L).

Chitooligomers Q2 (Figure 5K) and Q3 (Figure 5L) decreased the growth rate of *R. stolonifer* by causing an initial period of fungistasis, which was eventually overcome by the fungus after 60 hours (Q2) and 48 hours (Q3).

TABLE 1 Average increase in absorbance of *A. alternata*, *B. cinerea* and *P. expansum* on the sixth day and *R. stolonifer* on the third day on complete medium amended with chitooligomers. The results are means based on three replicate values for absorbance at 405nm. Numbers after ± indicate standard error.

Chitooligomer	**Concentration** ($\mu g \times mL^{-1}$)	**Average absorbance (λ=405nm)**			
		A. alternata	***B. cinerea***	***P. expansum***	***R. stolonifer***
Control	0	1.79±0.11	3.25±0.54	3.63±0.38	0.42±0.06
Q1	100	2.10±0.54	3.46±0.31	3.83±0.04	0.61±0.11
	400	2.12±0.77	3.27±0.96	3.54±0.29	0.59±0.14
	1,000	2.13±0.21	3.07±0.44	3.14±0.94	0.69±0.23
Q2	100	1.68±0.91	3.56±0.51	3.86±0.01	0.53±0.06
	400	3.09±0.25	2.42±0.28	3.90±0.04	0.41±0.07
	1,000	3.28±0.80	3.47±0.65	3.62±0.47	0.23±0.12
Q3	100	2.49±0.35	2.78±1.01	3.37±0.46	0.45±0.02
	400	1.05±0.34	1.63±0.14	2.85±0.58	0.83±0.39
	1,000	2.06±0.50	1.51±0.22	3.22±0.76	1.22±0.59

Chitooligomer Q1 (DP 5-8) did not affect the growth of any of the fungi tested in a concentration range from 100 to 1,000 $\mu g \times mL^{-1}$, while only a slight induction activity was observed for *A. alternata*.

Previous studies had investigated the relationship between chitosan and chitooligosaccharide DP (or Mm) and bioactivity against fungi. Kendra and Hadwiger (1984) showed that chitooligosaccharides with DP 7 or greater were required for bioactivity equivalent to that of chitosan against *F. solani*, whereas Uchida et al. (1988) described a mixture of chitosan oligomers of DP 2 to 8 (average DP≈5) as inactive against three *Fusarium* pathogens at a concentration of 1.0% (m/v). Zhang et al. (2003) reported that chitooligosaccharides with an average DP of 20 showed good growth inhibitory effects against sixteen plant pathogenic fungi. Torr et al. (2005) suggested that higher antifungal activity against certain fungi can be obtained with chitooligosaccharides (DP 9 and DP 14) than with DP 5 and chitosans (Mm 310 to higher than 375kDa; DP≈1,925 to 2,329).

Chitosan acetate and the chitooligosaccharide mixtures mentioned above were assayed against *Leptographium procerum*, *Sphaeropsis sapinea* and *Trichoderma harzianum*. *Leptographium procerum* and *S. sapinea* growth was prevented by chitosan acetate and chitosan oligomers at concentrations of 0.3–0.4% (m/v). The differences can be related to different fungi strains and species, media, methods and chitooligomer samples used.

Further studies will be required before generalizations can be made about the relationship between the DP of chitosans, chitooligomers and bioactivity. Our results suggest that chitooligomers Q1 (DP 5 to 8), Q2 (DP 2 to 12) and Q3 (DP 2 to 11) showed no or a lower antifungal effect against *A. alternata, B. cinerea, P. expansum* and *R. stolonifer*, than chitosan polymers with low F_A 0.1 and high DP 3,800 (details will be published elsewhere) under the experimental conditions adopted in this study.

Table 2 shows the degradation products, chitooligomers in the fungal spent media that had been previously amended with 1,000 $\mu g \times mL^{-1}$ of chitooligomers Q1, Q2 and Q3, identified by MALDI TOF MS. MALDI TOF MS spectra generally showed smaller chitooligomers for each fungus than Q1, Q2 and Q3, whereas depending on the fungus, different chitooligomers were produced. The chitooligomer DP ranges found in the fungal spent media were from 2 to 7 in case of *Alternaria alternata*, from 2 to 11 in the case of *Botrytis cinerea*, from 2 to 3 in the case of *Penicillium expansum* and from 2 to 7 in the case of *Rhizopus stolonifer*. Of these four phytopathogenic fungi, the *Penicillium expansum* showed a higher resistance than the other fungi in the presence of chitooligomers, as its growth was not affected by the oligomers Q1, Q2 and Q3 (Figure 5 (G, H and I)). The high resistance of the *Penicillium expansum* was also observed in the presence of chitosans with different DP and F_A (data not shown). MALDI TOF MS of the fungal spent medium of the *Penicillium expansum* revealed that disaccharides (A_2 and D_1A_1) and trisaccharides (A_3 and D_2A_1) were present in the spent fungal medium after six days of growth (Figures 7, 8 and 9). This result shows that the fungus was able to grow in the presence of the chitooligomers studied, and consequently it produced hydrolytic enzymes that degraded the chitooligomer mixtures into smaller ones used by the fungus as a source of growth.

Using gels containing glycol chitin and chitosan as substrates, enzymes showing chitinolytic and chitosanolytic activity were observed on the spent media of the fungi tested (Figure 6). Spent medium of *A. alternata* showed an intense band of chitosanolytic activity

(chitosan substrate), as can be seen in the lines (1) desalted and (2) not desalted, while no bands were found on the gel containing glycol chitin as substrate. Chitosanolytic and chitinolyitic activity were detected in the spent media, both desalted (line 3) and not desalted (line 4), from *P. expansum* (Figure 6). In the case of the *B. cinerea* no activity was detected on spent medium using both substrates (line 5). A low-intensity band on gel containing chitosan as substrate was detected on spent medium of *R. stolonifer* (line 6; column A), while chitinolytic activity (glycol chitin as substrate) was not observed (line 6; column B).

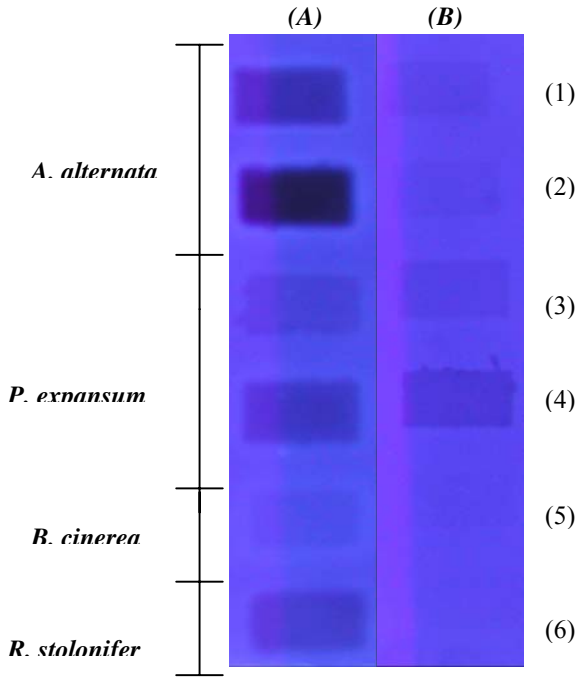

FIGURE 6 Detection of proteins showing chitinolytic (glycol chitin as substrate in column A) and chitosanolytic (chitosan F_A 0.22; DP 1,383 as substrate in column B) activities by using gels containing 0.01% (m/v) of substrate in the fungal spent media of *A. alternata*, *B. cinerea*, *P. expansum* and *R. stolonifer*. Spent media on lines 1 and 3 were desalted in prepacked Sephadex G-25 columns (PD-10, Amersham Biosciences, Uppsala, Sweden), while the spent media on lines 2, 4, 5 and 6 were not.

Chitinolytic and chitosanolytic activities of the four spent fungal media were analyzed by isoelectric focusing gel (IEF) (data not shown), and the results showed that *Alternaria alternata* and *Penicillium expansum* fungi produced hydrolytic enzymes of chitosans with different F_A, while *Botrytis cinerea* and *Rhizopus stolonifer* fungi were unable to degrade the substrates including glycol chitin.

The ability of the fung to degrade chitosans with different F_A had no correlation with their sensitivities in the presence of chitosan and chitooligomers, whereas both sensitive (*Alternaria alternata*) and resistant (*Penicillium expansum*) fungi showed chitosanolytic activity. Allan and Hadwiger (1979) suggested that chitosan reduces the *in vitro* growth of numerous fungi with exception of Zygomycetes, i.e. the fungi containing chitosan as the main component of their cell walls including *Mucor* spp. are more resistant to the antimicrobial action of the externally added chitosan. However, results reported by Roller and Covill (1999) showed inhibition of *Mucor racemosus* growth at 1 g × L^{-1} of chitosan. Roller and Covill´s report did not support Allan and Hadwiger´s proposition.

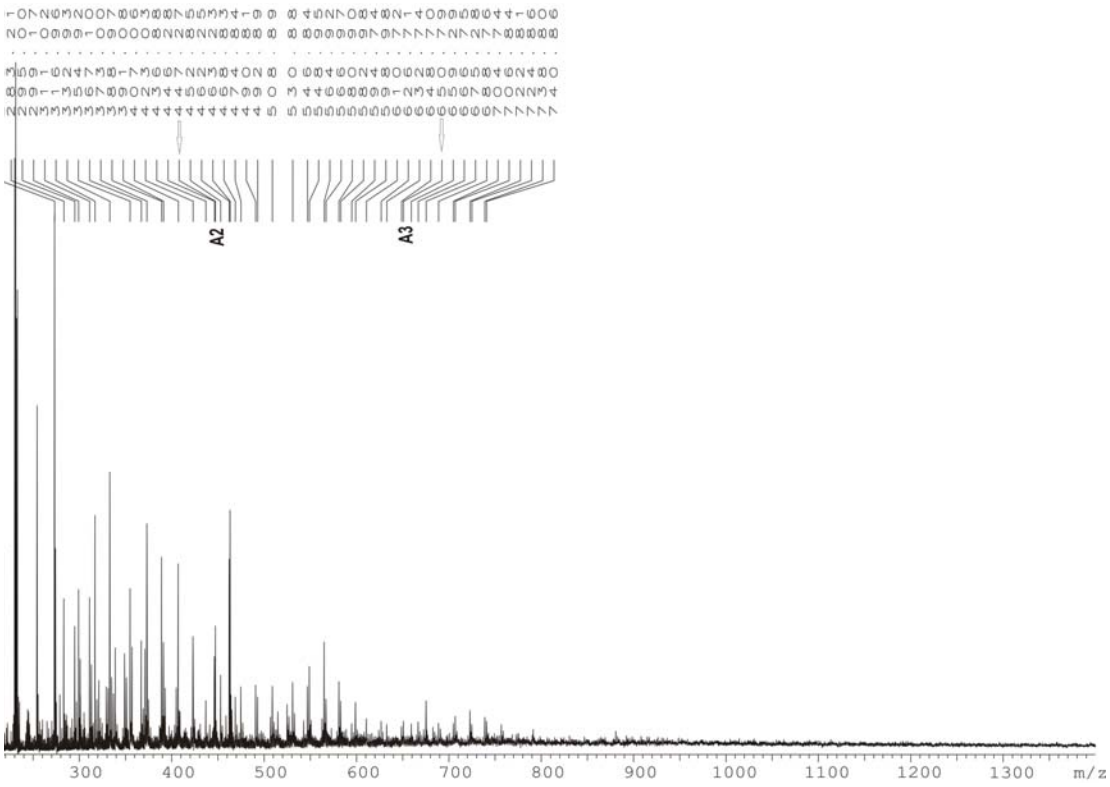

FIGURE 7 MALDI TOF MS of the spent medium of *Penicillium expansum* grown for six days in liquid complete medium previously amended with 1,000µg × mL^{-1} of chitooligomer sample Q1.

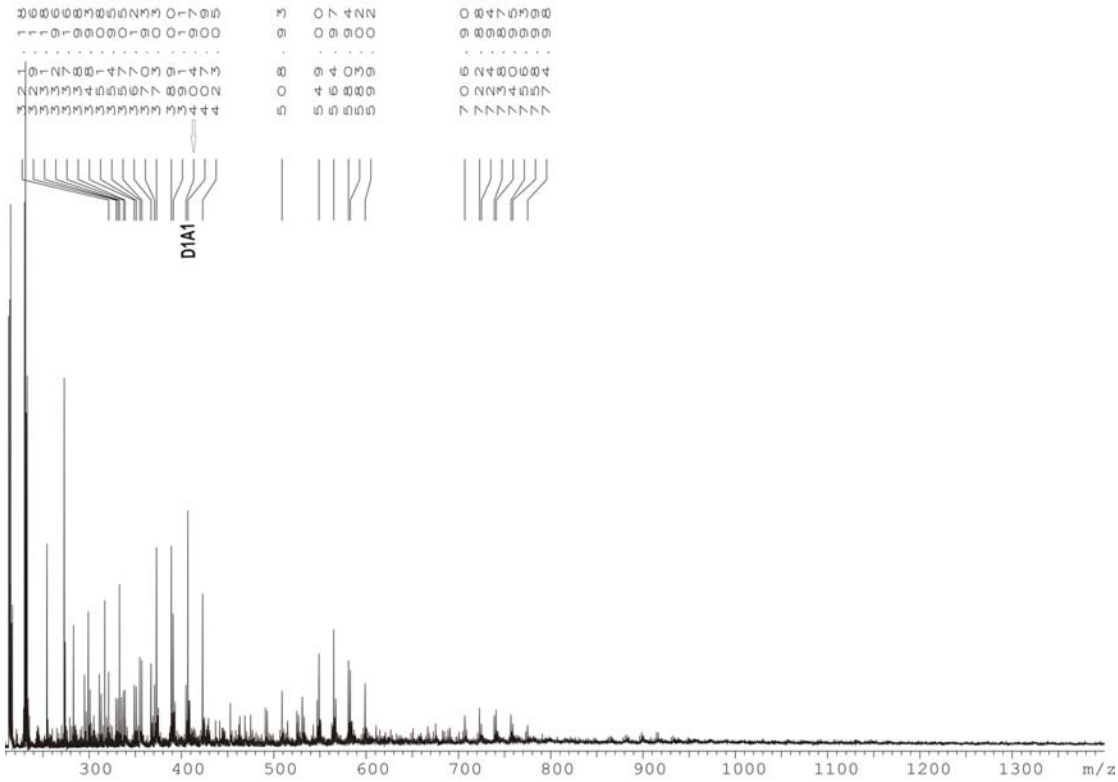

FIGURE 8 MALDI TOF MS of the spent medium of *Penicillium expansum* grown for six days in liquid complete medium amended with 1,000 µg × mL^{-1} chitooligomer sample Q2.

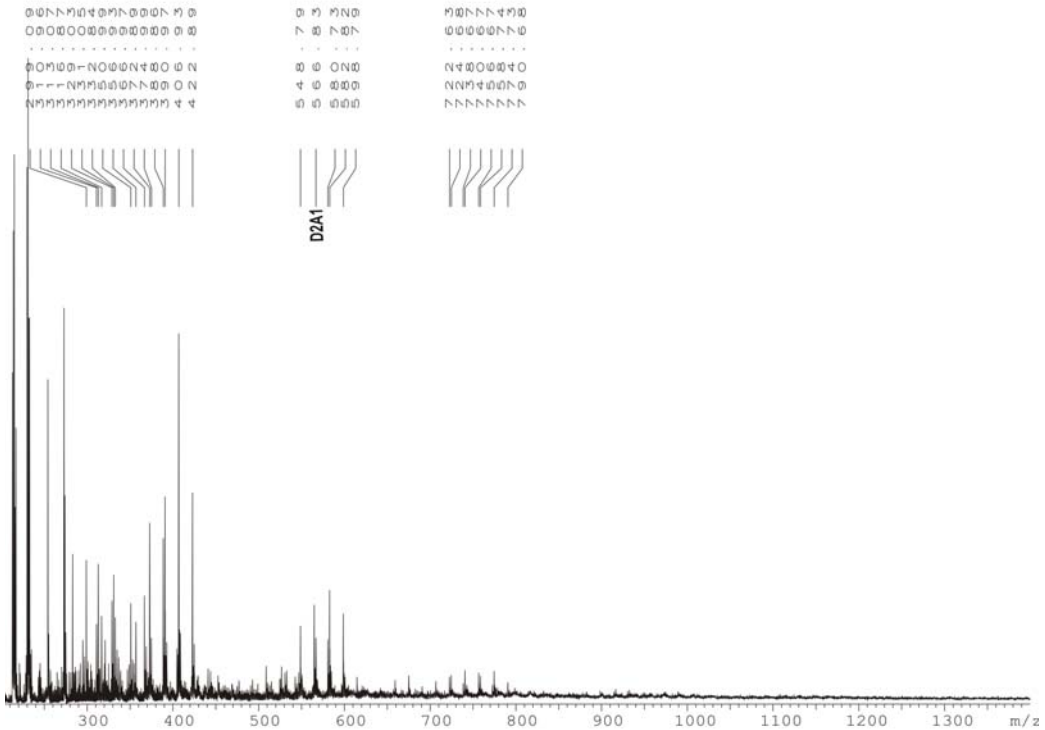

FIGURE 9 MALDI TOF MS of the spent medium of *Penicillium expansum* grown for six days in liquid complete medium amended with 1,000 µg × mL^{-1} chitooligomer sample Q3.

Although the cell wall components of the fungi studied had not been determined, their contents can be related to fungal sensitivity in the presence of chitosan and chitooligomers. Chitin is widely found in fungi, occurring in *Basidiomycetes*, *Ascomycetes* and *Phycomycetes*, where it is a component of the cell walls and structural membranes of mycelia, stalks and spores. The amounts vary between traces and up to 45% of the organic fraction, the remainder being mostly proteins, glucans and mannans (Roberts, 1992). However, not all fungi contain chitin, and the polymer may be absent in a species, even though it is closely related to another that contains it. Variations in the amount of chitin may depend on physiological parameters in natural environments as well as on the fermentation conditions in biotechnological processing or in cultures of fungi (Peter, 2002b).

Scanning electron microscopy micrographs (details will be published elsewhere) showed that morphological anomalies on the fungal mycelial surface were caused by the presence of chitosan in the growth medium. The micrographs showed that chitosan amendment caused aggregation of mycelia that were coated with chitosan. Similar morphological anomalies, caused by chitosan coating on the mycelial surface of the fungi studied, suggest that the chitosan layer around the spore or mycelium may render difficult the entrance of nutrients in the cells. Protonation of amino groups is believed to be an important factor in the antifungal activity of chitosan and chitooligosaccharides, although the exact mechanism of action is still unknown. Further studies will be required before generalizations can be made about the relationship between fungal sensitive, chitosans and chitooligomers.

TABLE 2 MALDI TOF MS peaks of chitooligosaccharides $[M+Na]^+$ and $[M+K]^+$ identified in the complete medium amended with the Q1, Q2 and Q3 before and after growth of *Alternaria alternata* (*Aa*), *Botrytis cinerea* (*Bc*), *Penicillium expansum* (*Pe*) and *Rhizopus stolonifer* (*Rs*). (+) Main components. $+^a$ Minor components.

DP		**Q1**	***Aa***	***Bc***	***Pe***	***Rs***	**Q2**	***Aa***	***Bc***	***Pe***	***Rs***	**Q3**	***Aa***	***Bc***	***Pe***	***Rs***
DP_2	D_1A_1		(+)					(+)	+	(+)			(+)			
	A_2		(+)		(+)		(+)				(+)	(+)				+
DP_3	D_2A_1		(+)	+		+		(+)			(+)		(+)		(+)	(+)
	D_1A_2		(+)	+			(+)	(+)	(+)		(+)	(+)	(+)	(+)		(+)
	A_3				+		(+)				(+)	+				
DP_4	D_4		(+)					+					+			
	D_4					+										
	D_3A_1					+					(+)		+			(+)
	D_2A_2		(+)	+				(+)	+		(+)	(+)	+	+		(+)
	D_1A_3			+			(+)	(+)	+			(+)		+		(+)
DP_5	D_5												+			
	D_4A_1					+					+					+
	D_3A_2		+	+							+	+	+	+		+
	D_2A_3		+	(+)			(+)	+				(+)		+		+
	D_1A_4	+					+									
DP_6	D_6												+			
	D_5A_1					+										
	D_4A_2			+		+			+					+		+
	D_3A_3	+		+			+	+	+			(+)		+		
	D_2A_4	(+)		+			+		+			(+)		+		
DP_7	D_7					+		+								
	D_5A_2													+		
	D_4A_3	(+)		+					+			+		+		
	D_3A_4	(+)		+			+		+			+		+		
DP_8	D_6A_2			$+^a$												
	D_5A_3	+		$+^a$					$+^a$					$+^a$		
	D_4A_4						+		$+^a$			+		$+^a$		
	D_3A_5						+									
DP_9	D_9			$+^a$												
	D_6A_3								$+^a$					$+^a$		
	D_5A_4						+		$+^a$			+		$+^a$		
	D_4A_5						+		$+^a$							
DP_{10}	D_7A_3													$+^a$		
	D_6A_4						+							$+^a$		
	D_5A_5													$+^a$		
DP_{11}	D_7A_4													$+^a$		

4.0 Conclusions

Our results show that chitooligomer mixtures of DP 5 to 8, DP 2 to 12 and DP 2 to 11 had little or no effect on the fungal growth of *A. alternata, B. cinerea, P. expansum* and *R. stolonifer* at a concentration of 1,000 μg × mL^{-1}. *A. alternata* growth was slightly induced in the presence of 1,000 μg × mL^{-1} of chitooligomers with DP from 5 to 8 and DP

from 2 to 12 for up to six days, while the sample with DP from 2 to 11 did not significantly affect growth in the presence of chitosan. Growth of the *B. cinerea*, one of the most resistant fungi, was unaffected by the presence of chitooligomers Q1 and Q2 at a concentration of 1,000 μg × mL^{-1} and was slightly retarded in the presence of Q3. *P. expansum* growth was largely unaffected by the presence of 1,000 μg × mL^{-1} of the three chitooligomers used. Chitooligomers Q2 and Q3 decreased the growth rate of the *R. stolonifer* by causing an initial period of fungistasis, which was eventually overcome by the fungus. Chitinolytic and chitosanolytic activity were detected in the fungal spent media of *Alternaria alternata* and *Penicillium expansum*, while only slight chitosanolytic activity was found in the spent medium of *Rhizopus stolonifer*. For *Botrytis cinerea* no activity was detected on spent medium using both substrates. Further studies will be required before generalizations can be made about the relationship between DP of chitosans or chitooligomers and bioactivity as well as the relationship between fungal sensitivity and chitosans or chitooligomers.

Acknowledgments

We would like to express our gratitude to Dr. Sophie Haebel and Msc. Sven Bahrke for characterizing the chitooligomer samples by MALDI TOF MS. Research funding was provided by FAPESP, CNPq, DAAD, ALFA POLYLIFE and CAPES-PROBRAL n° 219/05.

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CAPÍTULO V

Neste capítulo é apresentado o artigo submetido:

E. N. Oliveira Junior, I. S. Melo, and T. T. Franco. Scanning electron microscopy of changes of fungal morphology due to the effect of chitosan. ***Brazilian Journal of Microbiology*****.**

No presente artigo foram avaliadas as mudanças morfológicas micelianas *in vitro*, causadas pela adição de quitosana aos meios de cultura dos fungos: *Alternaria alternata*, *Botrytis cinerea*, *Penicillium expansum* e *Rhizopus stolonifer*. As mudanças morfológicas foram observadas em microscópio eletrônico de varredura Leo 982 com emissão de campo (Zeiss + Leica). A preparação das amostras e as análises de microscopia eletrônica foram realizadas no Laboratório de Microbiologia Ambiental da Embrapa Meio Ambiente, localizada em Jaguariúna, S.P. contando com o auxílio da Bióloga Rosely dos Santos Nascimento e com a colaboração do Dr. Itamar Soares de Melo pesquisador da Embrapa Meio Ambiente.

As micrografias obtidas por microscopia eletrônica de varredura com emissão de campo revelaram que a adição de quitosana aos meios de cultura promoveu agregação miceliana e mudanças estruturais morfológicas como ramificações excessivas, inchamento da parede celular e redução do comprimento das hifas.

Os resultados indicaram que quitosana retarda o crescimento *in vitro* dos fungos filamentosos *Alternaria alternata*, *Botrytis cinerea*, *Penicillium expansum* e *Rhizopus stolonifer* por meio de efeito fungistático.

Scanning electron microscopy of changes of fungal morphology due to the effect of chitosan

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Abstract – The growing consumer demand for foods without chemical preservatives has focused efforts in the discovery of new natural antimicrobials. In this context, the antimicrobial activity of chitosan against fungi has received considerable attention in recent years. Scanning electron microscopy (SEM) observations revealed that chitosan has a direct effect on the morphology of the chitosan-treated fungi reflecting its potential by causing a delay of the growth of the spoilage fungi: *Alternaria alternata*, *Botrytis cinerea*, *Penicillium expansum* and *Rhizopus stolonifer*. Mycelial aggregation and structural changes such as excessive branching, swelling of the cell wall and hyphae size reduction were observed in the micrographs.

Keywords: chitosan; antifungal activity; phytopathogenic fungi, fungal morphology.

1.0 Introduction

Chitin and chitosan (Figure 1) and oligomers of glucosamine (GlcN) and N-acetyl glucosamine (GlcNAc) shown in the Figure 2, have been of interest in the past few decades due to their broad range of potential industrial applications. However, only limited attention has been paid to food application of these versatile polymers, oligomers and monomers (Shahidi, 1999).

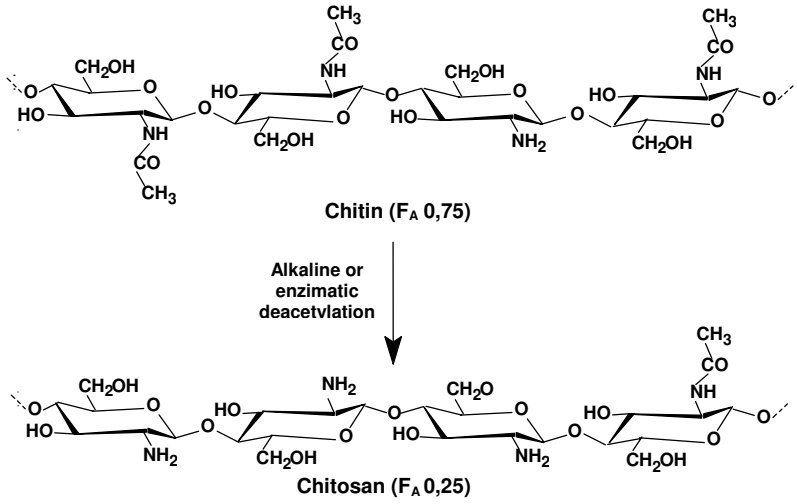

FIGURE 1 Structure of chitin and chitosan

Chitin and chitosan are aminoglucopyranans composed of N-acetylglucosamine (GlcNac) and glucosamine (GlcN) residues. The polymers may be distinguished by their solubility in 1% aqueous acetic acid. Chitin, containing ca. > 40% GlcNac residues (F_A>0.4) is insoluble, whereas soluble polymers are named chitosan (Peter, 2002a). Chitosan has three types of reactive functional groups, an amino group as well as both primary and secondary hydroxyl groups at the C-2, C-3 and C-6 positions respectively (Furusaki et al., 1996). Method mostly used by the industry to produce chitosan from chitin is chemical deacetylation (alkaline hydrolysis), however chitosan can be obtained from enzymatic deacetylation of chitin (Figure 1).

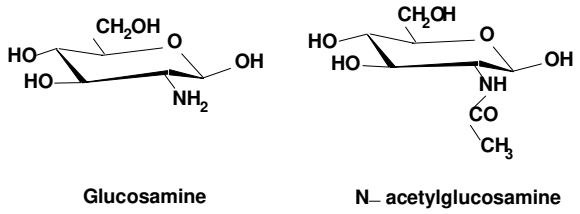

FIGURE 2 Structures of glucosamine and N-acetylglucosamine monomers

Chitin is a natural biopolymer and it is a structural polysaccharide found in the exoskeleton of marine crustaceans (crab and shrimp shells) and insects. It is also widely found in fungi, occurring in *Basidiomycetes*, *Ascomycetes*, and *Phycomycetes,* where it is a component of cell walls and structural membranes of mycelia, stalks, and spores (Peter, 2002b). After the discovery of antimicrobial activities of chitosans and its derivatives by Allan & Hadwiger (1979), Kendra & Hadwiger (1984) and Uchida & Ohtakara (1989), many researches have continued studies in this field.

The purpose of our research was to study the effect of chitosan on the fungal morphology of *Alternaria alternata, Botrytis cinerea, Penicillium expansum* and *Rhizopus stolonifer* analyzed by scanning electron microscopy.

2.0 Materials and Methods

2.1 Chitosans samples

Chitosan (molar fraction of acetyl groups F_A 0.16 and degree of polymerization DP 1,089) produced from alkaline deacetylation of chitin (details will be published elsewhere) and chitosan (F_A 0.18 and DP 1,242) from Primex (Siglufjordur, Iceland) were used. Chitosan F_A 0.16 and DP 1,089 was labeled as chitosan D and chitosan F_A 0.18 and DP 1,242 was labeled as chitosan P.

2.2 Microorganisms and cultivation

The filamentous fungi used in this study included *Alternaria alternata* (CCT 2816), *Penicillium expansum* (CCT 4680) and *Rhizopus stolonifer* (CCT 2002); all of these strains were purchased from André Tosello Foundation Research and Technology (Campinas, Brasil). *Botrytis cinerea,* an isolate from spoiled grape fruit, was provided by Institute of Food Technology (Campinas, Brasil). *Botrytis cinerea* and *Penicillium expansum* were cultured on potato dextrose agar (PDA) and in malt extract agar (MEA), supplemented with 2% (m/v) of glucose and peptone, respectively, while the *Rhizopus stolonifer* and *Alternaria alternata* were both cultured on MEA. In order to achieve the sporulation, the fungi were incubated in Petri dishes (Ø=9cm) for 8 days for *A. alternata*, *B. cinerea* and *P. expansum* and for 4 days for *R. stolonifer* at 25°C at 100 cm beneath Hg lamps with a 12 hours photoperiod. Spores were harvested by pouring sterile water into the slant and stirring with a vortex for 20 seconds. Suspensions of spores and mycelia were filtered in cotton. The concentration of spores was assessed by using a hemocytometer (Fuchs-Rosenthal Hell Linie) under optic microscopy (magnification 400×). Concentration of spores of *Rhizopus stolonifer* was adjusted to the 10,000 spores × mL^{-1} and for *B. cinerea*, *A. alternata* and *P. expansum* to the 20,000 spores × mL^{-1}.

2.3 Chitosan solution and media preparation

Stock solutions of chitosans D and P (10 g × L^{-1}) were transferred to 1 mL of acetic acid solution (40 mmol × L^{-1}) in Eppendorf vials of 2 mL and shaken in a Vortex mixer for

1 hour. The medium used was liquid malt extract (2% m/v) from Merck (Darmstadt, Germany). The chitosan concentrations in the media were 500 μg × mL^{-1} for the fungi *A. alternata* and *R. stolonifer* and 1,000 μg × mL^{-1} for the fungi *B. cinerea* and *P. expansum*. The final pHs were: 4.9 (control medium), 5.4 (acetic acid, chitosan and medium) and 4.4 (acetic acid and medium). In Eppendorf vials of 2 mL previously autoclaved were added appropriate volume of chitosan stock or acetic acid solutions (50 μL for *A. alternata* and *R. stolonifer* and 100 μL for *B. cinerea* and *P. expansum*). The Eppendorf vials containing volume of 50 μL had their volumes completed to 100 μL with autoclaved distilled water. In all Eppendorf vials were added 890 μL of malt extract medium. The spore suspension was first vortex-mixed vigorously and 10 μL of spore suspensions were transferred to the Eppendorf vials. After inoculation the Eppendorf vials were closed and incubated at 25 °C under stirring of 200 o.p.m (orbits per minute) for three days for *R. stolonifer* and five days for *A. alternata*, *B. cinerea* and *P. expansum*. The experiments were carried out with triplicate.

2.4 Scanning electron microscopy (SEM)

Scaning electron microscopy was carried out in accordance to the methodology described by Melo and Faull (2004) on the materials described below. In case of control suspension the hyphae was removed from the media by using clamp and the suspension treated with chitosans were filtered in Millipore membrane PTFE hydrophilic of pore size 0.45μm and ∅=13mm (São Paulo, Brasil). Samples were fixed by immersion in 2.5% (v/v) glutaraldehyde in 0.1 mol L^{-1} sodium cacodylate buffer pH 7.0 for 1h, washed three times in 0.1 mol L^{-1} sodium cacodylate buffer pH 7.0, post-fixed with 1% (m/v) osmium tetroxide in the same buffer for 1h and washed three times again in 0.1 mol L^{-1} sodium cacodylate buffer pH 7.0. The material was then dehydrated in an acetone series (10, 25, 40, 60, 75, 85, 95 and 100%v/v) with 15min per change. The materials were dried in a CO_2 critical point drying apparatus and sputter-coated with gold and viewed using a field emission scanning electron microscope, Leo 982 (Zeiss + Leica).

3.0 Results

Fungal mycelia of *A. alternata*, *B. cinerea*, *P. expansum* and *R. stolonifer* whose media were amended with chitosan were observed by means of a field emission scanning electron microscope. The micrographs, taken after five days of cultivation at 25°C for *A. alternata* (Figure 3 C and D), *B. cinerea* (Figure 4 C and D) and *P. expansum* (Figure 5 C and D) and after three days for *R. stolonifer* (Figures 6 C and D) showed that chitosan amendment caused aggregation and morphological changes of mycelia that were coated with chitosan. Aggregation, excessive mycelial branching and hyphae size reduction of all fungi studied whose media were amended with chitosan were observed.

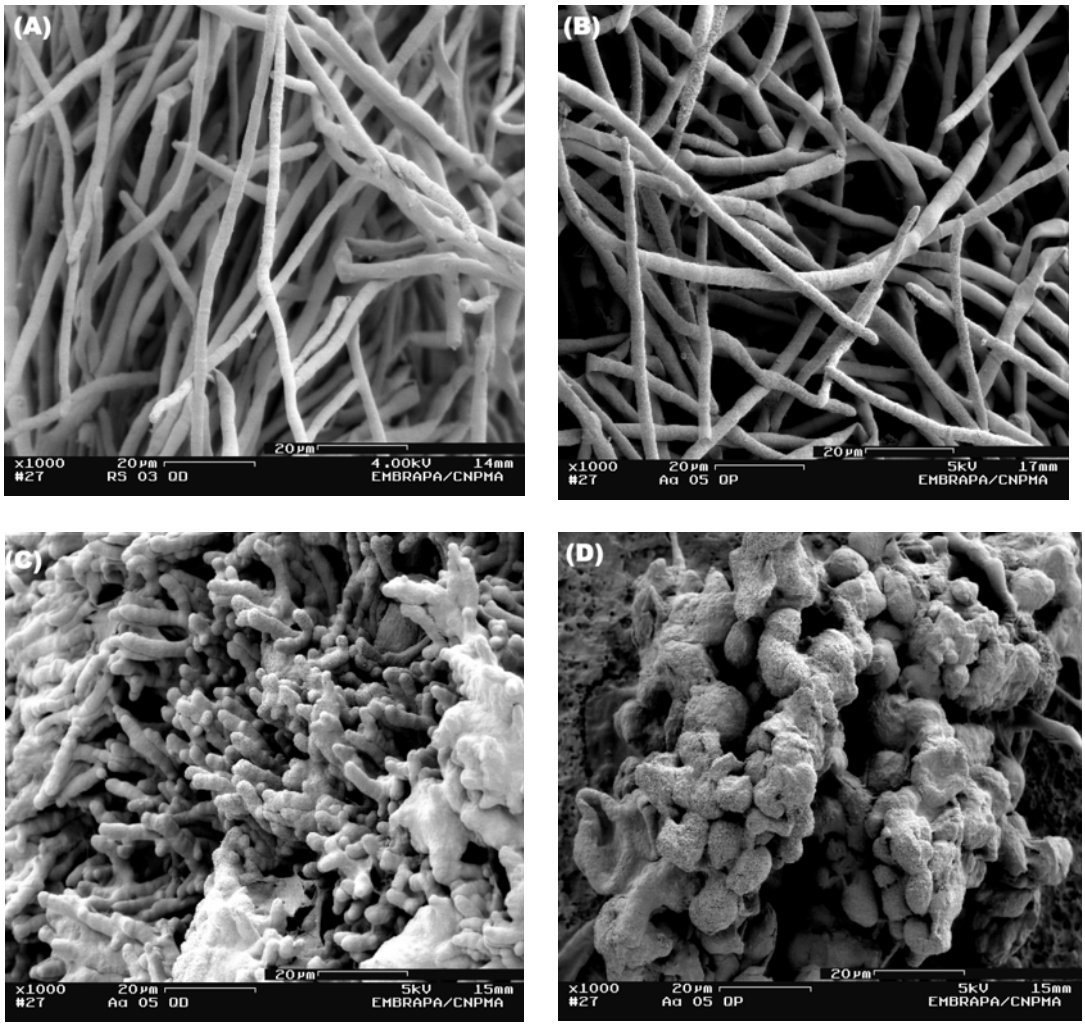

FIGURE 3 Scanning electron micrographs of mycelia of *Alternaria alternata* after 5 days of culture at 25 °C. (A) Control media, (B) acetic acid control (40 mmol × L^{-1}), (C) medium amended with chitosan D (500 mg × mL^{-1}) and (D) medium amended with chitosan P. Magnification at 1,000×.

A. alternata, *B. cinerea* and *R. stolonifer* treated with chitosan besides to have the morphological changes mentioned before, it was observed abnormal shapes and swelling of their mycelia.

El Ghaouth et al. (1992) reported that chitosan causes severe morphological changes in *Rhizopus stolonifer* that is characterized by excessive branching and swelling of the cell wall. Chitosans on the surface of *R. stolonifer* mycelia were observed in the scanning electron micrographs (Figures 6 C and D).

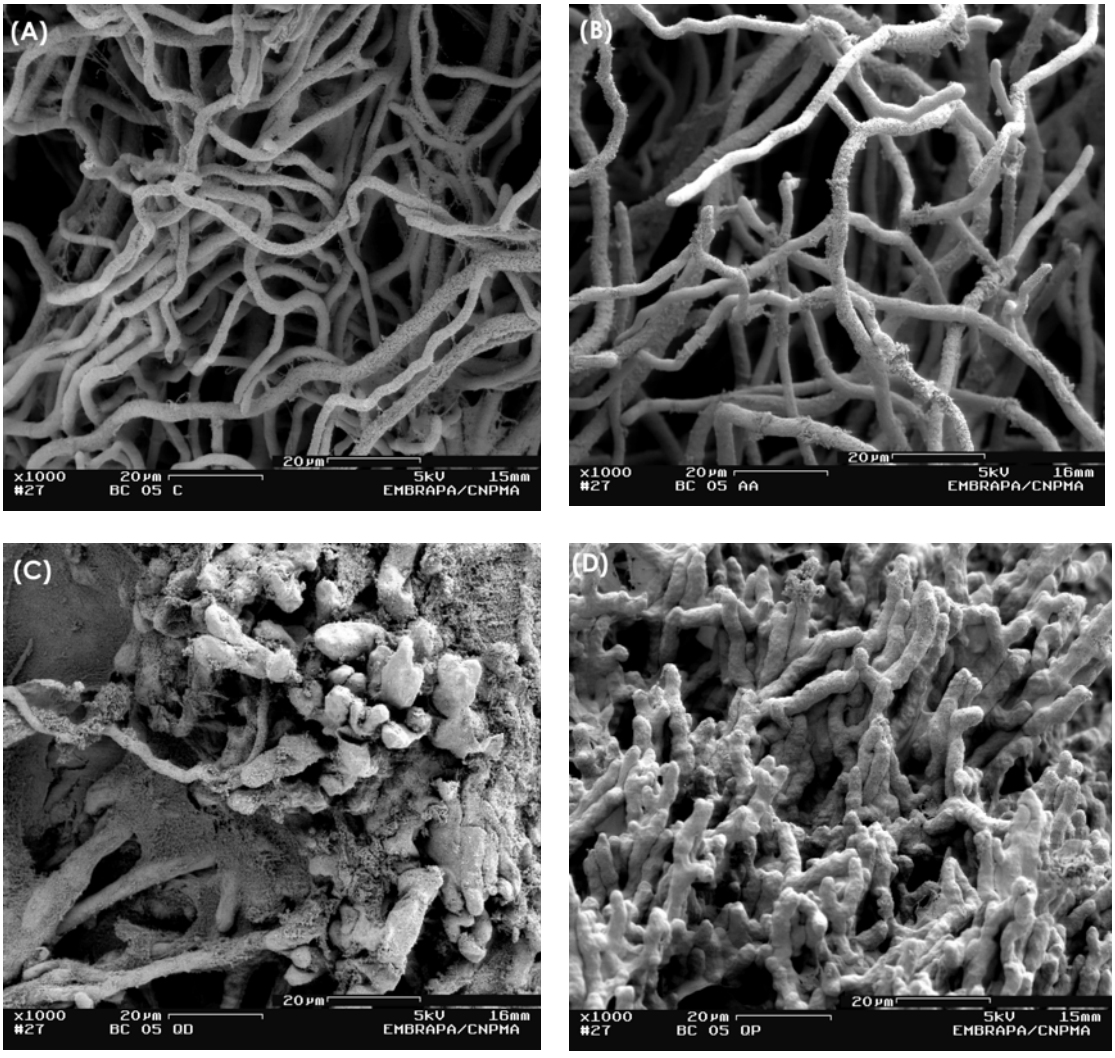

FIGURE 4 Scanning electron micrographs of mycelia of *Botrytis cinerea* after 5 days of culture at 25 °C. (A) Control media, (B) acetic acid control (40 mmol × L^{-1}), (C) medium amended with chitosan D (500 mg × mL^{-1}) and (D) medium amended with chitosan P. Magnification at 1000×.

In further studies, image analysis was used to measure the effect of chitosan on the morphology of fungi, such as *F. oxysporum* f. sp. *radicis-lycopersici*, and *S. sclerotiorum* treated with chitosan. These studies showed excessive mycelial branching, abnormal shapes, swelling, and hyphae size reduction (Benhamou, 1992; Cheah et al., 1997). Large vesicles or empty cells devoid of cytoplasm in the mycelium of *B. cinerea*, treated with chitosan, were observed by Ait Barka et al. (2004).

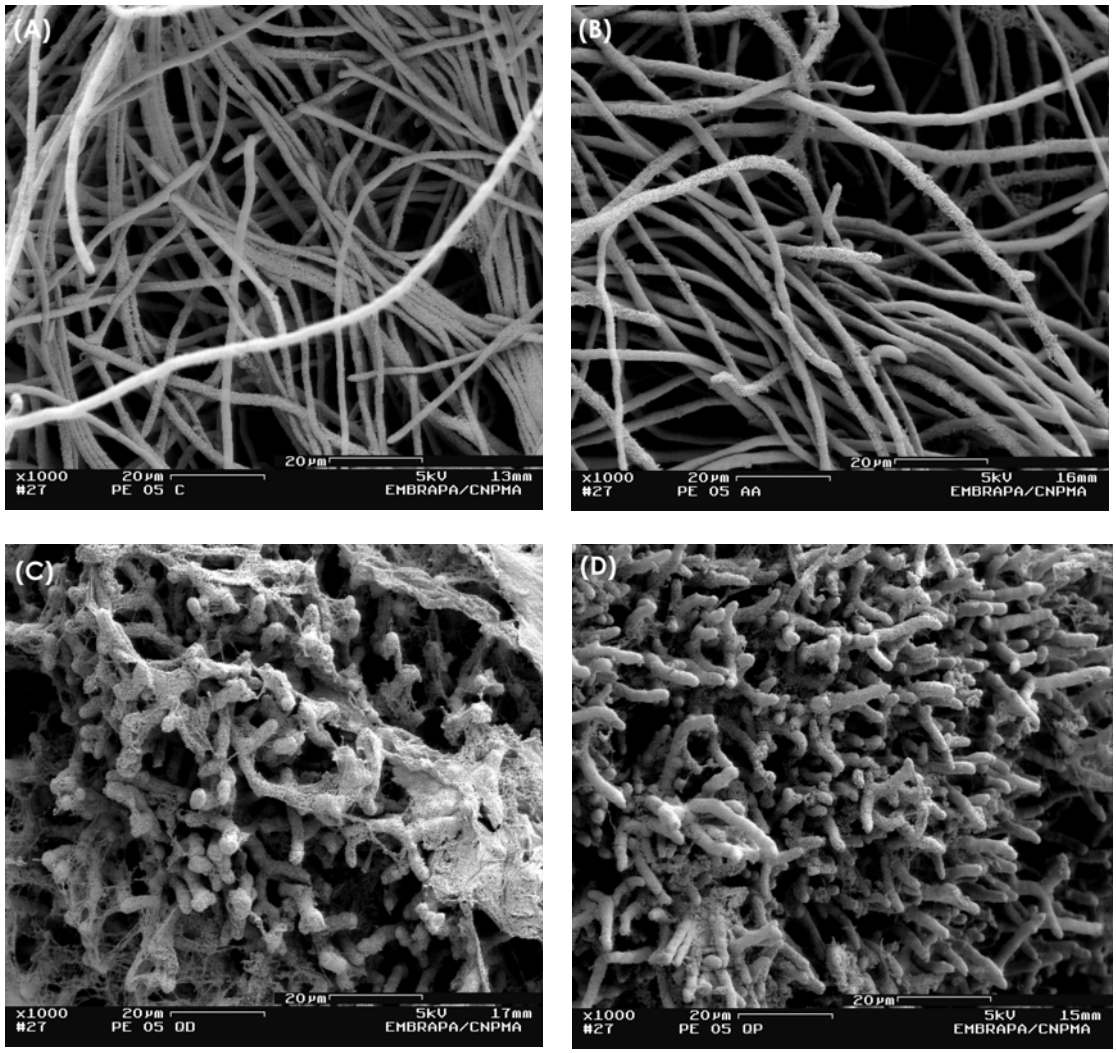

FIGURE 5 Scanning electron micrographs of mycelia of *Penicillium expansum* after 5 days of culture at 25 °C. (A) Control media, (B) acetic acid control (40 mmol × L^{-1}), (C) medium amended with chitosan D (500mg × mL^{-1}) and (D) medium amended with chitosan P. Magnification at 1,000×.

The micrographs showed that chitosan amendment caused aggregation of mycelia that were coated with this polymer. Similar morphological anomalies caused by chitosan coating on the mycelial surface of fungi studied, suggest that chitosan layer around the spores or hyphae, may become difficult the entrance of nutrients in the cells.

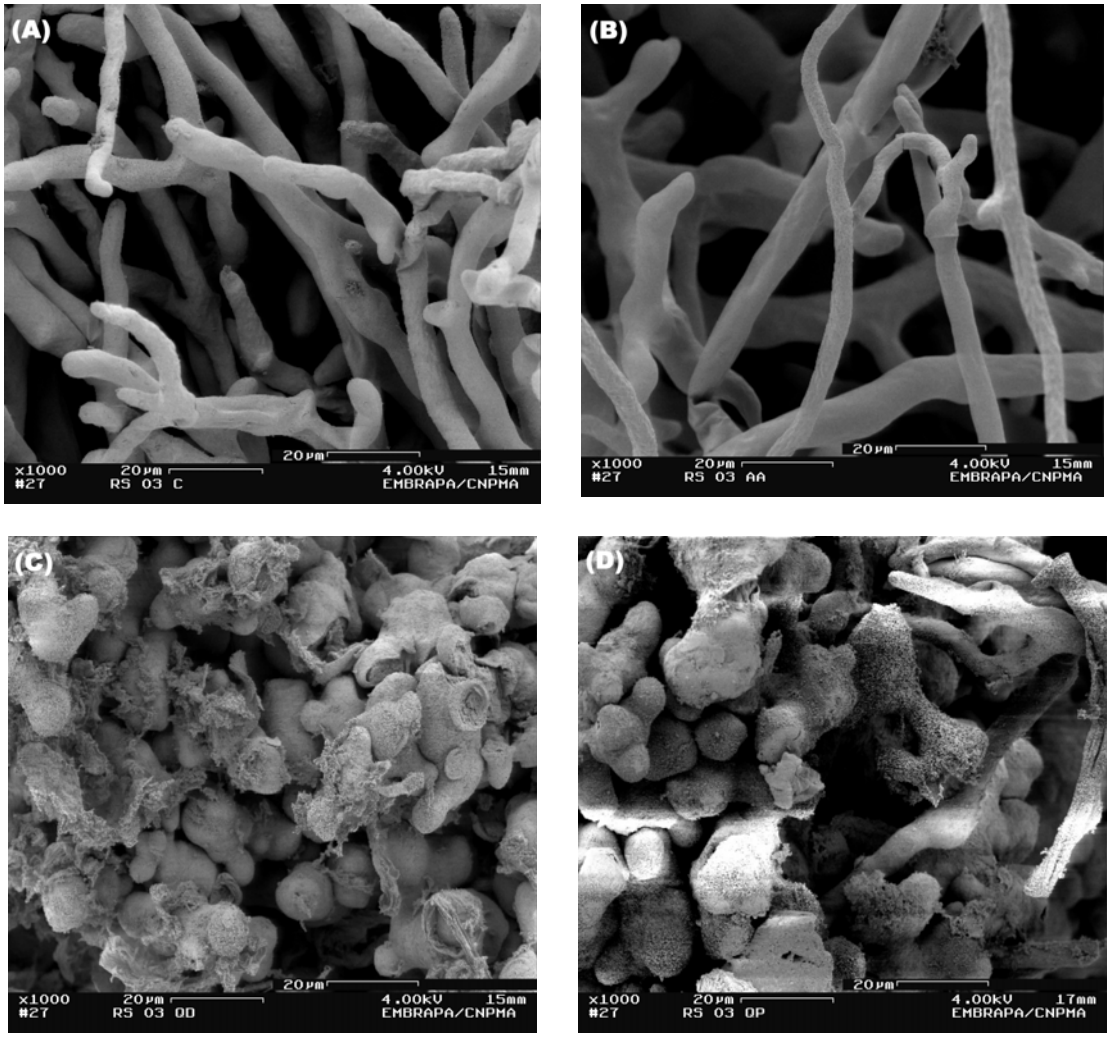

FIGURE 6 Scanning electron micrographs of mycelia of *Rhizopus stolonifer* after 3 days of culture at 25 °C. (A) Control media, (B) acetic acid control (40 mmol × L^{-1}), (C) medium amended with chitosan D (500 µg × mL^{-1}) and (D) medium amended with chitosan P. Magnification at 1,000×.

Protonation of amino groups is believed to be an important factor in the antifungal activity of chitosan and chitooligosaccharides, although the exact mechanism of action is

still unknown. A polycationic chitosan or oligomer can potentially interact with negatively charged fungal cell membrane components (i.e., proteins, phospholipids) and/or selectively chelate trace metals, thus interfering with the normal growth and metabolism of the fungal cells (Fang et al. 1994; Roller and Covill 1999; Shahidi et al., 1999). Chitosan coating observed on surface of the mycelia suggests that fungal growth inhibition could be explained by a direct interaction of chitosan on the fungal cell wall as a consequence of polycationic nature of chitosan. Fungal growth inhibition was directly proportional to concentration of chitosan (details will be published elsewhere).

The pH value of mycelial suspension, before its fixation and post-fixation, was about 5.4 (item 2.3) and after this, the pH value was changed to 7.0 (item 2.6). In the pH 5.4 chitosan was soluble in the mycelial suspension that was filtered in Millipore membrane (pore size 0.45 μm). Bound chitosan on the fungal cell wall was precipitated at a pH 7.0 and, then, chitosan coating was formed. This observation was supported by scanning electron microscopy for all fungi treated with chitosan. The micrographs of *Penicillium expansum* previously treated with chitosan viewed in high magnification of 10,000× show the chitosan coating formed on surface of the mycelia (Figure 7).

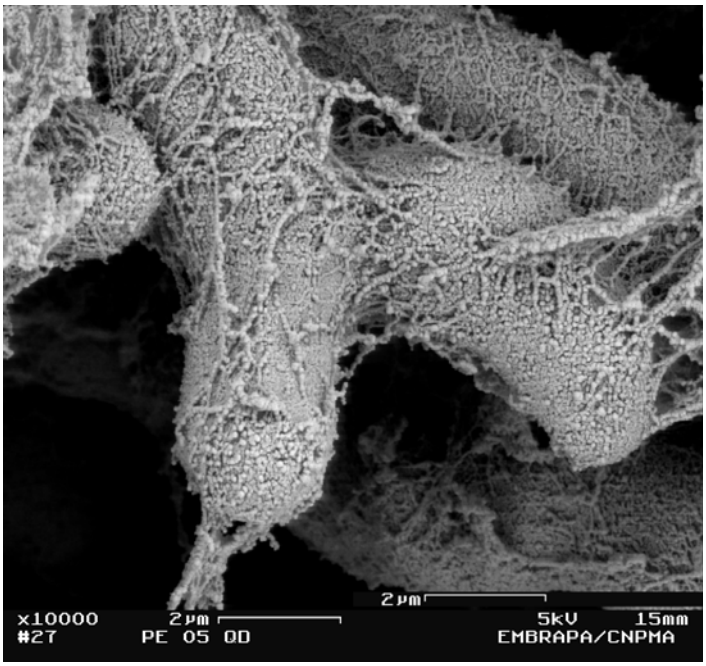

FIGURE 7 Scanning electron micrograph of mycelia of *Penicillium expansum* after 5 days of culture at 25 °C with medium amended with chitosan D (1,000 mg × mL^{-1}). Magnification at 10,000×.

Our results demonstrated that chitosan acetate was effective in restricting the fungal growth of filamentous fungi (details will be published elsewhere) by causing a fungistatic inhibition effect as observed by scanning electron microscopy. In case of *Alternaria alternata*, it was common to observe some spores with germ tubes inhibition as shown in Figure 8.

Aggregates of chitosans were observed in micrographs on media amended with chitosans D and P on surface of Millipore membranes that were fixed and post-fixed as described in the item 2.6 (Figure 9 A and C). As shown in the Figures 9 B and D, different aggregates of chitosans were observed in micrographs on media amended with the same chitosans on Millipore membrane surface. These chitosans were not fixed and post-fixed, they were filtered and dried at room temperature for one day. It is believed that the difference observed in the aggregate of chitosans can be related to the pH=7.0 of the sodium cacodylate buffer used for SEM preparation, that became chitosan insoluble.

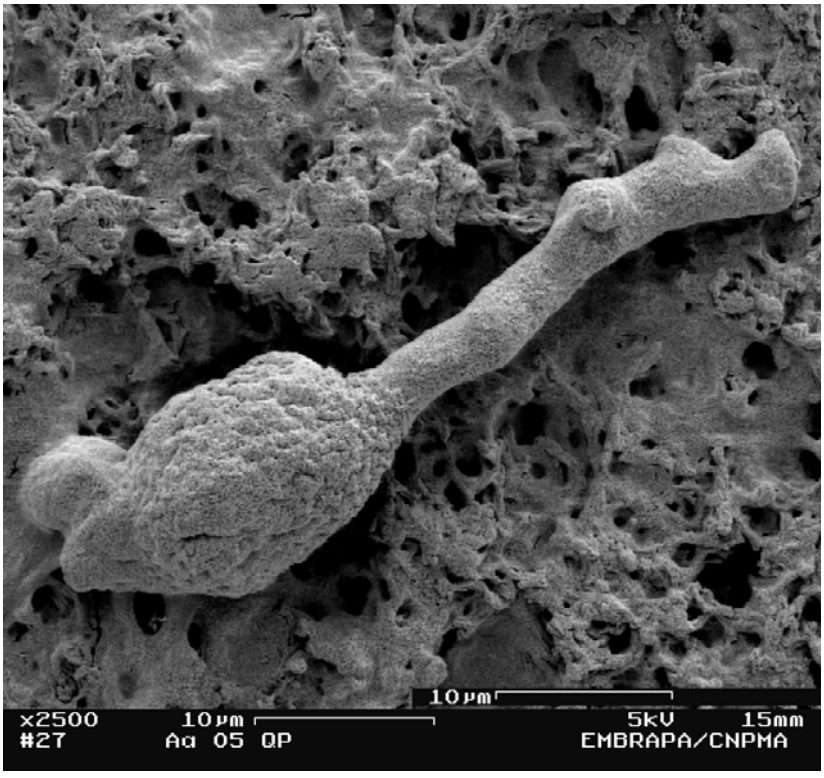

FIGURE 8 Scanning electron micrograph of spore and germ tube of *Alternaria alternata* after 5 days of culture at 25 °C with medium amended with chitosan P (1,000 mg × mL^{-1}). Magnification at 2,500×.

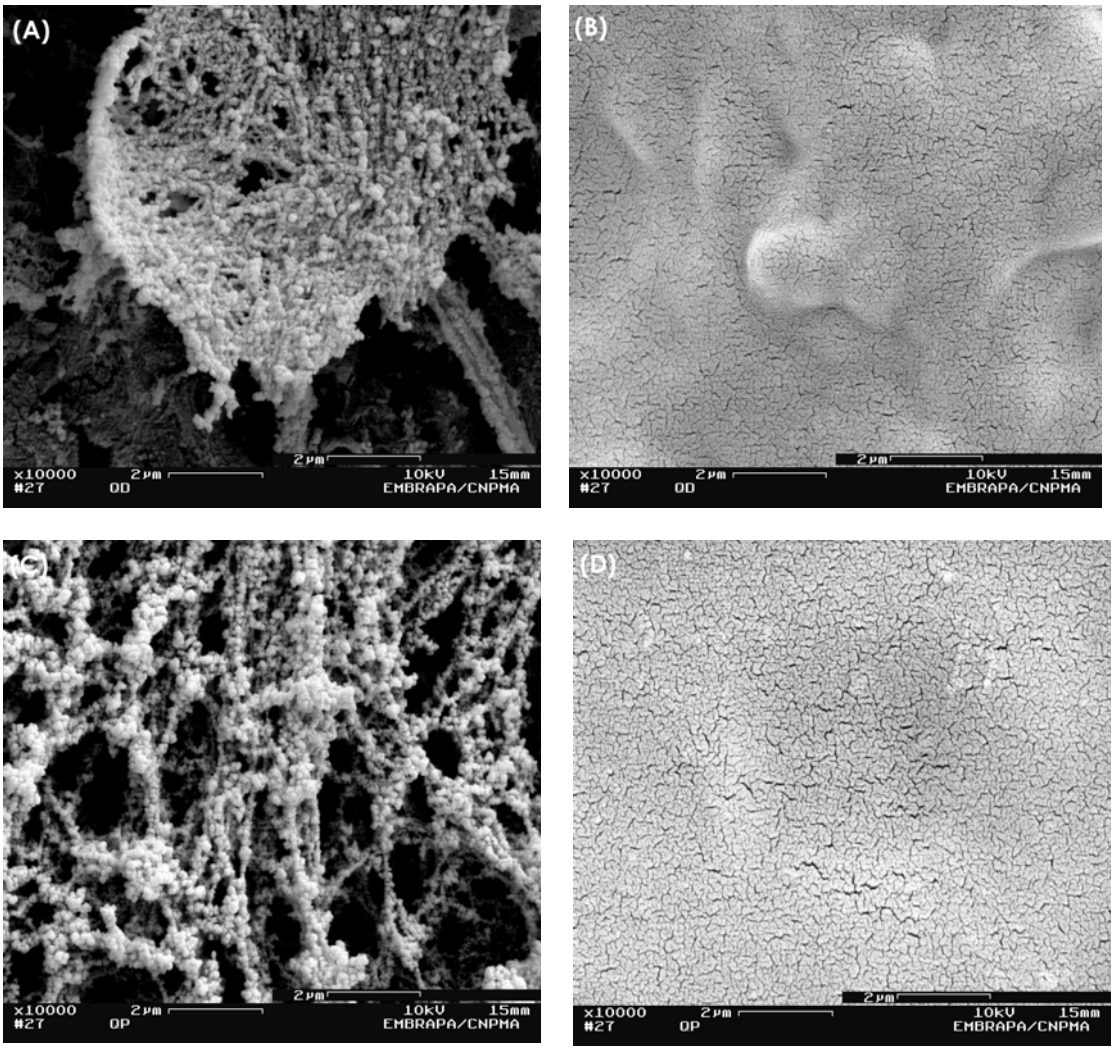

FIGURE 9 Scanning electron micrographs of medium amended with 1,000 µg × mL^{-1} of chitosan D (A) filtered and fixed as described in the item 2.6 (B) only filtered; 1,000 µg × mL^{-1} of chitosan P (C) filtered and fixed as described in the item 2.6 and (D) only filtered. Magnification at 10,000×.

4.0 Conclusions

SEM revealed that chitosan can be used to delay the fungal growth of the following filamentous fungi: *Alternaria alternata*, *Botrytis cinerea*, *Penicillium expansum* and *Rhizopus stolonifer*. The micrographs showed that chitosans caused mycelial aggregation and structural changes as excessive branching, swelling of the cell wall and hyphae size reduction.

Acknowledgments

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5.0 References

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CAPÍTULO VI

CONCLUSÕES

Os resultados deste estudo mostraram que quitosanas de diferentes massas molares determinadas por viscosimetria e GPC-RI e com mesmos graus de acetilação determinados por RMN ^{1}H e por titulação potenciométrica, resultaram da degradação térmica a 100 °C por 10 horas. Seis horas de tratamento térmico foram suficientes para se obter quitosanas com massas molares 90% menores, comparada a massa molar da quitosana sem tratamento.

Microplacas plásticas com 96 reservatórios foram bastante eficientes para o crescimento dos fungos filamentosos *Alternaria alternata, Botrytis cinerea, Penicillium expansum* e *Rhizopus stolonifer.*

Inibições completas dos fungos *A. alternata*, *B. cinerea*, *R. stolonifer* e redução do crescimento do fungo *P. expansum* foram obtidas com quitosanas de DP 45 a 1.460 e de F_A 0.08 a 0.22.

Quitosanas com menores valores de F_A 0.1 e maiores valores de DP 3.780, promoveram a máxima atividade fungistática contra os fungos *A. alternata* e *B. cinerea*.

Mudanças morfológicas e formação de agregados micelianos fúngicos foram observados por microscopia ótica nos fungos tratados com quitosana.

F_A de quitosana mostrou ser um importante fator envolvido na atividade antifúngica, cuja máxima atividade foi observada para os menores valores de F_A, destacando a importância dos grupos amino protonados (NH_3^+) na atividade antifúngica.

Misturas de quitoligômeros de DP 5 a 8, DP 2 a 12 e DP 2 a 11, promoveram menor efeito no crescimento fúngico de *A. alternata, B. cinerea, P. expansum* e *R. stolonife*r na concentração de 1,000 μg × mL^{-1} do que as quitosanas em geral. O crescimento do fungo *A. alternata* foi levemente induzido na presença de 1,000 μg × mL^{-1} das misturas de quitoligômeros DP 5 a 8 e DP 2 a 12, enquanto a mistura de quitoligômeros de DP 2 a 11

não afetou significativamente o crescimento. Os fungos mais resistentes tiveram seus crescimentos fracamente inibidos (*B. cinerea*) ou não afetados (*P. expansum*) pela presença de 1,000 $\mu g \times mL^{-1}$ dos quitoligômeros testados.

Micrografias obtidas por microscopia eletrônica de varredura com emissão de campo revelaram agregação miceliana e mudanças estruturais morfológicas como ramificações excessivas, inchamento da parede celular e redução do comprimento das hifas.

Nossos resultados indicaram que quitosana retarda o crescimento dos fungos filamentosos *Alternaria alternata*, *Botrytis cinerea*, *Penicillium expansum* e *Rhizopus stolonifer* por meio de efeito fungistático.

CAPÍTULO VII

SUGESTÕES PARA TRABALHOS FUTUROS

1) Determinação da composição da parede celular dos fungos testados com a finalidade de esclarecer as diferentes sensibilidades fúngicas observadas na presença de quitosana.

2) Avaliação da capacidade de quitosana em formar complexos com os sais e nutrientes presentes nos meio de cultivo usado para o crescimento dos fungos.

3) Estudos de campo *in vivo* pré e/ou pós-colheita de vegetais com o intuito de se avaliar a viabilidade econômica de aplicação de quitosana como agente inibidor de fungos.

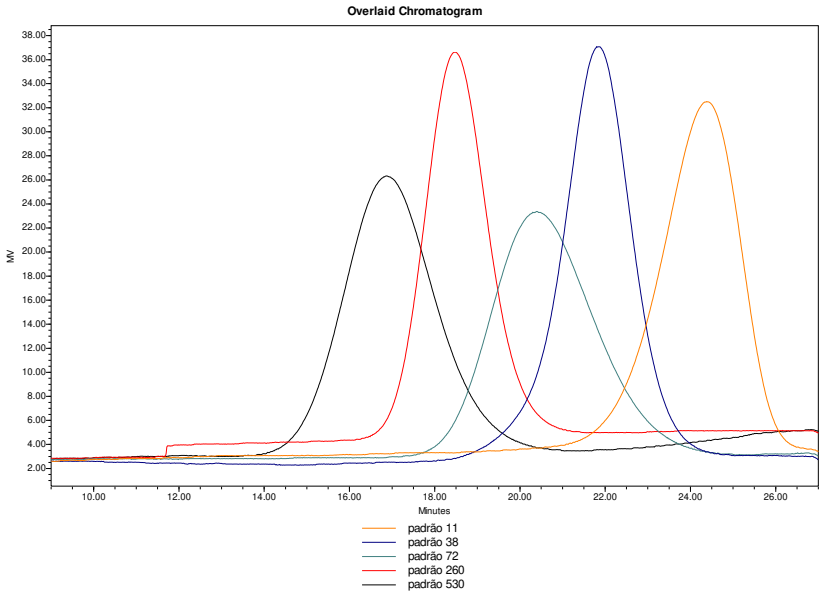
4) Associação de quitosana a outros métodos alternativos de controle de fungos em vegetais, por exemplo: tratamento térmico pós-colheita, atmosfera controlada, contole biológico pré-colheita entre outros.

ANEXO A

Gráficos e tabelas gerados no software Millenium

Curva de calibração 30/10/2003

(curva do tipo $Y = A + BX + CX^2 + DX^3$)



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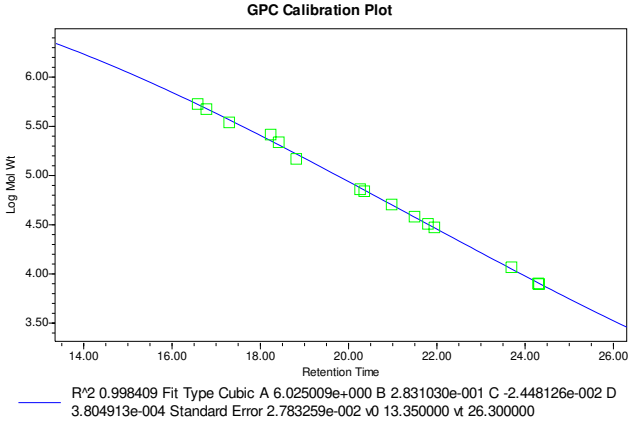
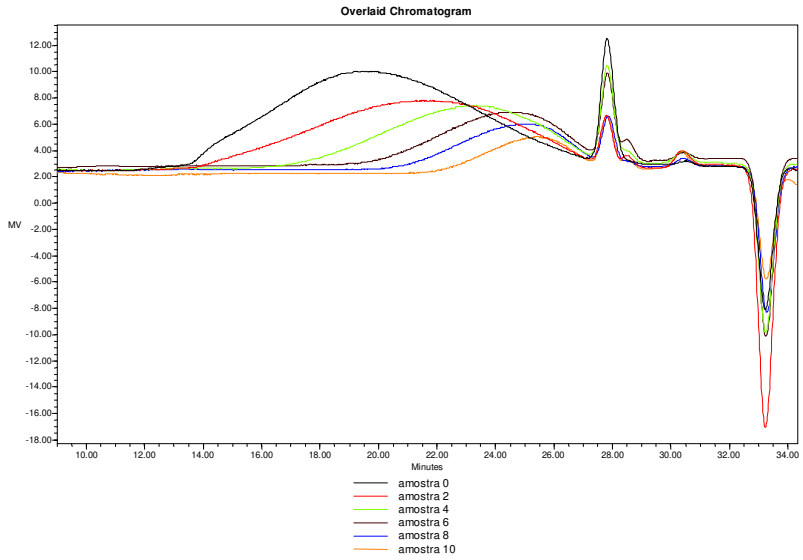


Resultados 30/10/2003 das amostras de quitosana controle e tratadas termicamente



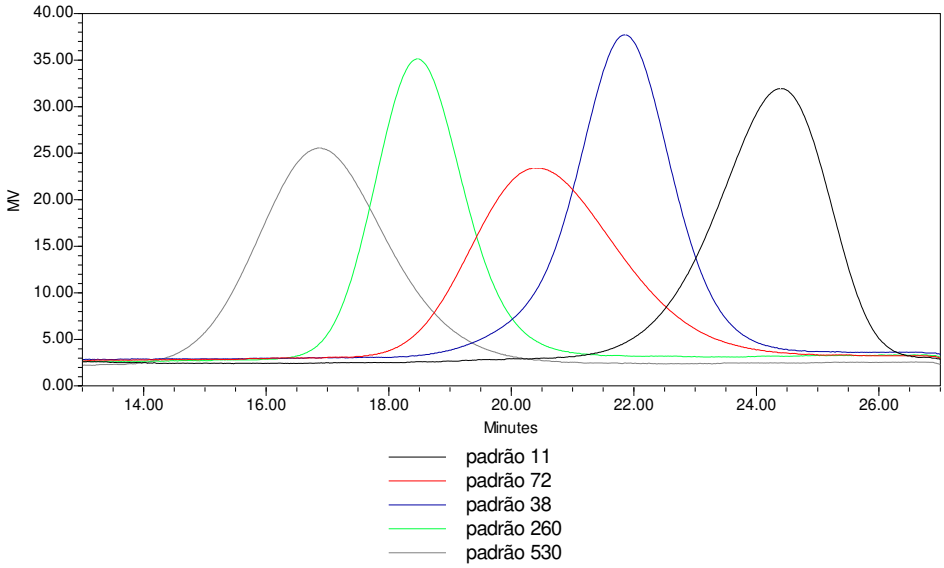
Distribuição das massas molares das amostras de quitosana controle e tratadas termicamente durante 10 horas

Vial	Sample name	Injection	Retention time	Mn	Mw	MP	Mz	Mz+1	Polydispersity
6	amostra 0	1	19.217	33865	242244	138572	709213	1055983	7.153254
6	amostra 0	2	19.266	33528	227858	134872	674000	1021164	6.795956
7	amostra 1	1	19.997	28030	229196	89866	732579	1086590	8.176711
7	amostra 1	2	20.305	27366	221415	75659	724512	1085976	8.090963
8	amostra 2	1	21.3	20866	152669	43403	587737	964999	7.316675
8	amostra 2	2	21.088	21839	159152	48846	588792	957343	7.287512
9	amostra 3	1	22.671	14733	62737	20293	209179	376296	4.258172
9	amostra 3	2	22.923	14726	62290	17677	211714	381692	4.229907
10	amostra 4	1	23.267	12761	44242	14658	145686	281175	3.466977
10	amostra 4	2	23.037	12674	41257	16607	117302	203324	3.255211
11	amostra 5	1	23.732	9789	22895	11405	53499	91326	2.338909
11	amostra 5	2	24.159	9432	21062	9080	46769	76880	2.233062
12	amostra 6	1	24.101	8452	16222	9366	32205	52018	1.919251
12	amostra 6	2	24.211	8188	15461	8836	30586	49630	1.888222
13	amostra 7	1	24.355	7685	13610	8189	25240	39971	1.7709
13	amostra 7	2	24.683	7476	12728	6897	22581	34655	1.702536
14	amostra 8	1	24.812	6949	11122	6453	18750	28239	1.600665
14	amostra 8	2	24.522	6993	10805	7503	17286	25040	1.545132
15	amostra 9	1	24.985	6526	10126	5901	18580	35391	1.551683
15	amostra 9	2	24.804	6551	9597	6477	14611	20668	1.465052
16	amostra 10	1	25.329	5688	7497	4951	10285	13746	1.317921
16	amostra 10	2	25.327	5869	7881	4957	11065	15147	1.342773

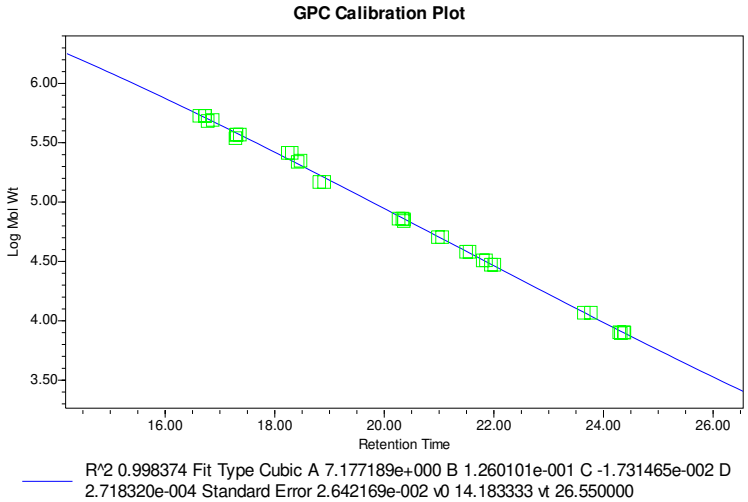
Gráficos e tabelas gerados no software Millenium

Curva de calibração GPC 08/11/2004

(curva do tipo $Y = A + BX + CX^2 + DX^3$)



Current Date 08/11/2004

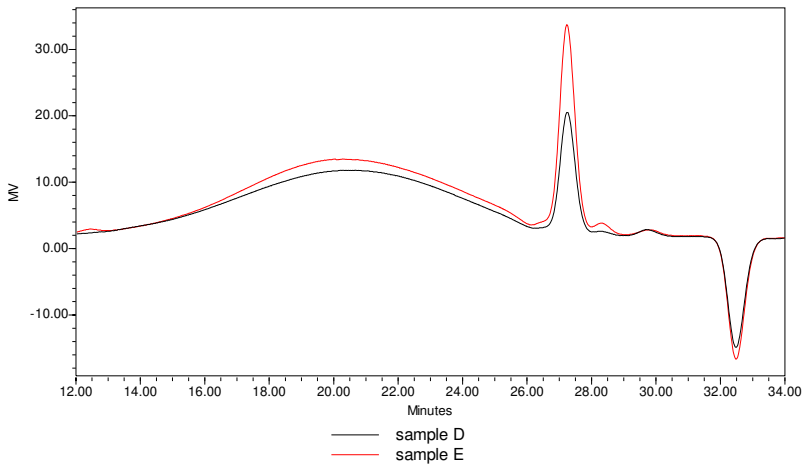


GPC Calibration Table

	Mol Wt (Daltons)	RT	Calculated Weight (Daltons)	% Residual
1	534000	17.3	515761	3.536
2	534000	16.774	544470	-1.923
3	534000	16.868	515161	3.657
4	260600	18.47	222954	16.885
5	260600	18.417	232045	12.306
6	223716	18.47	204428	9.435
7	72700	20.355	76402	-4.846
8	72700	20.359	73617	-1.245
9	71639	20.359	72416	-1.073
10	38200	21.856	37215	2.648
11	38200	21.797	38535	-0.87
12	32332	21.856	31504	2.627
13	11700	24.316	11770	-0.592
14	11700	24.383	10976	6.596
15	8000	24.383	7921	0.992

GPC Results

Vial	Sample Name	Retention Time	Mn	Mw	MP	Mz	Mz+1	Polydispersity
1	padrão 11	24.383	8000	11700	9900	25025	77761	1.4625
2	padrão 38	21.856	29700	38200	32000	51288	72583	1.286195
3	padrão 72	20.359	50700	72700	62900	101851	155451	1.433925
4	padrão 260	18.47	148100	260600	234200	402746	606336	1.759622
5	padrão 530	16.868	371000	534000	490000	716481	909672	1.439353



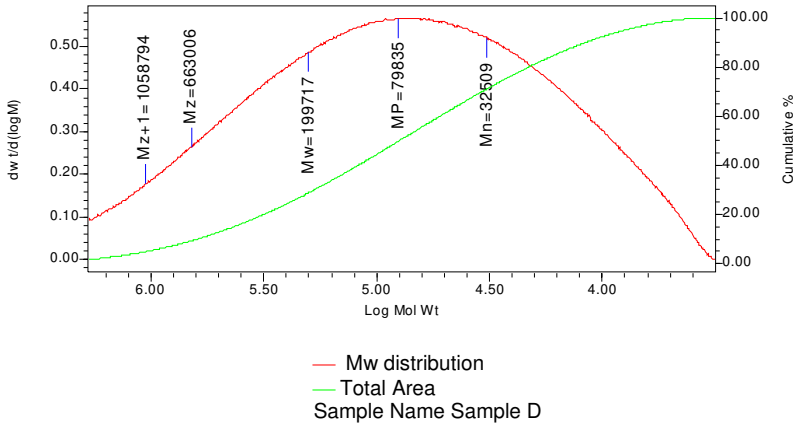
Cromatogramas das amostras D e E

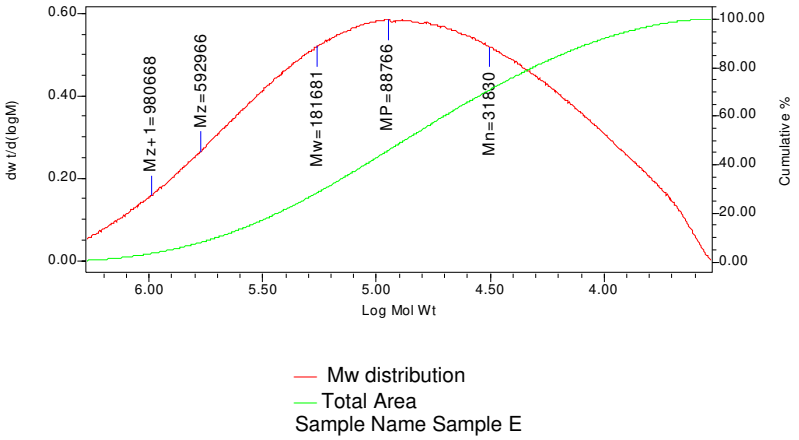
Tempos de retenção, valores das massas molares e polidispersividades das amostras de quitosana D e E

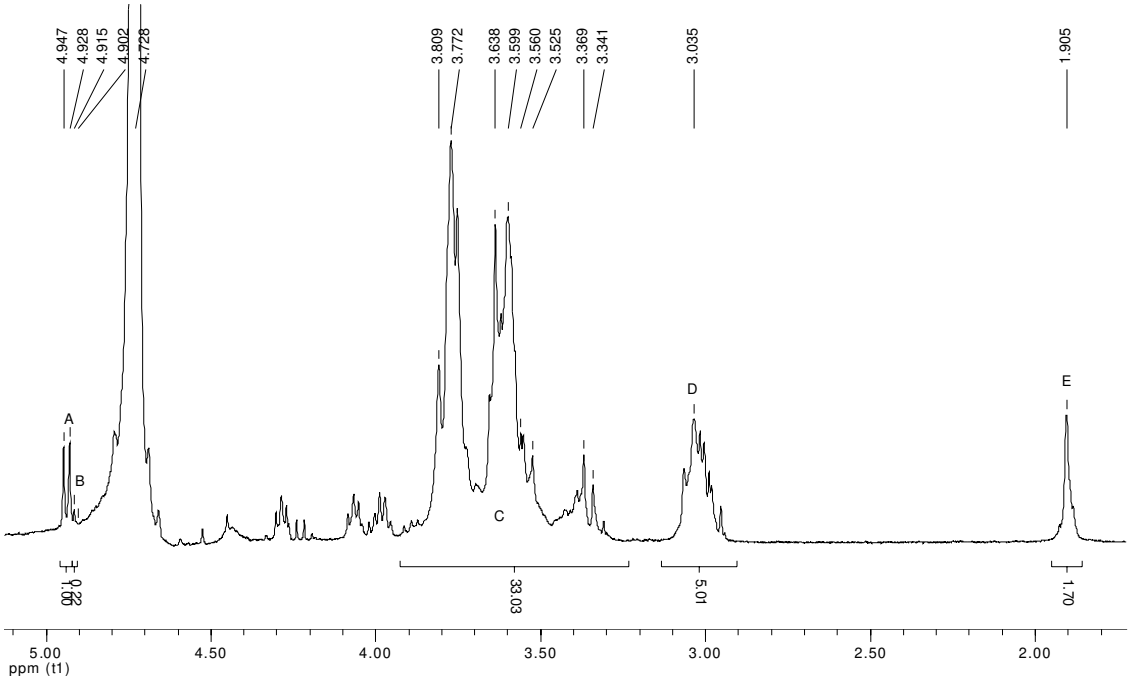
GPC Results

Vial	Sample Name	Retention Time	Mn	Mw	MP	Mz	Mz+1	Polydispersity
1	sample D	20.222	32016	192944	78137	629754	1002815	6.026536
2	sample E	20.032	31506	183962	86816	596283	969629	5.838918

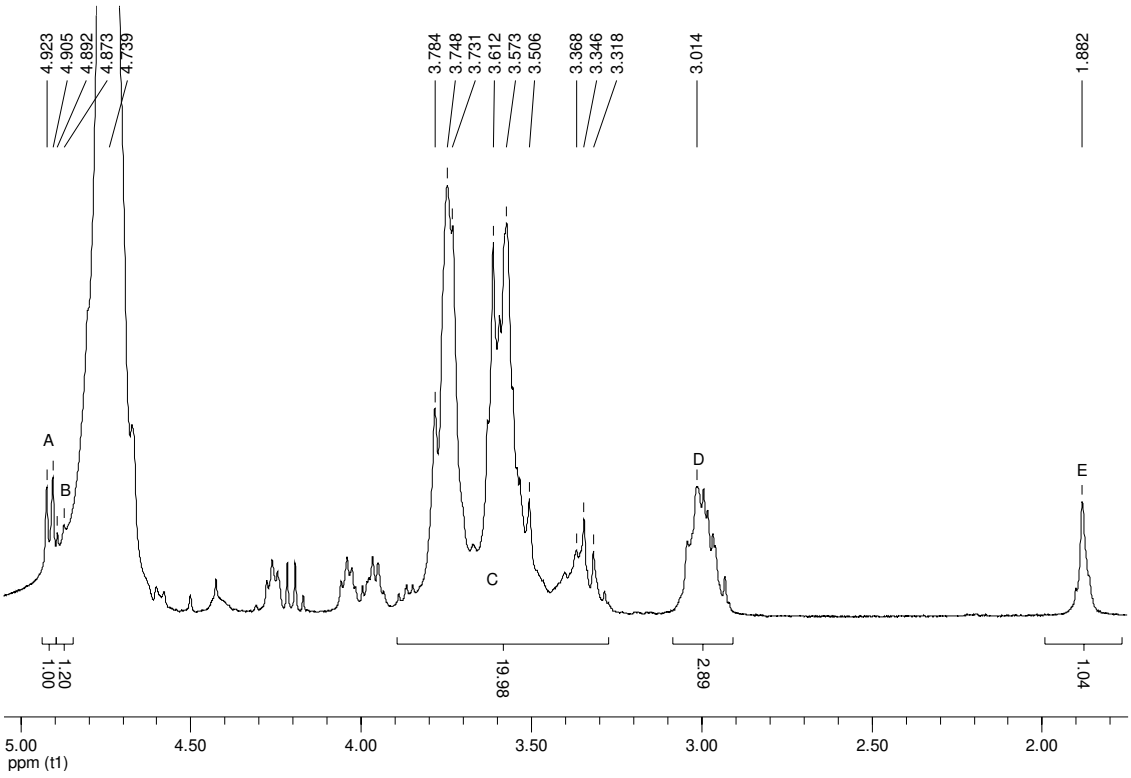
Distribuição das massas molares das amostras de quitosana D e E







Espectro de RMN ^{1}H de amostra de quitosana D e respectivos valores de integração dos picos A, B, C, D e E usados no cálculo do grau de acetilação da amostra.



Espectro de RMN ^{1}H de amostra de quitosana E e respectivos valores de integração dos picos A, B, C, D e E usados no cálculo do grau de acetilação da amostra.