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Produção e Caracterização de Quitina e Quitosana por *Rhizopus arrhizus* e *Cunninghamella elegans* e Aplicação em Membranas na Remoção de Cádmio.

Lúcia Raquel Ramos Berger

RECIFE - PE
DEZEMBRO DE 2013

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas, Universidade Federal de Pernambuco, como parte das exigências para obtenção do título de Doutora em Ciências Biológicas.

Orientadora: Profa. Dra. Galba Maria de Campos-Takaki

Co-orientadora: Profa. Dra. Thayza Christina Montenegro Stamford

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Banca Examinadora:

_____

Prof. Dra. Galba Maria de Campos-Takaki (Orientadora)

_____

Profa. Dra. Celuta Sales Alviano-UFRJ

_____

Prof. Dr. Camilo Enrique La Rotta Hernández-FACEPE/UNICAP

_____

Profa. Dra. Luciana de Oliveira Franco- UFRPE

_____

Prof. Dr. Ranilson de Souza Bezerra-UFPE

Suplentes:

_____

Prof. Dr. Marcos Antônio Barbosa de Lima-UFRPE

_____

Profa. Dra. Tereza Correia-UFPE

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RESUMO

Nos últimos anos, muitos estudos têm demonstrado o interesse em relação à produção e aplicação dos biopolímeros quitina e quitosana como materiais funcionais na medicina, farmácia, alimentação, biologia e engenharia. Especialmente devido a suas características únicas como biocompatibilidade, biodegradabilidade, não- toxicidade, atividade antimicrobiana, capacidade quelante e fácil produção. Após a celulose, a quitina é o segundo composto orgânico mais abundante na Terra, e é encontrado naturalmente como elemento estrutural em invertebrados e paredes celulares de fungos, principalmente na ordem Mucorales (classe Zygomycetes). A quitosana é obtida a partir da desacetilação da quitina (poli-(1-4)-2-acetamida-2-desoxi-β-D-glicosamina). A produção simultânea desses polímeros por via microbiológica mostrou ser vantajosa quando comparada com a extração tradicional a partir de crustáceos. A produção controlada por fermentação têm permitido o uso de resíduos agroindustriais como substratos de baixo custo, tais como: milhocina, manipueira, melaço e casca de mamão, o que corresponde a fontes de nutrientes alternativas para as culturas de fungos.Entre as várias aplicações da quitosana , destaca-se a sua capacidade de formar membranas, que podem ser aplicadas durante a remoção de metais pesados em ambientes aquosos . Esse método poderia ser considerado alternativa promissora, tendo em vista a inviabilidade operacional e o alto custo dos procedimentos habituais para remoção de metais. O objetivo deste estudo foi selecionar as melhores condições de cultivo para a produção satisfatória de quitina e quitosana a partir de *Cunninghamella elegans* e *Rhizopus arrhizus* e posterior aplicação de membranas de quitosana para a remoção de cádmio em soluções aquosas. Inicialmente, a produção de biomassa e de biopolímeros por estes fungos foi realizada através dos seguintes meios de cultura: meio específico modificado para Mucorales, e meios alternativos enriquecidos com milhocina (como fonte de nitrogênio) e suplementados manipueira, melaço ou suco de casca de mamão (como fontes de carbono). As caracterizações físico-químicas realizadas foram: infravermelho, difração de raios X, microscopia eletrônica de varredura, viscosidade, massa molecular, termogravimetria e densidade calorimétrica. Foi realizada a comparação entre as membranas obtidas utilizando quitosana fúngica com baixa massa molecular e quitosana comercial com massas moleculares baixa e média. Essas membranas de quitosana foram aplicadas para a remoção de Cd (II) a partir de soluções aquosas. E a remoção de cádmio foi estimada utilizando as análises de voltametria de redissolução anódica e voltametria de onda quadrada. Os resultados mostraram claramente as maiores produções de biomassa entre 16,00-24,60 g/L e produção de quitosana entre 77,78-77,76 mg/g obtidas utilizando os meios de cultura alternativos para *R. arrhizus* e *C. elegans*, respectivamente. Por outro lado, as produções mais elevadas de quitina de 137,17 e 235,00 mg/g foram obtidas usando os mesmos fungos e o meio de cultura padrão para Mucorales. A caracterização físico-química dos polímeros foi semelhante à encontrada para a quitina e quitosana comerciais utilizadas como padrões. A remoção de aproximadamente 100% de cádmio utilizando a membrana de quitosana de baixo custo sugere uma alternativa para complementar, ou até mesmo substituir, procedimentos de alto custo e baixa eficiência na descontaminação de efluentes industriais por metais pesados.

Palavras-chave: *Rhizopus arrhizus*, *Cunninghamella elegans*, quitina, quitosana, membranas, cádmio.

ABSTRACT

In recent years, many studies have shown interest regarding the production and application of the biopolymers chitin and chitosan as functional materials in medicine, pharmacy, food, biology and engineering. Specially due to their unique features of biocompatibility, biodegradability, non-toxicity, antimicrobial activity, chelating ability and the easy production. After cellulose, chitin is the second most abundant organic compound on earth, and it is naturally found as a structural element in invertebrates and fungi cell walls, mainly in order Mucorales (Zygomycetes class). The chitosan is obtained from chitin or (poly-(1-4)-2-acetamide-2-deoxy-β-D-glucosamine) throughout deacetylation. The simultaneous production of these polymers by microbiological means has shown to be advantageous when compared with the traditional extraction from crustaceans. The controlled production by fermentation have allowed the use of agroindustrial residua as low cost substrates, such as: corn steep liquor, cassava wastewater, molasses and papaya peel, which corresponds to alternative nutrient sources for the fungal cultures. Among the several applications stood out for chitosan, there is its ability to form membranes, which can be applied during the removal of heavy metals from aqueous environments. This method could be a promising for the current expensive and low practical ones. The aim of this study was to select the best growing conditions for a satisfactory production of chitin and chitosan from *Cunninghamella elegans* and *Rhizopus arrhizus* and the subsequent application of chitosan membranes to the removal cadmium from aqueous solutions. Initially, the production of biomass and biopolymers was performed using the following culture media: a specific modified medium for Mucorales, and an alternative media enriched with corn steep liquor (as nitrogen source), and media supplemented with cassava wastewater, molasses or papaya peel juice (as carbon sources). The physicochemical characterizations were performed based on: infrared, x-ray diffraction, scanning electron microscopy analysis, along with viscosity, molecular weight, thermogravimetric and calorimetric density determinations. A comparison between membranes obtained using fungal chitosan with low molecular weight and commercial chitosan with low and medium molecular weights, was performed. These chitosan membranes were applied to the removal of Cd (II) from aqueous solutions. And cadmium removal was estimated using anodic stripping voltammetry and square wave voltammetry electrochemical analysis. The results clearly showed the highest yields of biomass between 16.00 to 24.60 g/L and chitosan production between 77.78 to 77.76 mg/g were obtained using the alternative culture media for *R. arrhizus* and *C. elegans*, respectively. On the other hand, the highest chitin productions of 137.17 and 235.00 mg/g were obtained using the same fungi employing the standard culture media for Mucorales. The physicochemical characterization of the polymers was similar to the one found for commercial chitin and chitosan used as standards. A cadmium removal of almost 100% using the low cost chitosan membrane suggested an alternative as supplement, or even as replacement of the high cost and low efficiency methods used for the decontamination of industrial wastewaters polluted by heavy metals.

Keywords: *Rhizopus arrhizus*, *Cunninghamella elegans*, chitin, chitosan membranes cadmium.

1. INTRODUÇÃO GERAL

Os polímeros naturais apresentam ampla aplicabilidade em diversas áreas do conhecimento devido as suas propriedades e, sobretudo à facilidade de obtenção. Entre os polímeros com maior potencial biotecnológico destacam-se a quitina e o co-polímero quitosana (AZEVEDO *et al.*, 2007). A quitina é um polímero formado por unidades monoméricas repetitivas de β-1,4 N-acetil D-glicosamina, e está presente como elemento estrutural nos artrópodes e na parede celular de fungos em especial da classe Zygomycetes, ordem Mucorales (CAMPOS-TAKAKI, 2005; STAMFORD *et al.*, 2008; CAMPOS-TAKAKI & DIETRICH, 2009). A quitosana é um polímero de D-glicosamina derivada da desacetilação da quitina, com algumas características que favorecem sua aplicação em várias áreas destacando-se o potencial de utilização na descontaminação do meio ambiente (SHAHIDI *et al.*, 1999; SINGH *et al.*, 2008; ALBUQUERQUE *et al.*, 2009).

A fonte de quitina e quitosana mais utilizada atualmente é a carapaça de crustáceos, mas o processo de extração destes polímeros utiliza a desacetilação termoquímica que apresenta algumas desvantagens para a economia e o meio ambiente (AMORIN *et al.*, 2001; SANTOS *et al.*, 2003; ANTONINO, 2007). Por outro lado, a obtenção simultânea de quitina e quitosana a partir da biomassa de fungos da ordem Mucorales apresenta algumas vantagens em relação ao processo tradicional de obtenção pois requer menor custo de produção, ocassiona menos poluição devido a não utilização de altas temperaturas e soluções alcalinas fortes, e também possibilita o reaproveitamento de susbtratos ou resíduos agroindústrias como fonte de carbono e nitrogênio no cultivo destes microrganismos, em susbtituição aos meios de cultura sintéticos de alto custo. Muitos estudos sugerem a otimização das condições de cultivo destes microrganismos visando a maior obtenção da quitina e quitosana fúngica (AMORIN *et al.*, 2006; STAMFORD *et al.*, 2007; KLEKAYAI & SUNTORNSUK, 2010; FAI *et al.*, 2011).

Nos últimos anos, os meios de comunicação mostram um prognóstico preocupante quanto à disponibilidade da água para as futuras gerações, pois além do aumento no consumo existe o agravante da poluição doméstica e industrial. Desse modo, o processo de descontaminação por biorremediação que utiliza microrganismos livres, imobilizados ou apenas os produtos destes, entre eles a quitina e quitosana, é

considerado uma tecnologia ecologicamente correta, e alternativa para a remoção de metais pesados (FRANCO *et al.*, 2004; CARVALHO, 2006; BARRIADA *et al.*, 2008; ROSA & WALLAU, 2008). Nesse sentido, a aplicação de membranas de quitosana, em comparação com este biopolímero na forma de pó ou flocos, apresenta características mais promissoras porque possuem maior área superficial o que aumenta a velocidade e a capacidade de adsorção (DIAS *et al*., 2008). O uso desta alternativa também é mais interessante porque, ao contrário dos procedimentos habituais, apresenta baixa geração de resíduos, fácil recuperação dos metais e possibilidade de reutilização dos adsorventes (CARVALHO, 2006; ROSA & WALLAU, 2008).

Assim, é de fundamental importância a busca por alternativas que amenizem ou solucionem por completo os problemas de poluição ambiental, mas que também sejam favoráveis à economia e ao meio ambiente. A proposta deste trabalho é selecionar a melhor condição para obtenção de quitina e quitosana por via microbiológica, utilizando meios de cultura alternativos que resultem em redução dos custos finais de produção, e aplicar esta quitosana, na forma de membranas, na remoção de cádmio em soluções aquosas. Esse procedimento poderá se constituir em nova perpectiva na busca de soluções para as questões de despoluição ambiental e, consequentemente, melhoria da qualidade de vida.

2. OBJETIVOS

O presente estudo teve como objetivo geral a produção de quitina e quitosana por *Cunninghamella elegans* e *Rhizopus arrhizus* cultivados em meios de cultura com fontes alternativas de carbono e nitrogênio, para posterior aplicação de membranas de quitosana na remoção de cádmio. Para isso as seguintes etapas foram realizadas:

- Avaliar o crescimento e produção de quitina e quitosana em *C. elegans* e *R. arrhizus* a partir da otimização do meio proposto por HESSELTINE & ANDERSON (1957);
- Investigar o efeito de substratos alternativos de baixo custo na produção de biomassa, quitina e quitosana por *C. elegans* e *R. arrhizus*;
- Selecionar as melhores condições para produção de quitosana por *C. elegans* e *R. arrhizus;*
- Realizar a caracterização físico-química das quitosanas obtidas dos fungos *C. elegans* e *R. arrhizus;*
- Selecionar as melhores condições e parâmetros para produção de membranas de quitosana por *C. elegans* e *R. arrhizus;*
- Aplicar membranas produzidas a partir da quitosana de crustáceo e as membranas produzidas a partir da quitosana de fungo na remoção de cádmio dissolvido em água, comparando os resultados obtidos.

CAPÍTULO 1

REVISÃO DA LITERATURA

1. REVISÃO DA LITERATURA

1.1. Quitina e quitosana: Histórico e Ocorrência

O termo “quitina” é derivado da palavra grega “khitón”, que significa carapaça ou caixa de revestimento. Esse significado justifica-se pelo fato de a quitina apresentar função estrutural na natureza porque constitui a parede celular dos fungos, a carapaça dos crustáceos e o exoesqueleto dos artrópodes oferecendo revestimento e proteção para estes organismos (SANTOS, 2004).

Em 1811, a quitina foi descoberta pelo professor frânces Henri Braconnot durante um trabalho com fungos, sendo que neste momento foi denominada como fungina (SANTOS, 2004; CAMPANA-FILHO *et al.*, 2007; BHATNAGAR *et al.*, 2009). Em seu trabalho, Braconnot afirma que esses microrganismos continham uma nova susbtância que, em sua opinião, era completamente diferente da encontrada nas madeiras (KNORR, 1991). Por muitos anos, houve controvérsias se esse material encontrado em fungos poderia ser considerado um material novo porque o mesmo apresentava muitas semelhanças estruturais com a celulose. Apenas em 1823, esse material foi denominado como quitina pelo pesquisador Odier que isolou este biopolímero de insetos. Embora não tenha detectado nitrogênio na composição, Odier foi o primeiro a relatar a semelhança entre as substâncias suportes presente nos insetos e nos tecidos vegetais. Posteriormente, Odier também observou a presença de quitina na carapaça de caranguejo e sugeriu que ela seria o material básico na formação do exoesqueleto de todos os insetos e possivelmente dos aracnídeos. Entretanto, só em 1843, Payen detectou a presença de nitrogênio na quitina (ROBERTS, 1992).

Em torno de 1823, os pesquisadores Odier e Children relataram que isolaram a quitina através de múltiplos tratamentos com soluções de hidróxido de potássio concentrado. Entretanto, certamente foi isolado quitosana ao invés de quitina, uma vez que, a quitina em meio alcalino concentrado pode sofrer desacetilação. A quitosana foi descrita pela primeira vez em 1859 por Rouget. Essa denominação foi proposta por Hoppe-Seyler porque a quitosana possui quantidade de nitrogênio igual à quitina original (ROBERTS, 1992).

A quitosana foi produzida industrialmente por volta de 1971, no Japão e EUA, países líderes do mercado mundial. Por volta de 1993 o Brasil também passou a

comercializar quitina e quitosana em larga escala. Duas empresas brasileiras tem se destacado na produção e comercialização destes biopolímeros como Polymar Indústria Comércio Exportação e Importação, situada em Fortaleza-CE e Kito Química Fina, localizada em Palhoça-SC (SANTOS, 2004).

Após a celulose, a quitina é considerada o biopolímero mais abundante e largamente distribuído na natureza, estando presente como elemento estrutural em animais invertebrados, em algas diatomáceas, na maioria dos artrópodes e na parede celular de fungos das classes Ascomycetos, Basidiomycetos, Deuteromycetos, e principalmente Zygomycetes, ordem Mucorales (FRANCO *et al.*, 2005; CAMPANA-FILHO *et al.*, 2007; STAMFORD *et al.*, 2008). Lulas, caranguejos, camarões, lagostas e ostras apresentam em sua constituição 10 a 15 % de peso seco de quitina, já fungos da ordem Mucorales possuem na constituição da sua parede celular, além da quitina, o derivado desacetilado deste biopolímero denominado quitosana (22 a 44 %) (Andrade *et al.*, 2003). A quitosana, forma deacetilada da quitina, é encontrada naturalmente na parede celular de fungos, principalmente aqueles da classe Zygomycetes (STAMFORD *et al.*, 2007; FAI *et al.*, 2008), que podem apresentar até 50% deste polímero na sua estrutura (ZAMANI *et al.*, 2010).

1.2. Características físico-químicas da quitina e quitosana

A quitina é um biopolímero linear formado por unidades repetitivas de β- 1,4 N-acetilglicosamina (ANDRADE *et al.*, 2003) ou também descrito como unidades repetitivas de 2-acetamido-2-desoxi-D-glicopiranose com 5 a 10% de unidades 2-amino-2-desoxi-D-glicopiranose unidas por ligação glicosídica do tipo β(1→4) (CAMPANA-FILHO *et al.*, 2007) (Figura 1).

Esse polissacarídeo natural possui uma fase cristalina organizada, como comprovada por difração de raios-X. A estrutura da quitina ocorre em três diferentes formas denominadas α, β e γ (Figura 2), as quais diferem no arranjo de suas cadeias nas regiões cristalinas. As três estruturas polimórficas estão possivelmente relacionadas a diferentes funções no organismo. A forma α é encontrada onde é necessária uma extrema dureza (resistência), como em cutículas de artrópodes e frequentemente, é associada com proteínas e/ou materiais inorgânicos. As formas β e γ proporcionam flexibilidade e dureza. A forma dominante, α - quitina é mais estável que as forma β e γ,

entretanto estas últimas podem ser convertidas à forma α por tratamentos adequados (ANTONINO, 2007).

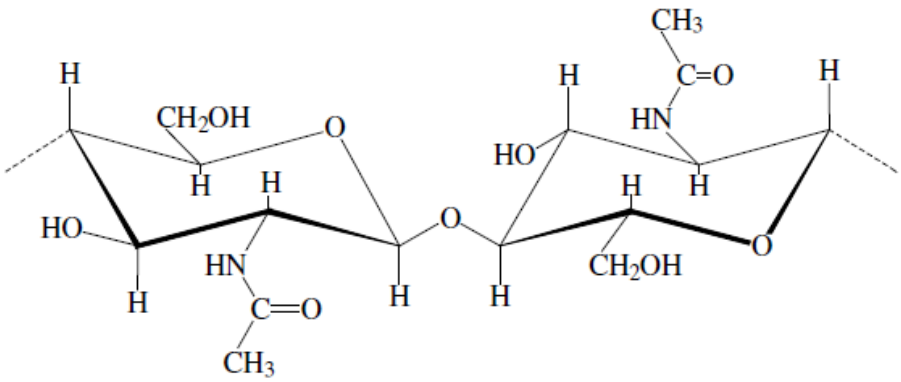

Figura 1. Estrutura da quitina. Fonte: DUTTA *et al.*, 2004.

As cadeias polimórficas de quitina correspondem a diferentes arranjos no estado sólido, decorrentes de disposições distintas das cadeias do polímero nas lamelas ou folhas que constituem os domínios cristalinos (Figura 2). A α-quitina corresponde a um empacotamento denso resultante da disposição antiparalela das cadeias poliméricas em diferentes lamelas ou folhas, o que favorece a existência de numerosas ligações hidrogênio inter- e intra-cadeias da mesma lamela e de lamelas vizinhas. Na β-quitina as cadeias pertencentes a diferentes lamelas dispõem-se paralelamente, o que dificulta o estabelecimento de ligações hidrogênio intermoleculares envolvendo cadeias de lamelas adjacentes e resulta em material menos densamente empacotado. Em γ-quitina parece ocorrer uma combinação dos dois arranjos presentes na α-quitina e β-quitina pois as cadeias de duas lamelas em disposição paralela são intercaladas por lamela em que as cadeias se dispõem antiparalelamente. Essa estrutura é a menos estudada e a literatura sugere que pode ser uma distorção das duas estruturas anteriores (CAMPANA-FILHO *et al.*, 2007).

Figura 2. Estruturas polimórficas da quitina. Fonte: ANTONINO, 2007.

A quitina é insolúvel em meio aquoso e na maioria dos solventes orgânicos, tem baixa reatividade química (LARANJEIRA & FÁVERE, 2009), é rígida, inelástica e de coloração branca (DUTTA *et al.*, 2004).

A quitosana é um polímero linear de β-1,4-D-glicosamina, ligado por resíduos de N-acetil-D-glicosamina (Figura 3), derivada da desacetilização da quitina, com uma configuração tridimensional helicoidal estabilizada por ligações de hidrogênio intramolecular (KAS, 1997). Esse polímero é solúvel em soluções aquosas diluídas de ácidos orgânicos e inorgânicos e dependendo do processo de desacetilação, da perda do grupo acetil, ela pode apresentar vários graus de acetilação (ANDRADE *et al.*, 2003; SYNOWIECKI & AL-KHATEEB, 2003). Através de estudos de raios-X, pode-se observar que a cristalinidade da quitina e da quitosana depende do grau de acetilação e do processo de extração utilizado. Em relação a estrutura espacial da quitosana, ela pode se apresentar na forma hidratada, anidra, como complexos ou sais de quitosana (ANTONINO, 2007).

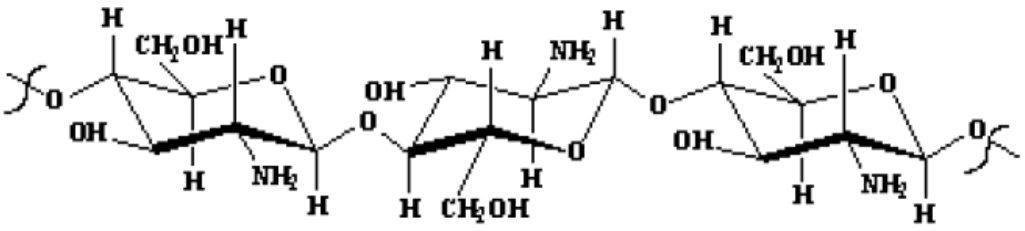

Figura 3. Estrutura da quitosana. Fonte: ANTONINO *et al.*, 2007.

1.3. Propriedades e formas de obtenção da quitosana

O polímero quitosana apresenta excelente biocompatibilidade; quase nenhuma toxicidade ao ser humano e animais; alta bioatividade; biodegradabilidade; reatividade do grupo amino desacetilado; permeabilidade seletiva; ação polieletrolítica; atividade antimicrobiana; habilidade em formar gel e filme; habilidade de quelação e capacidade adsortiva (SYNOWIECKI & AL-KHATEEB, 2003; THARANATHAN & KITTUR, 2003; SINGH *et al.*, 2008).

Devido as suas propriedades a quitosana vem sendo utilizada na preservação de alimentos (SHAHIDI *et al.*, 1999; FRANCO *et al.*, 2004; DUTTA *et al.*, 2004); na indústria farmacêutica; na biomedicina devido a sua capacidade antibacteriana e antifúngica contra microrganismos patógenos do homem e também à sua capacidade de inibir a proliferação de células cancerígenas (KUMAR *et al*., 2012); no clareamento de sucos; na fabricação de embalagens (SHAHIDI *et al.*, 1999; FRANCO *et al.*, 2004); na biorremediação por adsorção de corantes (PIRILLO *et al*., 2009; NESIC *et al*., 2012), metais pesados, e outros resíduos poluentes no meio ambiente; na agricultura (BERGER *et al*., 2011a), como antifúngico (SAJOMSANG *et al*., 2012; YANG *et al*., 2012), na preservação de frutas, legumes e sementes contra a deteriorização por microrganismos, para estimular o sistema imune da planta, proteger a planta contra o ataque de patógenos (GHAOUTH *et al.*, 1992; BADAWY & RABEA, 2008; HERNÁNDEZ-LAUZARDO *et al.*, 2008), favorecer o seu crescimento e consequentemente aumentar a produção vegetal (OTHA *et al.*,2000; RABEA *et al.*,2003; BOONLERTNIRUM *et al.* 2008; ABDEL-MAWGOUD *et al.*, 2010). Entretanto, a eficiência da quitosana depende da sua massa molecular, grau de desacetilação, cristalinidade, solubilidade e de seus produtos derivados (CHATTERJEE *et al.*, 2005).

Atualmente, a fonte tradicional de quitina e quitosana explorada a nível comercial tem sido a carapaça de caranguejos e cascas de camarão, oriundas de resíduos da indústria pesqueira que processam estes crustáceos (FAI *et al.*, 2008). Por outro lado, a alternativa de obtenção de quitina e quitosana extraídas da biomassa de fungos ainda permanece pouco utilizada pela indústria (ANDRADE *et al.*, 2003).

A quitosana pode ser obtida através do processo de desacetilação termoquímica responsável pela remoção do grupo acetamido da quitina extraída da carapaça de crustáceos. Devido à alta cristalinidade e insolubilidade da quitina, este processo utiliza etapas de desmineralização e desacetilação com ácidos e bases fortes que podem causar

parcial desacetilação da quitina e hidrólise do polímero devido ao acesso limitado aos seus sítios reativos, resultando em produtos finais com propriedades inconsistentes, ou seja, produtos heterogêneos com diferentes massas moleculares e graus de desacetilação; além disso, essas soluções alcalinas fortes são fontes de poluição para o meio ambiente (CAMPANA-FILHO *et al.*, 2007) e os resíduos de proteínas no produto final podem causar reações alérgicas no ser humano (FRANCO, 2000; AMORIN *et al.*, 2001). Desse modo, a natureza do produto obtido depende do processo químico utilizado (KAFETZOPOULOS *et al.*, 1993; ANDRADE *et al.*, 2003; CAI *et al.*, 2006).

O método de desacetilação enzimática da quitina tem sido estudado para obtenção do biolpolímero quitosana com características padronizadas e ideais para aplicação com determinada finalidade (KAFETZOPOULOS *et al.*, 1993; CHATTERJEE *et al.*, 2005). Esse processo desacetilação da quitina, ao contrário da desacetilação termoquímica, ocorre naturalmente em fungos da ordem Mucorales com a participação de duas enzimas, a quitina desacetilase e a quitina sintetase (SILVA *et al.*, 2007). A desacetilação enzimática utiliza condições mais brandas que supera a maioria das desvantagens encontradas no processo de desacetilação alcalino. Muitos trabalhos tem sido desenvolvidos sobre as características e atividade da quitina desacetilase e sua função na biossíntese de quitosana na parede celular do fungo, visando o aumento da produção microbiológica deste polímero (CAI *et al.*, 2006; PAREEK *et al.*, 2011a; PAREEK *et al.*, 2011b; SURESH *et al.*, 2011).

A produção de quitina e quitosana a partir da biomassa micelial de fungos da ordem Mucorales pode ser uma alternativa por apresentar algumas vantagens como: extração simultânea de quitina e quitosana, ser um processo fácil e economicamente viável, não apresentar contaminação por proteínas, independência dos fatores de sazonalidade, produção em larga escala, com fácil controle do pH e da concentração de nutrientes no meio fermentativo (CAMPOS-TAKAKI, 1984; CAMPOS-TAKAKI, 2005; AMORIN *et al.*, 2006; FRANCO *et al.*, 2005; STAMFORD, *et al.*, 2007).

1.4. Produção microbiológica de quitina e quitosana

O conteúdo de quitina presente na parede celular dos fungos vária entre as diferentes espécies (Tabela 1). Muitos estudos tem sido realizados para verificar a possibilidade de utilização da biomassa de fungos, principalmente da ordem Mucorales, classe Zygomycetes, como fonte alternativa de quitina e quitosana. Muitos destes

trabalhos testam modelos simples para verificar a produção de quitina e quitosana, ou seja, experimentos que adotam a abordagem univariada, estudando apenas uma variável de cada vez durante o processo fermentativo, como por exemplo, tempo de cultivo, agitação, pH, temperatura e concentração de determinado nutriente. Por outro lado, nos últimos anos o interesse por métodos científicos que diminuam os números de ensaios e aumentem a precisão dos resultados tem sido cada vez maior. Desse modo, abordagem multivariada, ou a seja, a utilização do planejamento fatorial, permite a observação dos efeitos sinérgicos entre as variáveis independentes analisadas, uma vez que, todas as variáveis são consideradas simultaneamente, resultando ao final na obtenção da condição otimizada (RODRIGUES & IEMMA, 2005).

O crescimento de várias espécies de fungos, principalmente os representantes da classe Zygomycetes, ordem Mucorales é avaliado em vários trabalhos que visam maior produção de biomassa e, consequentemente, incremento nos rendimentos obtidos de quitina e quitosana microbiológica. Para isso, muitos meios de cultura sintéticos e alternativos tem sido testados como mostra a tabela 1. Entre esses meios alternativos estão resíduos de indústrias, e produtos ou resíduos agroindustriais como jacatupé (STAMFORD *et al*., 2007; FAI *et al*., 2011); milhocina (LINS *et al*., 2010; BERGER *et al.*, 2011b; CARDOSO *et al*., 2012) e manipueira (BERGER et al., 2011b).

Tabela 1. Substratos, biomassa, quitina, quitosana e grau de desacetilação obtidos a partir de vários estudos.

Microrganismo	**Substrato**	**Biomassa**	**Quitina**	**Quitosana**	**Grau de Desacetilação**	**Referência**
Cunninghamella elegans	Hesseltine et Anderson (1957) modificado	10,41 g/L	288 mg/g (28,8 %)	NA	NA	Andrade *et al.* 2000
Mucor javanicus	Hesseltine et Anderson (1957) modificado	8,71 g/L	23,9% (239 mg/g)	NA	NA	Andrade *et al.*, 2003
Mucor rouxii	MSM, YPG, PDB	NA	NA	MSM (0,61 g/L) PDB (0,51 g/L) YPG (0,56 g/L)	PDB (89,9 %), MSM (87,2%) e YPG (82,2%),	Chatterjee *et al.*, 2005
Cunninghamella elegans	Meio a base de jacatupé (*Pachyrhizus erosus* L. Urban)	24,3 g/L	440mg/g	66mg/g	6,2% (quitina), 85% (quitosana)	Stamford *et al.* 2007
Rhizopus arrhizus	Meio Hesseltine et Anderson (1957)	NA	92mg/g	13mg/g	78 %	Cardoso *et al.* (2010)
Rhizopus arrhizus	Meio Andrade *et al.*(2000)	NA	94mg/g	14mg/g	95%	Cardoso *et al.* (2010)
Rhizopus arrhizus	Milhocina 4%	13,1 g/L	30,4 mg/g	12,85 mg/g	95%	Lins *et al.* (2010)
Rhizomucor miehei	Sabouraod dextrose (SDB)	4,1 (g^{-1})	NA	13,67% (136,7 mg/g)	98,6%	Tajdini *et al.* (2010)
Mucor racemosus	Sabouraod dextrose (SDB)	3,8 (g^{-1})	NA	11,72% 117 mg/g)	97,1%	Tajdini *et al.* (2010)
Rhizopus arrhizus	Milhocina e manipueira	8,80 g/L	54,38 mg/g	20,51 mg/g	82% (quitosana), 25% (quitina)	Berger *et al.* (2011b)
Mucor circinelloides	Yam bean (jacatupé)	20,7 g/L	500 mg/g	64 mg/g	83% DD	Fai *et al.*, 2011
Rhizopus arrhizus	Milhocina e mel de abelha	20,6 g/L	NA	29,3 mg/g	86%	Cardoso *et al.* (2012)

*MSM: Molasses salt medium, YPG: Yeast peptone glucose medium, PDB: Potato dextrose broth

1.4.1. Produção de quitina e quitosana microbiológica com substratos sintéticos

ANDRADE *et al.*, 2000 e ANDRADE *et al.*, 2003 observaram o crescimento e produção de quitina por *Cunninghamella elegans* e *Mucor javanicus* (Zygomycetes), respectivamente, através de um planejameto fatorial 2^4, variando o tempo, e as concentrações de D-glicose, L-asparagina e tiamina dos meios de cultivo. Em ambos os experimentos conduzidos com *Cunninghamella elegans* e *Mucor javanicus,* não foi observada nenhuma interação significativa entre as variáveis, ou seja, os efeitos principais podem ser interpretados separadamente. O rendimento de quitina aumentou quando qualquer uma das quatro variáveis testadas foi alterada do menor para o maior nível. Desse modo, foi observado nestes experimentos que a condição que mais proporcionou o crescimento de *M. javanicus* (8,71 g/L) e *C. elegans* (10,41 g/L) e aumentou o rendimento da quitina de 23,9% e 28,8% extraídas de *M. javanicus* e *C. elegans*, respectivamente, foi a condição que apresentou todas as variáveis independentes no maior nível. *M. javanicus* e *C. elegans* podem ser consideradas promissoras fontes de quitina sendo alternativas para substituir a extração deste biopolímero por resíduos da indústria pesqueira.

CHATTERJEE *et al.*(2005) realizou experimento de abordagem univariada, estudando apenas uma variável de cada vez durante o processo fermentativo, neste caso o meio de cultura. Outra espécie do gênero *Mucor, M. rouxii,* também foi avaliada quanto ao seu crescimento e potencial para produção de quitosana em três diferentes meios de cultivo, sendo: meio salino com melaço (MSM); meio com extrato de levedura, peptona e glicose (YPG); e meio com batata, dextrose e ágar (BDA). O meio MSM para *M. rouxii* foi considerado mais promissor por ser mais viável economicamente e o rendimento e a qualidade da quitosana são superiores em relação à quitosana dos outros dois meios. Assim, segundo os autores o meio de cultura e as condições de fermentação podem ser manipulados para melhor produção e qualidade físico-química da quitosana.

LOGESH AR *et al.* (2012) sugerem o fungo *Aspergillus niger* como um promissor microrganismo produtor de quitosana. Os autores observaram que *A. niger* cultivado em meio BD (batata dextrose) sem e com diferentes concentrações de glicose de 2 a 12%, e em 12 dias apresentou maior rendimento de quitosana equivalemte a 1,34 g/L. Entretanto, maior produção desse biopolímero (1,93 g/L) foi obtida com esse fungo cultivado em meio BD acrescido de 10% glicose. Os autores colocam que a presença de

glicose no meio de cultivo influencia a produção de quitosana e que o crescimento desse fungo e a produção de quitosana foram favorecidos com o aumento da concentração de glicose no meio. LOGESH AR *et al.* (2012) sugerem que as condições de cultivo influenciam a produção desse biopolímero.

1.4.2. Produção de quitina e quitosana microbiológica com susbtratos alternativos

Apesar da utilização de meios de cultura sintéticos para fermentação de fungos e consequente produção de quitina e quitosana como demonstardo por AMORIN *et al.* (2001); POCHANAVANICH & SUNTORNSUK (2002); FRANCO *et al.* (2005); SILVA *et al.* (2006); SILVA *et al.* (2007); CARDOSO *et al.* (2010), a substituição destes meios por substratos alternativos de baixo custo mostram-se favoráveis para uma futura produção em larga escala destes biopolímeros (AMORIN *et al.*, 2006; STAMFORD *et al.*, 2007; KLEKAYAI & SUNTORNSUK, 2010; FAI *et al.*, 2011).

Um dos trabalhos envolvendo meios economicamente viáveis foi realizado por FAI *et al.*, (2011) com a utilização de jacatupé (*Pachyrhizus erosus* L. Urban) como substrato alternativo para o crescimento e produção de quitina e quitosana por *Mucor circinelloides.* Os autores observaram rápido crescimento do fungo em 48 h, com maior produção de biomassa (20,7 g/L) após 72 h de cultivo. Após 48 h de cultivo obteve-se a maior produção de quitosana (64 mg/g ou 6,4%) e após 72 h para a quitina (500 mg/g or 50%). A maior produção de quitosana em 48h de crescimento de *Mucor circinelloides* sugeriu que durante o início do crescimento a quitina é menos cristalina e, consequentemente, mais susceptível à ação da quitina desacetilase. Durante essas 48h o pH entre 5-4 do meio de cultivo provavelmente favoreceu a atividade desta enzima, assim como já confirmado por AMORIN *et al.* (2005) que considerou o pH ótimo de 4,5 para a atividade da quitina desacetilase de Zygomycetes.

CHATERJJE *et al.* (2005) consideraram que a composição do meio de cultura influencia na produção de quitosana, por outro lado, FAI *et al.* 2011 propõe que o crescimento de microrganismos é fortemente dependente das condições de cultivo, inclusive do tempo de incubação. De acordo com esses autores, a influencia dessas variáveis na produção de quitosana também foram observadas por NADARAJAH *et al.* (2001), POCHANAVANICH & SUNTORNSUK (2002), AMORIN *et al.* (2006), e STAMFORD *et al.* (2007).

A produção de quitosana também foi observada em fermentação com substrato sólido a base de batata doce (*Manihot esculenta*) e solução mineral suplementada com diferentes quantidades de uréia. O fungo *Gongronella butleri* apresentou aumento na produção de biomassa de 40 g/kg de batata doce suplementado com 7,2 g de ureia porém, quantidades maiores de uréia afetaram negativamente o crescimento desse fungo. O mais alto rendimento de quitosana (11,4g/100g de micélio) foi obtido na presença de e 14,3 g de uréia/kg de batata doce, mas 7,2 g de uréia/kg de batata doce também apresentou rendimento de quitosana similar, assim como, o grau de acetilação de 11% em ambas as condições. A quantidade de uréia adicionada influenciou no pH do meio de cultura e, consequentemente, no crescimento de *G. butleri*, bem como no rendimento e massa molecular da quitosana (NWE *et al.*, 2004).

Outros meios alternativos de baixo custo também foram testados por vários autores (AMORIN *et al.*, 2006; STAMFORD *et al.*, 2007; ZAMANI *et al.*, 2007; KLEEKAYAI & SUNTORNSUK, 2010; LINS *et al.*, 2010; BERGER *et al.*, 2011b; BERGER *et al.*, 2012). Entre os substratos utilizados para composição desses meios, estão os resíduos agroindústrias como, por exemplo, a milhocina, resíduo da indústria de processamento do milho que possui grande quantidade de aminoácido, vitaminas e minerais (CARDOSO, 2007); e a manipueira, líquido amarelado obtido da mandioca durante o seu processamento nas fábricas de farinha de mandioca, o qual é rico em potássio, nitrogênio, magnésio, fósforo, cálcio e enxofre (PONTE, 2006).

BERGER *et al.* (2011b), através da análise estatística multivariada, realizou planejamento fatorial 2^2 para observar a influência das concentrações de milhocina e manipueira no crescimento e produção de quitina e quitosana de *Rhizopus arrhizus* UCP 402. A maior produção de biomassa (8,80 g/L), quitina (54,38 mg/g) e quitosana (20,51 mg/g) foram obtidos na condição experimental que apresentava maiores concentrações de milhocina e menores concentrações de manipueira. Através desses resultados os autores observaram que, estatisticamente, a milhocina influenciou positivamente no aumento da produção das variáveis dependentes (biomassa, quitina e quitosana) e a manipueira mostrou efeito contrário. Desse modo, as concentrções do meio de cultivo podem ser manipuladas para a maior da produção deste biopolímeros.

Trabalho semelhante ao de BERGER *et al.* (2011b) foi realizado por CARDOSO *et al.* (2012) com o mesmo microrganismo, *Rhizopus arrhizus* UCP 402, no entanto, foi utilizado milhocina e mel de abelha como fontes agroindustrias de carbono e nitrogênio, e como variáveis independentes em planejamento factorial 2^2. Os maiores

rendimentos de biomassa (20,6 g/L) e quitosana (29,3 mg/g) foram obtidos nas condições com milhocina 8% e mel de abelha 6%, e com milhocina 6% e mel de abelha 13,24%, respectivamente. E através da análise estatística foi observado que a interação entre milhocina e mel de abelha favoreceu a maior produção de quitosana. Desse modo, a realização de experimentos com abordagem multivariada pode facilitar a obtenção do meio de cultura otimizado para produção destes biopolímeros.

TAJDINI *et al.* (2010) avaliaram a produção de quitosana para outros dois fungos, *Rhizomucor miehei* e *Mucor racemosus* da ordem Mucorales, classe Zygomycetes. *Rhizomucor miehei* apresentou maior produção de biomassa e quitosana (13,67%). As análises de espectro de infravermelho das quitosanas dos dois fungos foram semelhantes aos da quitosana de crustáceo e de outras quitosanas de fungos de outros trabalhos. Além da extração de quitosana, também foi realizada a purificação e a desacetilação da quitosana extraída utilizando NaOH e $NaBH_4$. A finalidade da purificação e desacetilação foi alcançada, uma vez que, as quitosanas dos dois fungos tiveram seu grau de desacetilação aumentado com este processo de 80,6% para 98,6% de desacetilação e 84,4% para 97,1% de desacetilação para *R. miehei* e *M. racemosus*, respectivamente.

De acordo com TAJDINI *et al.* (2010) o método de extração de quitosana fúngica simples e rápido é mais viável que o método habitualmente utilizado para extração de quitosana de crustáceo, considerado dispendioso e demorado; e que os fungos podem ser considerados uma fonte alternativa e promissora de quitosana. Além disso, é possível observar que a realização de um prático procedimento de purificação e desacetilação desse biopolímero, após sua extração, proporciona maior grau de desacetilação, o que pode favorecer suas futuras aplicações em várias áreas; como relatado por CHATTERJEE *et al.* (2005) que considera a eficiência da quitosana depende de várias de suas próprias características, inclusive do seu grau de desacetilação.

O método de extração de quitina e quitosana fúngica também pode influenciar no rendimento final destes biopolímeros como observado no trabalho desenvolvido por LINS *et al.* (2010). Esses autores observaram que entre os fungos *Mucor javanicus*, *Mucor circinelloides*, *Rhizopus oryzae* e *Rhizopus arrhizus*, estes últimos mostraram maiores valores de biomassa (8,0 e 13,1 g/L, respectivamente) em meio de cultura com milhocina 4%. Além disso, o método de extração de quitina e quitosana proposto por HU *et al.* (2004) proporcionou maior rendimento de quitosana, já o método descrito por

ZAMANI *et al.* (2007) extraiu, da maioria das biomassas, elevada quantidade de quitina.

1.5. Metal pesado no meio ambiente

O meio ambiente cada vez mais tem sido exposto à poluição ocasionada pelo crescimento industrial e populacional. Um exemplo é a exploração desordenada dos recursos hídricos por atividades humanas que poluem a água com pesticidas, metais pesados, corantes, derivados do petróleo, esgotos domésticos e resíduos indústriais (MORAES, 2009).

Os metais pesados são elementos traços presentes na litosfera em concentrações menores do que 0,1%. São designados metais pesados aqueles elementos que apresentam peso específico maior que 6 g/ cm^3 ou que possuem número atômico maior que 20. Alguns dos metais mais tóxicos são mercúrio (Hg), chumbo (Pb), cádmio (Cd), cobre (Cu), níquel (Ni) e cobalto (Co). O que distingue os metais pesados dos outros elementos tóxicos são sua não biodegradabilidade e sua toxicidade que depende de suas propriedades físicas e químicas, como por exemplo o estado de oxidação dos metais que determina a mobilidade, biodisponibilidade e toxicidades destes elementos (COSTA *et al.*, 2006).

Os metais pesados são necessários na manutenção à saúde dos seres vivos em concentrações mínimas (são denominados oligoelementos ou micronutrientes). Entretanto, são considerados poluentes conservativos porque não são degradados por microrganismos ou por qualquer outro processo, o que facilita seu acúmulo desordenado no meio ambiente (IGWE & ABIA, 2006**).** Quantidades em excesso destes elementos (normalmente acima de dez vezes a quantidade não tóxica) provocam efeitos deletérios na natureza e devido a toxicidade, tendência a acumulação, a solubilidade e mobilidade, eles podem interferir ao longo da cadeia alimentar atingindo, consequentemente, a saúde humana (TOREM *et al.*, 2005).

Entre os 59 elementos considerados na literatura como metais pesados, nove (Ag, Cd, Cu, Hg, Ni, Pb, Sb, Sn, Zn) tem recebido atenção especial devido ao seu grau de toxicidade e elevadas taxas de liberação dos mesmos no meio ambiente (TOREM *et al.*, 2005). Os metais pesados podem provocar sérios danos a saúde humana como, por exemplo: desidratação, problemas estomacais, náuseas, desmaios, destruição do sistema

nervoso, irritação no pulmão e nos olhos, erupção na pele, dor abdominal, insuficiência pulmonar e danos ao fígado (HAIDER & PARK, 2009).

Os procedimentos habituais de remoção desses metais são processos físico-químicos de precipitação, troca iônica, floculação, eletrólise, cristalização, e extração por solventes. Contudo, essas técnicas convencionais são inviáveis para a descontaminação de grandes volumes de efluentes contendo metais pesado em baixas concentrações, devido à sua reduzida eficiência operacional e aos elevados custos de extração; e também estas técnicas podem contribuir para a formação de novos contaminantes prejudiciais ao meio ambiente, e consequentemente, à saúde humana (FRANCO *et al.*, 2000; TOREM *et al.*, 2005; QUINTELAS, 2007; QUINTELAS *et al.*, 2008).

Diante da crescente geração de efluentes que poluem o meio ambiente, torna-se imprescindível a realização de estudos visando o aperfeiçoamento e a elaboração de novas técnicas de despoluição. Em se tratando da poluição provocada por metais pesados em águas residuais, a técnica de adsorção é geralmente considerada a mais apropriada porque consegue remover poluentes orgânicos e inorgânicos. O adsorvente mais utilizado é o carvão ativado mas, sua aplicação em grande escala nos tratamentos de adsorção de poluentes apresenta alto custo sendo inviável economicamente; assim, é importante a busca por adsorventes de baixo custo (FRANCO *et al.*, 2004; BHATNAGAR & SILLANPAA, 2009).

Os métodos biológicos baseados no uso de microrganismos têm sido desenvolvidos e considerados uma alternativa promissora para a remoção e recuperação de metais pesados (FRANCO *et al.*, 2004,COLLA *et al.*, 2012). Um exemplo é a técnica de biorremediação através da adsorção dos metais pesados com biosorventes que, ao contrário dos outros procedimentos, apresenta baixa geração de resíduos, fácil recuperação dos metais e a possibilidade de reutilização do adsorvente. A biorremediação utiliza microrganismos livres, imobilizados, como biomassas de bactérias (WIERBA, 2010), de fungos (FRANCO et al., 2004; COLLA *et al.*, 2012), ou apenas os produtos obtidos a partir destes, como os biopolímeros.

Desse modo, a busca por tecnologias ecologicamente corretas, menos agressivas ao ambiente tem se mostrado uma alternativa promissora, como por exemplo, as pesquisas com polímeros naturais (CARVALHO, 2006; ROSA & WALLAU, 2008). Entre esses destaca-se a quitosana que apresenta capacidade de adsorver metais pesados, como foi comentado anteriormente.

1.6. Aplicação de várias formas de quitosana na biorremediação de metal pesado

Atualmente, muitas pesquisas vêm sugerindo que a quitosana pode ser material alternativo para aplicações na biorremediação de metais pesados na água, por apresentar a habilidade de quelar íons devido atração eletrostática dos ânions pelos grupos aminos protonados em solução ácida (GUIBAL, 2004). Os grupos amino também possuem pares de elétrons disponíveis para coordenação dos íons metálicos, atuando como uma base de Lewis (BENGUELLA & BENAISSA, 2002). Além disso, a quitina e a quitosana possuem grupos hidroxilas livres nas suas moléculas também disponíveis para interagir com os metais pesados. Portanto, o grau de desacetilação e a massa molecular são características indicadoras da quantidade de grupos amina na molécula desses polímeros, o que interfere diretamente nas suas capacidades para maior remoção de íons metálicos (BATISTA, 2011). Ao contrário da quitina, a quitosana apresenta superior capacidade para adsorção de metais pesados devido ao seu maior conteúdo de gupos amino (WU *et al*., 2010).

O complexo quitosana-metal pesado pode ser descrito como dois ou mais grupos amina ligando-se ao mesmo íon metálico, modelo conhecido como modelo de ponte. De acordo com este modelo de ponte, a complexação intra ou intermolecular pode ocorrer entre o íon metálico e o grupo amina de cadeias iguais ou diferentes. Outro experimento sugere que apenas um grupo amina está envolvido na ligação e o íon metálico está ligado ao grupo amina como um pendente, este é o modelo pendente (EMARA *et al.*, 2011).

FRANCO *et al.* (2004) observou que a quitosana extraída de *Cunninghamella elegans* apresentou alta capacidade para adsorção de cobre e que este processo é influenciado pelo pH da solução metálica, com o melhor resultado observado em condições com pH 4. Além da utilização da quitosana pura no processo de adosrção de íons metálicos, alguns trabalhos procuram otimizar esta capacidade através de diferentes modos de aplicação deste polímero: quimicamente modificado (CÁRDENAS *et al*., 2001; TABAKCI & YILMAZ, 2008; BHATNAGAR & SILLANPAA, 2009; EMARA *et al.*, 2011; WU *et al*., 2010); aderido à superfície de esferas de vidro (LIU *et al*., 2002); na forma de nanotubos (SALAM *et al.*, 2011); microesferas (ZHOU *et al.*, 2009); nanofibras (DESAI *et al.*, 2009) e membranas (BARONI et al., 2008).

Algumas modificações químicas da quitosana podem ocasionar, ou não, menor capacidade de adsorção de íons metálicos, mas também podem proporcionar maior durabilidade do polímero em soluções ácidas (pH menor que 2). Muitas vezes isso ocorre porque certos grupos $–NH_2$ da cadeia polímerica estão envolvidos nestas modificações, e ficam impossibilitados de interagir com os íons metálicos. Entretanto, isso pode ser revertido através do uso de agentes que apresentam grupos com a mesma capacidade de adsorção, como: hidroxila (OH^-), carboxila (-COOH) e amino ($-NH_2$). A interação da quitosana com gluteraldeído (presença de $-NH_2$) resultou na maior adsorção de Cd^{2+} em comparação com a quitosana sem modificações, como observado no estudo desenvolvido por WU *et al*., 2010. Outra modificação da quitosana com oligômeros de Calix [n] arenos formando calix [4] areno-base de quitosana (C[4]BCP) apresentou maior rendimento de sorção dos cátions metálicos (Co^{2+}, Ni^{2+}, Cu^{2+}, Cd^{2+} , Hg^{2+} , Pb^{2+}) e estabilidade para meios ácidos (TABAKCI & YILMAZ, 2008).

Os nanotubos de carbono modificados com quitosana também podem adsorver e remover metais pesados do ambiente aquoso, como por exemplo, íons de cobre, zinco, cádmio, e níquel presentes em solução aquosa. Esse nanocomposto apresentam características favoráveis para adsorção de metais como aumento da área superficial e maior quantidade de sítios ativos para ligação destes íons (EMARA *et al.*, 2011).

A quitosana na forma de nanofibras também são de considerável interesse nos processos de adsorção de íons metálicos (WANNGAH *et al.*, 2002) porque apresentam características desejáveis, como grande área de superfície específica, alta porosidade (MIN *et al.*, 2004; SHELMA *et al.*, 2008), e capacidade para serem recuperadas após o processo de remoção destes poluentes (HAIDER & PARK, 2009; JAYAKUMAR *et al.*, 2010; KUO, TSAI, 2010). Além disso, a aplicação de nanofibras de quitosana não acarreta perda das propriedades originais deste biopolímero como biocompatibilidade, hidrofilicidade, bioatividade, e não toxicidade (HAIDER & PARK, 2009). As nanofibras de quitosana também são mais eficientes na remoção de microrganismos patogênicos e partículas contaminantes do ar e água, devido a sua maior área superficial, quando comparada com as nanofibras de polímeros sintéticos (DESAI *et al*., 2009).

As microesferas de quitosana também são consideradas alternativas mais eficientes para aplicação na adsorção de metais pesados, devido a sua grande área superficial de aproximadamente 100 vezes maior do que a quitosana em flocos. Elas também possuem cinética de adsorção mais rápida e maior facilidade de manuseio e

operação (DIAS *et al.*, 2008; LEITE *et al.*, 2005). Essas microesferas ainda podem ser quimicamente modificadas com tioureia, apresentando eficiência na biorremediação de Hg^{2+}, Cu^{2+}e Ni^{2+}. (ZHOU *et al.*, 2009), e aquelas produzidas com N-carboximetil-quitosana foram eficientes na adsorção de Cd^{2+} (LEITE *et al.*, 2005).

Além da utilização direta das membranas de quitosana em processos de remoção de metais em água, estes materias também podem ser produzidas com a incorporação de íons metálicos como cobre, zinco e magnésio, sem alteração visível dos grupos funcionais deste polímero, mas com maior redução da flexibilidade e maior fragilidade deste material devido a formação de ligações intermoleculares entre a quitosana e os metais (CARDOSO et al., 2012).

Dentre os metais pesados o cádmio apresenta alta toxicidade em baixas concentrações e polui os recursos hídricos através das descargas de efluentes industriais, principalmente os produzidos pelas galvanoplastias, minerações, produção de pigmentos, plásticos, pilhas, soldas, fabricação de alumínio, equipamentos eletrônicos, lubrificantes, inseticidas e acessórios fotográficos. Os efeitos prejudiciais do cádmio na saúde humana incluem várias desordens crônicas, como a doença de *itai-itai*, danos renais, enfisemas, hipertensão, câncer, e atrofia testicular (COLLA *et al.*, 2012).

A recuperação de águas contaminadas por esse metal pode ser realizadas com a utilização de quitosana na forma de membranas (ALIABADI *et al*., 2013), nanopartículas (HEIDARI *et al*., 2013), quitosana em pó e misturada com carvão ativado (HYDARI et al., 2012) e quitosana em gel ou em flocos; estudos demonstraram que formas de uso da quitosana com maior área superficial proporcionam maior adsorção de íons de cádmio (DZUL EROSA *et al*., 2001).

Desse modo, o biopolímero quitosana apresenta características e propriedades promissoras que favorecem sua aplicação em várias áreas, destacando-se neste trabalho de tese o seu potencial para remoção do metal pesado cádmio em água. As membranas de quitosana oferecem maior área de superfície específica o que potencializa a velocidade e a capacidade de interação com este íon metálico. Portanto, a seleção de melhores condições de cultivo dos fungos *R. arrhizus* e *C. elegans* para maior produção de quitosana microbiológica favorece a aplicação deste polímero como membrana para remoção de cádmio em soluções aquosas, podendo se tornar uma alternativa promissora para a economia e o meio ambiente.

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CAPÍTULO II

Produção e Caracterização Físico-Química de quitina e quitosana produzidas por *Cunninghamella elegans* e *Rhizopus arrhizus* em Meio Padrão para Mucorales – Análise Fatorial

Trabalho aceito para publicação como capítulo do livro: “Industrial, Medical, and Environmental Applications of Microorganisms: Current Status and Trends".

PRIMEIRO ARTIGO

Comparative Study of growth, chitin and chitosan production by *Cunninghamella elegans* and *Rhizopus arrhizus* using the ratio carbon/nitrogen - A factorial design

L. R. Ramos Berger[*,1,2] ; T. C. Montenegro Stamford[2,3] and G. M. Campos-Takaki[1,2]

[1] Post-Graduate Program in Biological Sciences, University Federal of Pernambuco, Avda. Prof. Moraes Rego, 1235, Cidade Universitária, 50670-420, Recife, PE, Brazil

[2] Nucleus of Research in Environmental Science, Catholic University of Pernambuco, Nunes Machado Street, 42,
Boa Vista, 50050-900, Recife, PE, Brazil

[3] University Federal of Pernambuco, Center for Health Sciences, Department of Tropical Medicine, Avda Professor Moraes Rêgo, 1255,Cidade Universitária, 50670-420, Recife, PE - Brasil

*Corresponding author: e-mail: quelberger@hotmail.com, Phone: +55 +81 89015729

Chitin and chitosan are copolymers consisting of units *N*-acetil-D-glucosamine and D-glucosamine in varying proportions, with the first type of these units are predominant in the case of chitin, while chitosan is composed predominantly of D-glucosamine units. In this investigation was compared the growth, chitin and chitosan production by *Rhizopus arrhizus* and *Cunninghamella elegans* using different amount of carbon and nitrogen source, represented by the concentrations of D-glucose, thiamine and L-asparagine. These parameters were varied symmetrically around the central point according to the 2^3 factorial design. The extraction process involved deproteinization using 1M NaOH solution, followed by treatment with acetic acid 2% solution. Different carbon and nitrogen concentrations of the culture medium influenced the biomass, chitin and chitosan yields, consequently, these independent variables can be manipulated during the fermentation to possibility the optimal production of these biopolymers. The best yield of chitin by *R. arrhizus* (135.17 mg/g) was inferior to *C. elegans* (235mg/g). However, the higher chitosan production by *R. arrhizus* (68.17 mg/g) was superior *C. elegans* (46.18 mg/g).

Keywords Chitin; chitosan; production; extraction

1. Introduction

Chitin is a biopolymer of N-acetyl-d-glucosamine (GlcNAc) and d-glucosamine residues linked by β-(1-4) glycosidic bonds [1], which is present as structural element in the exoskeleton of crustaceans, mollusks, annelids, coelenterates, insects and fungal cell walls, particularly of Zygomycetes [2]. The derivative form of chitin is the chitosan

obtained by the deacetylation of chitin [3]. Chitosan is a cationic and linear polymer, essentially composed of β-1,4 D-glucosamine (GlcNAc) linked to N-acetyl-D-glucosamine residues [4].

Chitin and chitosan has been extensively studied as due to unique properties such biodegradability, biocompatibility, biological activites, chemical applications [5], ability to form films, metal chelations, optical and structural characteristics. Chitosan has been used in medical applications, food, pharmaceutical, cosmetics, agricultural, textile, waste water treatment, and other industries [6].

Most studies suggested the fungi as an alternative source for the chitin and chitosan production, especially the order of Mucorales, Zygomycetes class [6] since these biopolymers were found to be the main components of its cell wall [7], with homogenous characteristics, a more consistent and desirable physiochemical property [8]. The characteristics and yields of the chitin and chitosan may be optimized by controlling fermentation and processing parameters such as pH, nutrient concentration in the fermentation medium and length of incubation time. Generally, the composition of culture media for chitosan production from fungi contain yeast extract, D-D-glucose, and peptone [9]. The use of synthetic culture media to the growth of fungi and chitin and chitosan production can be optimized through the use of multivariate approach, where all independent variables are considered simultaneously. In this investigation, a study was carried out to compare the growth, chitin and chitosan production by *Rhizopus arrhizus* and *Cunninghamella elegans* using different amount of carbon and nitrogen source, represented by the concentrations of D-glucose, L-asparagine and minimal concentrations of thiamine. These independent variables were chosen as the basis in the medium proposed by Hesseltine and Anderson (1957) [10]. These parameters were varied symmetrically around the central point according to the 2^3 factorial design.

3. Methodology

2.1. Microorganism and growth conditions

Cunninghamella elegans UCP 542 and *Rhizopus arrhizus* UCP 402, registered at the World Federation for Culture Collection – WFCC, were grown in Petri dishes containing PDA, pH 5.6, and incubated at 28°C until sporulation (5 days). The spore

solution (10^7 spores/ mL) were inoculated in Petri dishes with PDA for 18 hours. A total of 20 discs with 1 cm in diameter were inoculated with the fungus, removed and placed in 50mL of the desired medium used as pre-inoculum. After 24 hours, the pre- inoculum was transferred to the flasks of 500 mL capacity with 150 mL of the same medium and again incubated in shaker at 150 rpm, 28°C for 120 hours. The mycelia were harvested, washed with destilled water by filtration and lyophilized.

2.2. Extraction and characterization of chitin and chitosan

The extraction of chitin and chitosan was carried out using dry biomass of *Cunninghamella elegans* UCP 542 and *Rhizopus arrhizus* UCP 402 following the methodology of Jin Hu *et al.* [11]. After the biomass drier, it was treated with 1M NaOH solution (1:30 w/v, 121°C, 15 min). Alkali-insoluble material was obtained by centrifugation (4000g, 20 °C, 10 min), and extracted using 2% of acetic acid (1:100 w/v, 100°C, 15 min) followed by centrifugation at 4000 g, 20°C, 15 min. The pH of supernatant was adjusted to 10, and maintained overnight at 5°C, until the chitosan fraction was precipitated. The chitosan was obtained by centrifugation at 4000 g, and washed with distilled water four times, freeze-dried, and kept in a dissecator until constant weight.

The degree of deacetylation for microbial chitin and chitosan were determined using the infrared spectroscopy according to Baxter et al. [12] using the absorbance ratio A1655/A3450.

2.3. Experimental design

Cunninghamella elegans UCP 542 and *Rhizopus arrhizus* UCP 402 were grown in synthetic media with compositions defined by variations around the medium proposed by Hesseltine and Anderson (1957)[10] with the following composition: D-glucose 40 g; L-asparagine 2 g; chloridrate of thiamine 0.5 mg; potassium phosphate 0.50 g and magnesium sulphate 0.25 g per litre of distilled water, pH 5.2. This medium was used as central point, except for the concentration of thiamine which, in this experiment was hundred times smaller (0,005 mg/L) than the concentrations of Hesseltine and Anderson (1957) [10] (0,5 mg/L). The D-glucose, L-asparagines and thiamine were varied symmetrically around the central point according to the 2^3 factorial design given in Table 1.An estimate of pure experimental error was calculated from four replicates run

corresponding to a central point of the complete factorial. The response recorded were biomass, chitin and chitosan yield by *C. elegans* and *R. arrhizus*. The analyses of the dates and graphs were made with the Statistical 7.0 software package and significance of the results was tested at the $p<0.05$ level.

Table 1. Design matrix for the factorial experiments used to study the of three factors on biomass, chitin and chitosan production by *Cunninghamella elegans* UCP 542 and *Rhizopus arrhizus* UCP 402, with experimental conditions set in the average of two extreme levels. An estimate of pure experimental error was calculated from four replicates run corresponding to a central point of the complete factorial (assay 9).

Assay	D-glucose g/L	L-asparagine g/L	Tiamine mg/L
1	-	-	-
2	+	-	-
3	-	+	-
4	+	+	-
5	-	-	+
6	+	-	+
7	-	+	+
8	+	+	+
9*	0	0	0

Level (-1): D-glucose 20 g/L, L-asparagine 1,0 g/L, Thiamine mg/L 0,002; Central point (0): glucose 40 g/L, L-asparagine 2,0 g/L, Thiamine mg/L 0,005; Level (+1): glucose 60 g/L, L-asparagine 3,0 g/L, Thiamine mg/L 0,008.

3. Results and discussion

Table 2 shows biomass production, chitin and chitosan yields by *C. elegans* and *R. arrhizus* obtained in the experiments. The experimental condition 4 (D-glucose 60 g/L; L-L-asparagine 3 g/L and thiamine 0.002 mg/L) and the condition 8 (D-glucose 60 g/L; L-L-asparagine 3 g/L and thiamine 0.008 mg/L) obtained the higher biomass production, 7.55 and 6.9 g/L, respectively, and these results are not statistically different. This suggests that the increase in the thiamine concentrations not favor the growth by *C. elegans.* On the other hand, the biomass production obtained by *R.*

arrhizus was lower than the biomass of *C. elegans*. The experimental condition 1 (20g/L D-glucose, 1g/L L-asparagine and 0.002 mg/g thiamine) and experimental condition 8 (20g/L D-glucose, 1g/L L-asparagine and 0.002 mg/g thiamine) resulted in the higher biomass production of 2.93 g/L and 2.85 g/L by *R. arrhizus*, respectively. These values of biomass production are not statistically different, consequently, the same yield could be obtained using the higher or the lower level of D-glucose, L-asparagine and thiamine. These results suggest a culture medium optimized and economic to increase the biomass production by *C. elegans* and *R. arrhizus*.

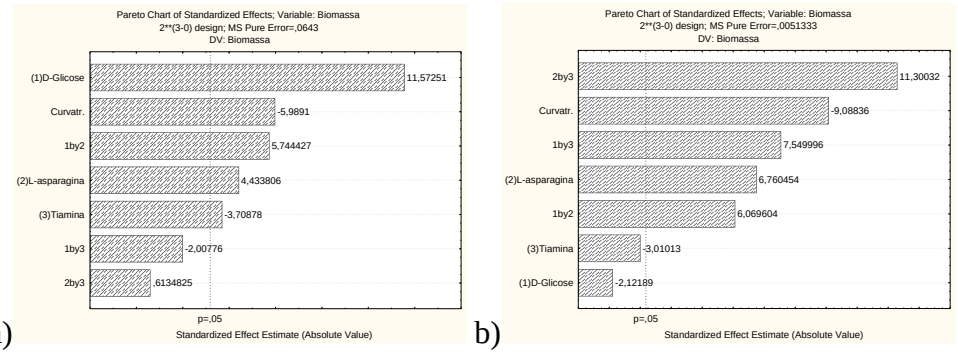
The best yield of chitin by *C. elegans*, 235mg/g (23.5%), was obtained in experimental condition 9, the central point, (40g/L D-glucose, 2g/L L-asparagine and 0,005mg/g tiamine) and for chitosan the best yields, 46.18 mg/g (4.61%), was obtained in condition 5 (20g/L D-glucose, 1g/L L-asparagine and 0.008 mg/g tiamine). On the other hand, this higher yield of chitosan (46.18 mg/g) did not differ statistically from the value (45.00 mg/g) obtained at the condition 1 (20g/L D-glucose, 1g/L L-asparagine and 0.002 mg/g tiamine). The same influence of thiamine occurred to the biomass production. The values of chitin yield were similar to Franco et al [13] and Andrade et al. [2], but inferior to Stamford et al. [5]. The values of chitosan yield were inferior to the results describe by Pochanavanich et Suntornsuk [14], Franco et al. [13], Stamford et al. [5]; but were superior to Amorim et al [15].

The best yield of chitin by *R. arrhizus*, 135.17 mg/g, obtained in the experimental condition 4 (D-glucose 60 g/L; L-L-asparagine 3 g/L and thiamine 0.002 mg/L) was inferior to the production of this biopolymer by *C. elegans* (235mg/g). However, the higher chitosan production by *R. arrhizus*, 68.17 mg/g, (condition 8 (20g/L D-glucose, 1g/L L-asparagine and 0.002 mg/g thiamine) was superior to the chitosan yield obtained by *C. elegans* (46.18 mg/g). These results propose that the chitosan and biomass productions were directly proportional, because the condition 8 showed the highest values of biomass and chitosan. These data are in agreement with Pochanavanich and Suntornsuk [14] that reported chitosan production by the microorganisms is strongly dependent of the culture conditions, including carbon source. The value of chitin yield (135.17 mg/g) was superior to the values describe by Berger et al. [16], Cardoso et al. [17]; but inferior to the value describe by Stamford et al. [5]. The production of chitosan obtained in this work (96 mg/g or 9,6 %) was similar to the values describe by Franco *et al.* [13], Stamford *et al.* [5] and superior to Amorim *et al.* [15].

Table 2. Design matrix for the factorial experiments used to study the influence of 3 factors, D-glucose, L-asparagine and thiamine, varied symmetrically around the central point (*) according to the 2^3 factorial. The response recorded were biomass, chitin and chitosan yield by *Cunninghamella elegans* and *Rhizopus arrhizus.*

	Biomass (g/L)		Chitin (mg/g)		Chitosan (mg/g)	
Run	*Cunninghamella elegans*	*Rhizopus arrhizus*	*Cunninghamella elegans*	*Rhizopus arrhizus*	*Cunninghamella elegans*	*Rhizopus arrhizus*
1	4.43	2.93	115.94	116.91	45.00	49.68
2	6.1	1.97	77.13	92.95	13.52	50.87
3	4.35	2.23	146.67	104.91	15.29	66.14
4	7.55	2.21	101.06	135.17	5.23	46.22
5	4.28	1.66	131.52	53.19	46.18	35.92
6	4.70	1.79	194.31	110.94	6.18	29.26
7	3.89	2.43	193.88	129.03	34.97	44.57
8	6.90	2.85	85.65	67.69	9.78	68.17
9*	4.64	1.82	235.00	74.95	22.85	44.72
10*	4.43	1.91	214.33	76.92	27.20	46.73
11*	4.27	1.78	210.70	77.57	25.23	47.65
12*	4.04	1.93	201.53	78.85	23.24	47.99

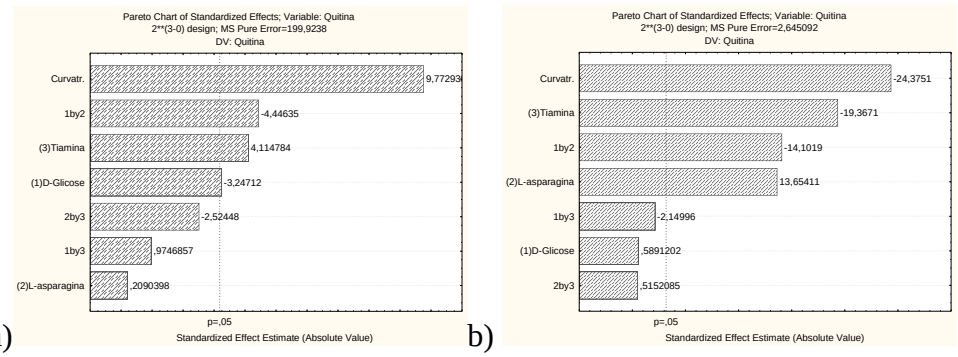
Figure 1a shows the influence of the D-glucose (1), L-asparagine (2) and thiamine (3) and the interaction between these factors in the biomass, production by *Cunninghamella elegans* (figure 1a) and *Rhizopus arrhizus* (figure 1b). The D-glucose (1), L-asparagine (2) and the interaction between these factors (1by2) were the independents variables that most influenced positively the biomass production by *C. elegans*. These variables were statistically significant at 95% confidence level ($p<0.05$). Figure 1b also shows the positive influence of L-asparagine (2), the interactions 1by2, 1by3, 2by3 in the biomass production by *R. arrhizus*, being statistically significant.



a) b)

Fig. 1 Pareto chart showing the effect of D-glucose, thiamine and L-asparagine concentration in the biomass production by *Cunninghamella elegans* a) and b) *Rhizopus arrhizus*.

The chitin production by *C. elegans* was influenced positively by thiamine (3) and negatively by D-glucose (1), and interaction 1by2 as showed in Fig. 2a. These result are totally contrary to the results shows in Fig. 1a for biomass production by this fungus. On the other hand, Figure 2b shows the negative effect of thiamine (3) and interaction 1by2 and the positive influence of L-asparagine (2) in the chitin production by *R. arrhizus.* These variables were statistically significant at 95% confidence level ($p<0.05$).

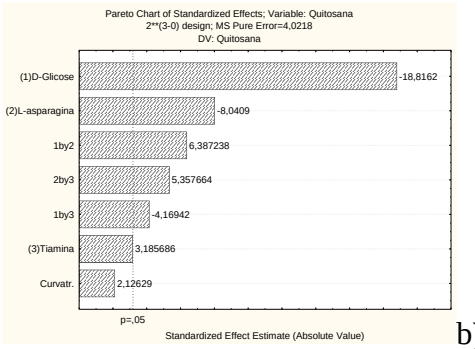


a) b)

Fig. 2 Pareto chart showing the effect of D-glucose, thiamine and L-asparagine concentration in the chitin production by *Cunninghamella elegans* a) and b) *Rhizopus arrhizus*.

The chitosan production by *C. elegans* (Fig. 3a) was influenced positively by the thiamine (3), interactions 1by2, 2by3, being statistically significant. The Pareto chart (Fig. 3a) also shows that the increase in the L-asparagine (2), D-glucose (1), and interaction (1by3) negatively influenced the chitosan production. Moreover, to improve the chitosan yield by *R. arrhizus* should be necessary to increase the concentrations of

L-asparagine, and interactions 1by3 and 2by3; and reduce the thiamine concentration, as shows in Fig. 3b.

a) 

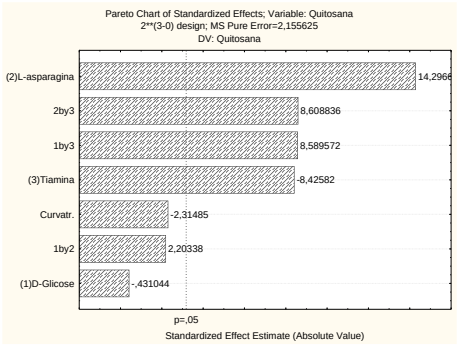
b) 

Fig. 3 Pareto chart showing the effect of D-glucose, thiamine and L-asparagine concentration in the chitosan production by *Cunninghamella elegans* a) and b) *Rhizopus arrhizus*.

The results suggest an increase to the concentrations of the D-glucose, L-asparagine and thiamine that positively influenced the dependet variables and the Decrease in the concentrations of these variables with negative effect on the biomass, chitin and chitosan production. Consequently, this modification could provide greater optimization to the dependent variables and economy during the fermentation process.

The obtained results point out to the influence of carbon and nitrogen concentrations of the culture medium in the biomass, chitin and chitosan yields and to the possibility of achieving an optimal production of these biopolymers by controlling these variables during the fermentation.

The characterization of chitin and chitosan obtained from *C. elegans* and *R. arrhizus* in the H&A medium by infrared spectra are similar to those reported in the literature [4, 6, 21]. The most significant parts of chitin and chitosan spectra are those showing the amide bands at approximately 1665, 1555 and 1313 cm-1, which could be assigned to the C = O stretching, the N-H deformation in the CONH plane and the CN bond stretching plus CH_2 wagging. In the present study, chitin and chitosan and presents 14,76% and 78% DD, respectively, obtained from *C. elegans* and 15,6% and 77% DD, respectively, obtained from *R. arrhizus*. These results are in accordance with Stamford et al [9], Pochanavanich and Suntornsuk [31].

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CAPÍTULO III

Produção e Caracterização Físico-Química de quitina e quitosana produzidas por *Cunninghamella elegans* e *Rhizopus arrhizus* em Meios de Cultura de Baixo Custo

SEGUNDO ARTIGO

Agroindustrial waste as alternative medium in the production of chitin and chitosan by *Rhizopus arrhizus* – A factorial design

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Agroindustrial waste as alternative medium in the production of chitin and chitosan by *Rhizopus arrhizus* – A factorial design

LÚCIA RAQUEL RAMOS BERGER [1,2]; ANTONIO CARDOSO [2,3]; THAYZA CHRISTINA MONTENEGRO STAMFORD [4]; HORACINNA Mª DE M. CAVALCANTE[5], RUI DE OLIVEIRA MACEDO[5], GALBA MARIA DE CAMPOS-TAKAKI[2]

[1]Doutoranda em Ciências Biológicas, Universidade Federal de Pernambuco-UFPE, PE, Brasil;

[2] Núcleo de Pesquisas em Ciências Ambientais, Pós-Graduação em Desenvolvimento de Processos Ambientais, Universidade Católica de Pernambuco -UNICAP, 50.050-590 Recife, PE, Brasil.

[3]Rede Nordeste de Biotecnologia – RENORBIO, Recife/PE, Brasil

[4] Departamento de Fisiologia e Patologia, Centro de Ciências da Saúde, Universidade Federal da Paraíba-UFPB, João Pessoa, PB, Brasil.

[5] Departamento de Ciências Farmacêuticas, Universidade Federal da Paraíba-UFPB, João Pessoa, PB, Brasil.

ABSTRACT

Chitin and chitosan hold a great economic value as due to their versatile biological activities and chemical applications. Studies were carried using factorial design on the production of these biopolymers by Rhizopus arrhizus grown on Agroindustrial waste, corn steep liquor and cassava wastewater. Chitin and chitosan were extracted by alkali-acid treatment, and characterized by Infrared spectroscopy, biocompatibility and viscosity. A higher production of biomass, 8.80 g/L, chitin (54.38 mg/g) and chitosan (20.51 mg/g) are obtained in experimental condition 3 (5% cassava wastewater, 8% corn steep liquor). Chitin and chitosan showed degree of deacetilation, respectively of 25% and 82%, viscosimetric molecular weight of chitin and chitosan 2.4 $_x$ 10^4 g/mol and 3.0 $_x$ 10^4 g/mol, respectively. Chitosan showed not irritating and biocompatibility. The results suggest that Agroindustrial waste improves chitin and chitosan production and the mycelial biomass of C. elegans may be used as an alternative source of these polymers.

Key words: *Rhizopus arrhizus, chitin, chitosan, corn steep liquor, cassava wastewater.*

INTRODUCTION

The commercial chitin and chitosan are originated from waste product of the crustacean exoskeletons, such as crab and shrimp shells, obtained after industrial processing of seafood [1,2]. This chitosan is produced from the chemical deacetylation of the crustacean chitin with strong alkaline solution and high temperatures. The chitosan obtained by this method can be heterogeneous and inconsistent due to the variability of raw materials, and the harshness of the isolation and conversion processes. The caustic effects of the chemicals used in the isolation process can cause variability in the levels of deacetylation and protein contamination in the chitosan [3-6].

The fungal production of chitin and chitosan can be a viable alternative to obtain these biopolymers with homogenous characteristics [4,6]. The characteristics and yields of the chitin and chitosan may be optimized by controlling fermentation and processing parameters such as pH, nutrient concentration in the fermentation medium and length of incubation time [7-10]. There are more considerable advantages in the use of biomass as a source of fungal chitin and chitosan such as: simultaneous extraction of the biopolymers, independence of seasonal factors, large-scale production, simple and economic process, absence of protein contamination that can cause allergic reactions in humans [7-9,11].

Generally, the composition of culture media for chitosan production from fungi contains yeast extract, D-glucose, and peptone [9]. However, the use of synthetics or complex cultures media to the growth of fungi can increase the cost of chitin and chitosan production. Therefore it is important to obtain economic culture media that promote the growth of fungi and stimulate the production of the polymers [8]. Among the agroindustrial substrates, stand out the corn steep liquor and cassava wastewater. The corn steep liquor is a residue from the corn processing industry that has a large amount of amino acids, vitamins and minerals. This residue can be considered an alternative substrate for the production of fungal chitin and chitosan [12].The manipueira (cassava wastewater) is a yellowish liquid obtained from cassava during the manufacturing process of cassava flour, rich in many nutrients such as potassium (K), nitrogen (N), magnesium (Mg), phosphorus (P), calcium (Ca) and sulfur (S) [13]. Nowadays there are discharged of cassava wastewater into rivers or on soil without any kind of treatment, causing damage for the environment and human health[14,15] was observed that *Aspergillus niger* showed no difference in their growth and production of citric acid in

the presence of alternative substrate cassava wastewater as compared to the synthetic medium.

This research describes an experimental study of the influence of the agroindustrial waste, corn steep liquor and cassava wastewater concentration on chitin and chitosan production by *R. arrhizus*. The effect of these factors or the interaction effects between these will be observed by factorial design analysis.

EXPERIMENTAL

Microorganisms

Rhizopus arrhizus UCP 402 (Culture Collection of Catholic University of Pernambuco, Recife, Brazil) was isolated from mangrove sediment situated in Rio Formoso, PE, Brazil. The strain was maintained on Potato Dextrose Agar (PDA) medium at 5ºC.

Cultural Conditions

Rhizopus arrhizus was grown in Petri dishes (9 cm in diameter), containing PDA medium at 28 ºC for 8 days. A suspension was prepared and adjusted to 10^7 sporangioles/mL, using a hematocytometer for counting. Petri dishes with PDA was inoculated with 1 mL of the sporangioles suspension and maintained for 18 hours at 28 °C. At the end of the desired incubation period a total of 20 discs PDA medium (1 cm diameter) with mycelium of *R. arrhizus* were inoculated in Erlenmeyer flask containing 50 mL of the alternative medium, pH 5.6 , varying corn steep liquor and cassava wastewater concentration. These parameters were varied symmetrically around the central point according to the 2^2 factorial design. The flasks were incubated at 28ºC in an orbital shaker at 150 rpm, during 24 hours. After this time, the culture used as pre-inoculum was transferred to the Erlenmeyer flasks with 150 mL of the alternative medium, pH 5.6 and incubated at 28 °C in orbital shaker at 150 rpm during 72 hours. The mycelia were harvested, washed twice in destilled water by filtration, utilizing a silkscreen nylon membrane (120 F), and were submitted to lyophilization process. After lyophilization the biomass was maintained in a vacuum dissecator until constant weight.

Experimental factorial design

R. arrhizus was grown, for chitin and chitosan production, in a agroindustrial wastewater medium varying the cassava wastewater (5-10%) and corn steep liquor (4-8%) concentrations according to the according to the 2^2 factorial design as shown in table 1. An estimate of pure experimental error was calculated from four replicates run corresponding to a central point of the complete factorial. The response recorded were biomass, chitin and chitosan yield by *R. arrhizus*. The analyses of the dates and graphs were made with the Statistical 7.0 software package and significance of the results was tested at the $p<0.05$ level.

Table 1. Design matrix for the factorial experiments used to study the influence of 2 factors on biomass, chitin and chitosan production by *Rhizopus arrhizus* UCP 402, with experimental conditions set in the average of two extreme levels. An estimate of pure experimental error was calculated from four replicates run corresponding to a central point of the complete factorial (assay 5).

Run	**Factor levels**	
	Cassava wastewater[1]	Corn steep liquor[2]
1	-1	-1
2	+1	-1
3	-1	+1
4	+1	+1
5	0	0

[1] Concentration of cassava wastewater (%, v/v): 5.00 at level -1; 7.50 at level 0; 10.00 at level +1
[2] Concentration of corn steep liquor (%, v/v): 4.00 at level -1; 6.00 at level 0; 8.00 at level +1

Chitin and Chitosan Extraction

The extraction of chitin and chitosan was carried out using dry biomass of *Rhizopus arrhizus* following the methodology of Hu *et al.* [16]. After the biomass drier, it was treated with 1M NaOH solution (1:30 w/v, 121°C, 15 min). Alkali-insoluble material was obtained by centrifugation (4000g, 20 °C, 10 min), and extracted using 2% of acetic acid (1:30 w/v, 100°C, 15 min) followed by centrifugation at 4000 g, 20°C, 15 min. The supernatant was obtained and the pH was adjusted to 10, and maintained overnight at 5°C, until the chitosan fraction was precipitated. The chitosan was obtained by centrifugation at 4000 g, and washed with distilled water four times, freeze-dried, and kept in a dissecator until constant weight.

Characterization of chitin and chitosan

The degree of deacetylation for microbial chitin and chitosan were determined by means of infrared spectroscopy in accordance with Baxter *et al.*[17], using the absorbance ratio A1655/A3450. A two milligram sample of fungal chitin and chitosan, which had been dried overnight at 60°C under reduced pressure, was thoroughly blended with 100mg of KBr, to produce 0.5mm thick disks. The disks were dried for 24hr at 110°C under reduced pressure. Infrared spectroscopy was recorded with a Bruker 66 Spectrometer, using a 100mg KBr disk for reference. The intensity of the maximum absorption bands was determined by the baseline method.

The molecular weights of chitin and chitosan were determined by viscosity, using the procedure described by Santos *et al.*[18]. The viscosity of chitosan was determined using an AVS-350 viscometer (Schott-Geräte), type/capillary: Cannon-Fenske d_{inside}= 1.01mm, at 25ºC. After obtaining the intrinsic viscosity from tables, K and a, were obtained for HAc/NaAc. K = 0.076, a = 0.76. The flow time was determined in seconds. Using the Mark-Houwinks equation, the average viscosimetric molecular weight was expressed in g/mol.

Evaluation of biocompatibility of chitosan

The chick embryo, specifically its chorioallantoic membrane (CAM), can be used to evaluate the activity or toxicity of a drug on both the CAM[19].

To the test were used eggs at 10º days of incubation, obtained from Guaraves Guarabira Aves Ltda and incubation at 37ºC. Five eggs were used for each test substance. On day 10 of incubation, the egg shell above the air space was removed. The exposed membrane was moistened with a drop of 0.9% physiological saline was carefully removed, exposing the CAM (Chorioallantoic membrane of chick embryo). An aliquot of 200 µl of the chitosan was applied on the CAM and observed vasoconstriction, hemorrhage and coagulation for 5 minutes to evaluate the potential for irritation according to the method of HET-CAM. It was also observed signs of inflammation, edema or neovascularization to evaluate the biocompatibility of chitosan.

RESULTS AND DISCUSSION

Biomass, chitin and chitosan production by Rhizopus arrhizus

The table 2 shows biomass production, chitin and chitosan yields obtained in the experiments. The experimental condition 3 (5% cassava wastewater, 8% corn steep liquor) obtained the higher biomass production, 8.80 g/L, chitin, 54.38 mg/g, and chitosan, 20.51 mg/g, yields. This suggests that the increase in the corn steep liquor concentrations and the cassava wastewater in the lower level could favor the growth and production of chitin and chitosan by *R. arrhizus*. The corn steep liquor consists of amino acids and carbohydrates which influence the growth of Mucoralean fungi [20]. On the other hand, the higher yield of chitosan (20.51 mg/g) did not differ statistically from the values obtained at the central point (20.43 mg/g). These results are compared with the literature as verified in Table 3.

Table 2. Design matrix for the factorial experiments used to study the influence of 2 factors, cassava wastewater and corn steep liquor concentrations, varied symmetrically around the central point according to the 2^2 factorial. The response recorded were biomass production, chitin and chitosan yield by *R. arrhizus*.

Run	Cassava wastewater (%)	Corn steep liquor (%)	Biomass (g/L)	Chitin Yield (mg/g)	Chitosan Yield (mg/g)
1	5.00	4.00	5.80	47.63	13.98
2	10.0	4.00	5.76	31.89	10.86
3	5.00	8.00	8.80	54.38	20.51
4	10.0	8.00	4.02	35.85	13.57
5-8*	7.50	6.00	6.4±0.3	34.4±1.0	18.43±2.0

*Four replicates run: central point

Table 3. Biomass, chitin and chitosan production by *Rhizopus arrhizus* grown on Agroindustrial waste compared results obtained by the literature.

Microorganism	Substrate	Biomass ($g.L^{-1}$)	Chitin ($mg.g^{-1}$)	Chitosan ($mg.g^{-1}$)	Reference
Rhizopus arrhizus	Corn steep liquor and cassava wastewater	8.80	54.38	20.51	This study
Rhizopus arrhizus	Synthetic medium for Mucorales	-	92	13	21
Rhizopus arrhizus	Modified Synthetic medium for Mucorales	-	94	14	21
Rhizopus arrhizus	Corn steep liquor 4%	13.00	30.40	12.85	20
Mucor circinelloides	Yam bean	20.70	500	64	22
Cunninghamella elegans	Yam bean	24.30	440	66	8
Cunninghamella elegans	Hesseltine and Anderson-factorial design added glucose, sucrose and NaCl	-	415	183	23
Cunninghamella elegans	Hesseltine and Anderson added of 5%NaCl and 6%glucose	24.40	388	70	24
Cunninghamella bertholletiae	Sugar cane juice	7.70	-	128	9
Aspergillus niger	Potato Dextrose Broth	9.00	-	107	25
Lentinus edodes	Potato Dextrose Broth	1.4	-	33	25
Zygosaccharomyces rouxii	Yeast Malt Extract Broth	4.4	-	36	25
Candida albicans	Yeast Malt Extract Broth	1.8	-	44	25
Mucor racemosus	YPD medium	15.0	-	35.1	26
C. elegans	YPD medium	25.0	-	20.5	26
C. elegans	Andrade *et al.*	11.6	238	78.0	7
Rhizopus arrhizus	Corn steep liquor 8%	16.8	575	416	12

- Data not shown

The Figure 1 showed the influence of the cassava wastewater, corn steep liquor and the interaction between these factors in the biomass production by *R. arrhizus.* Biomass production was influenced negatively, being statistically significant, by cassava wastewater concentration and the interaction between this factor and corn steep liquor. However, the corn steep liquor had a positive effect on biomass production, but no statistically significant. This indicates that biomass production increase when the corn steep liquor concentration is at the higher level.

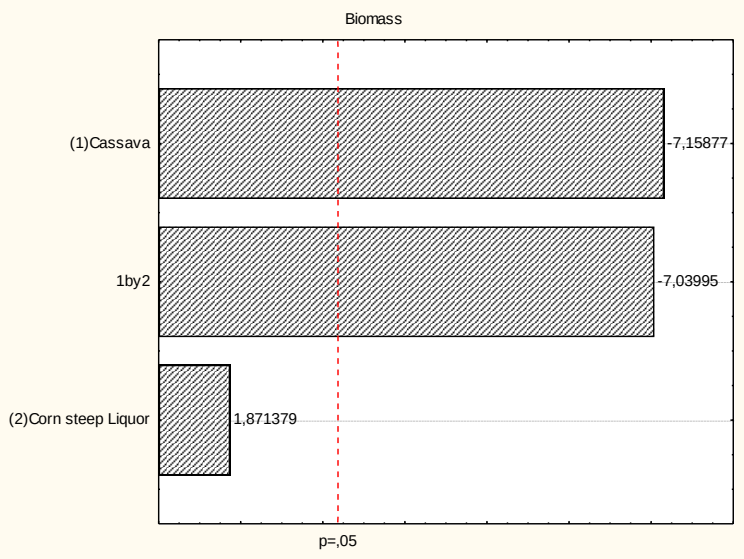

Figure 1. The Pareto chart shows the effect of corn steep liquor and cassava wastewater concentrations and the effects of the interaction between these factors on the production of biomass by *Rhizopus arrhizus* (UCP 402).

The corn steep liquor concentration also tends to favor, the chitin (figure 2) and chitosan (figure 3) yields, being statistically and no statistically significant, respectively. The interaction between the agroindustrial wastewater (1 by 2, in the figure 2 and 3) provide negative influence in the chitin and chitosan production. These biopolymers yields were influenced negatively by cassava wastewater. On the other hand, chitin and chitosan production increases when the corn steep liquor is at highest level and, probably, a decrease in the cassava wastewater concentration could favor the growth and production of chitin and chitosan by *R. arrhizus*.

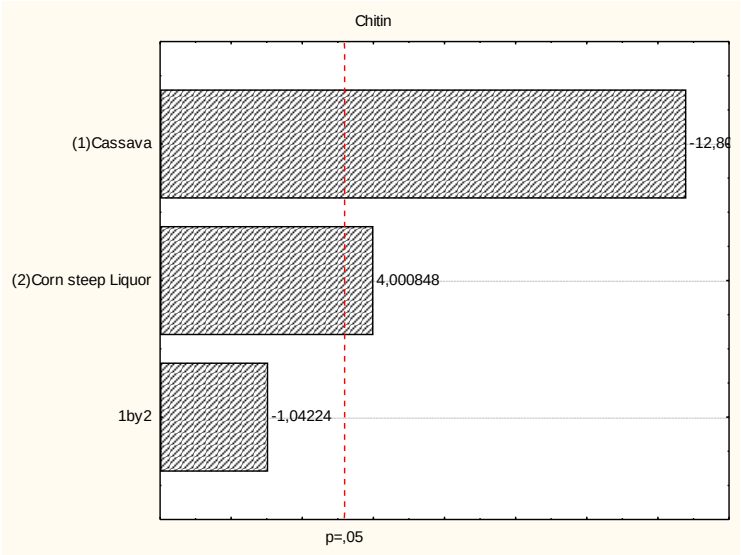

Figure 2. The Pareto chart shows the effect of corn steep liquor and cassava wastewater concentrations and the effects of the interaction between these factors on the chitin yield by *Rhizopus arrhizus* (UCP 402).

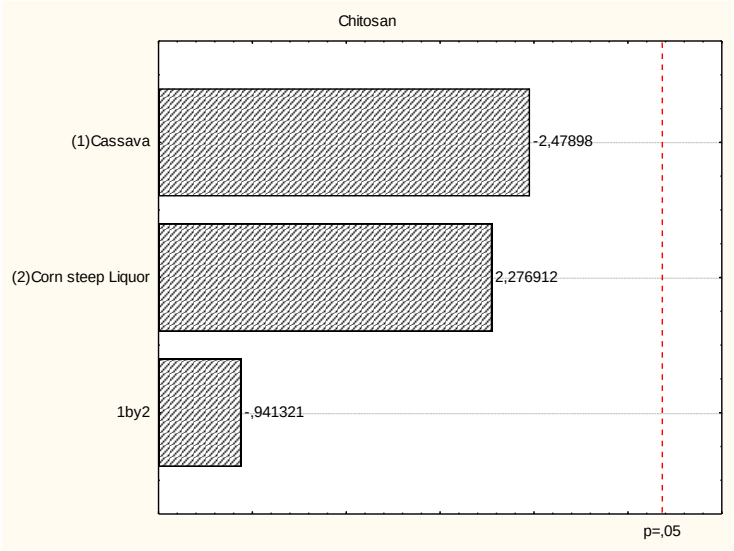

Figure 3. The Pareto chart shows the effect of the corn steep liquor and cassava wastewater concentrations and the effects of the interaction between these factors on the chitosan yield by *Rhizopus arrhizus* (UCP 402).

Chitin and chitosan characterization

The characterization of chitin and chitosan obtained from *Rhizopus arrhizus* using corn steep liquor and cassava wastewater as substrates by infrared spectra are similar to those reported in the literature[8,9,22,23]. The most significant parts of chitin and chitosan spectra are those showing the amide bands at approximately 1665, 1555 e 1313 cm^{-1}, which could be assigned to the C=O stretching, the N– H deformation in the CONH plane and the CN bond stretching plus CH_2 wagging. In a similar way, chitin from *R. arrhizus* shows bands in the amide II region, which were 1153, 1378, and 1558 cm^{-1}. The results are in agreement with Stamford *et al.*[8], Silva *et al.* [23] and Fai *et al.* [22] reported that the chitin structure contains two types of amide group and both form C=O···N-H intermolecular bonds, but one is also an acceptor for the CH_2OH group.

According to Santos *et al.*[18], deacetylation and the regeneration process cause disturbance in the initial crystalline reticulum of chitin, inducing a reordering of the hydrogen linking of chitosan. This can be observed in the central band at approximately 3483 cm^{-1} e 3305 cm^{-1}, in the region of: (i) the axial deformation of OH, which appears as overlapping the band of axial deformation of NH indicating an intermolecular hydrogen linking formation, and at (ii) the displacement of the higher frequency band indicating an increase in the structural order. The data are in accordance with those reported in literature when comparing both chitin and chitosan infrared spectra obtained by microbiological methods [17,27].

Deacetylation degree (%DD) is an important parameter associated with the physical–-chemical properties of chitosan, because it is linked directly to the chitosan cationic properties[22]. In the present study, chitosan obtained from *R. arrhizus* presents 82% DD and chitin present 25% DD. These results are in accordance with [8,25]. These authors reported the deacetylation degree of chitosan from fungi occurred between 80 to 90% DD.

The average viscosimetric molecular weight (M_V) of chitin and chitosan from *R. arrhizus* obtained in this study are 2.4×10^4 g/mol and 3.0×10^4 g/mol, respectively, which are considered relatively low[25]. The result is in agreement with the literature, which reports molar weights ranging between $1,0 \times 10^3$ to $9,0 \times 10^5$ g/mol [8,17]. Chitosan with a low molecular weight was reported to reduce the tensile strength and elongation of the chitosan membrane but to increase its permeability. Thus, fungal chitosan could have potential medical and agricultural applications[25].

Chorioallantoic membrane of chick embryo (CAM): toxicity and biocompatibility assay

The HET-CAM assay is based on macroscopic changes occurred in CAM as hyperaemia, hemorrhage and coagulation. The method is rapid, inexpensive and effective[28]. This assay was proposed as an alternative to the Draize rabbit eye irritation test [29]. According the method of HET-CAM, the chitosan tested shown not irritating.

No signs of inflammation, edema or neovascularization were observed, demonstrating biocompatibility. Similarly, the field of cosmetics has used the CAM model to evaluate the irritation properties of cosmetic formulations and ingredients [30].

CONCLUSION

The results obtained allow to indicate that corn steep liquor and cassava wastewater may be used as alternatives substrates for the growth, chitin and chitosan production by *Rhizopus arrhizus*. The use of agroindustrial wastes as source of nutrients for the large-scale production of these biopolymers is economically and environmentally viable. *Rhizopus arrhizus* may be used as an alternative source of chitin and chitosan.

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TERCEIRO ARTIGO

Effect of Corn Steep Liquor (CSL) and Cassava Wastewater (CW) on Chitin and Chitosan Production by *Cunninghamella elegans* and *Rhizopus arrhizus* and Physico-Chemical Characteristics and Cytotoxicity

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Article

Effect of Corn Steep Liquor (CSL) and Cassava Wastewater (CW) on Chitin and Chitosan Production by *Cunninghamella elegans* and Their Physicochemical Characteristics and Cytotoxicity

Lúcia Raquel Ramos Berger [1,2], Thayza Christina Montenegro Stamford [3], Thatiana Montenegro Stamford-Arnaud [3], Luciana de Oliveira Franco [4], Aline Elesbão do Nascimento [2]; Horacinna M. de M. Cavalcante [5], Rui Oliveira Macedo [5] and Galba Maria de Campos-Takaki [2,*]

1 Post-Graduate Program in Biological Sciences, Federal University of Pernambuco, Recife 50670420, PE, Brazil; E-Mail: quelberger@hotmail.com

2 Nucleus of Research in Environmental Science and Biotechnology, Catholic University of Pernambuco, Recife 50050590, PE, Brazil; E-Mail: elesbao@unicap.br (A.E.N)

3 Department of Tropical Medicine, Center of Health Sciences, Federal University of Pernambuco, Recife 50670420, PE, Brazil; E-Mails: thayzastamford@yahoo.com.br (T.C.M.S.); thatianaarnaud@hotmail.com (T.M.S.A.)

4 Center of Biology Sciences, Department of Microbiology, Federal Rural University of Pernambuco, Recife 52171900, PE, Brazil; E-Mail: lucianafranco@terra.com.br

5 Department of Pharmaceutical Sciences, Federal University of Paraíba-UFPB, João Pessoa, PB 58051-900, Brazil, E-Mails: horacinnammc@yahoo.com.br (H.M.M.C.); ruimacedo@ccs.ufpb.br (R.O.M.)

***** Author to whom correspondence should be addressed; E-Mail: galba_takaki@yahoo.com.br; Tel.: +55-81-2119-4044; Fax: +55-81-2119-4043

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Abstract: Microbiological processes were used for chitin and chitosan production with *Cunninghamella elegans* UCP/WFCC 0542 grown in different concentrations of two agro-industrial wastes, corn steep liquor (CSL) and cassava wastewater (CW) established using a 2^2 full factorial design. The polysaccharides were extracted by alkali-acid treatment and characterized by infrared spectroscopy, viscosity, thermal analysis, elemental analysis, scanning electron microscopy and X-ray diffraction. The cytotoxicity of chitosan was evaluated for signs of vascular change on the chorioallantoic membrane of chicken eggs. The highest biomass (9.93 g/L) was obtained in trial 3 (5% CW, 8% CSL), the greatest

chitin and chitosan yields were 89.39 mg/g and 57.82 mg/g, respectively, and both were obtained in trial 2 (10% CW, 4% CSL). Chitin and chitosan showed a degree of deacetylation of 40.98% and 88.24%, and a crystalline index of 35.80% and 23.82%, respectively, and chitosan showed low molecular weight (LMW 5.2×10^3 Da). Chitin and chitosan can be considered non-irritating, due to the fact they do not promote vascular change. It was demonstrated that CSL and CW are effective renewable agroindustrial alternative substrates for the production of chitin and chitosan.

Keywords: *Cunninghamella elegans*; chitin; chitosan; corn steep liquor; cassava; wastewater; characterization; chorioallantoic membrane

1. Introduction

In recent years, there has been an increasing interest in biopolymers, specially chitin and chitosan, due to the fact they are easy to obtain, their wide applicability and their promising features such as their absence of toxicity, biodegradability, biocompatibility and environmentally friendly nature and their wide range of potential industrial applications [1]. Chitin and chitosan are natural co-polymers, comprised of units of 2-amino-2-desoxy-D-glycopyranose and of 2-acetamide-2-desoxy-D-glycopyranose interconnected by glycosidic β-1,4 bonds in variable proportions. The first type of these units is frequently present in chitosan [2,3]. Chitin is widely distributed in Nature, being the main component of the exoskeleton of crustaceans and insects and it also occurs in nematodes and in the cell walls of yeast and fungi [4,5].

Chitosan is naturally found in the cell wall of fungi, mainly in the *Zygomycetes* [6,7]. Chitosan is formed by the deacetylation of chitin, and the N-acetyl group can undergo several degrees of deacetylation. Chitosan is a cationic and linear polymer, characterized according to its deacetylation level and its molecular weight which may influence its degradability and polysaccharide hydrolysis. According to its deacetylation level and molecular weight, chitosan's physical and chemical properties such as solubility, pKa and viscosity will vary. It is difficult to obtain highly deacetylated chitosan because upon hydrolysis the degree of degradation of the polymer increases, thus making the purification process more difficult [8,9]. Chitosan with various molecular weights and degrees of deacetylation has been found to have a wide spectrum of applications as chelating agents in wastewater treatment, an antibacterial and antifungal, and as an immobilizing agent for enzymes or for delivering drugs to their target. Consequently chitosan is widely used in the pharmaceutical, food, agriculture, cosmetics and textile industries [10].

Chitin is commercially obtained from the exoskeletons of marine crustaceans, and chitosan by alkaline deacetylation of chitin at high temperatures for long periods of time [2]. However, these traditional isolation methods of these polymers present some drawbacks and limited potential for industrial acceptance such as the seasonal and limited supply of the raw material while the process of demineralization and deproteinization is aggressive and causes changes in the final product, thereby often lowering its quality since this can cause chemical changes. Consequently these end-product biopolymers often have heterogeneous and inconsistent physiochemical properties. As a result,

filamentous fungi have been considered an attractive source of chitin and chitosan for industrial applications because their specific products can be manufactured under standardized conditions [3,7,10].

Advances in the fermentation technology for producing fungal chitin and chitosan have been considered as an alternative for overcoming the adverse effects of the traditional extraction of these polymers [5,11,12,13]. In addition to obtaining microbiological chitin and chitosan with homogenous characteristics and more consistent quality, the biopolymer yields may also be optimized by controlling fermentation and processing parameters such as pH, nutrient concentration in the fermentation medium and the length of incubation time [12,14]. The extraction of these biopolymers is simultaneous; independent of seasonal factors; and the final products do not have the protein contamination that can cause allergic reactions in humans [2,4]. The use of industrial wastes as an alternative nutritional source can favor obtaining a byproduct of high value added [6,15,16] and above all, it can decrease the total production costs by 38% to 73% [17].

Corn steep liquor (CSL) is a residue from the corn processing industry that has a large amount of amino acids, vitamins and minerals. This residue can be considered an alternative substrate for producing fungal chitin and chitosan. Manipueira (cassava wastewater (CW)) is a yellowish liquid obtained from cassava during the cassava flour manufacturing process, and is rich in many nutrients such as potassium, nitrogen, magnesium, phosphorus, calcium and sulfur. Currently, CW is discharged into rivers or released on soil without any kind of treatment, thus causing damage to the environment and human health [6].

On the other hand, for the fungal production of chitosan to be commercially viable, further process optimization at the laboratory and engineering levels is required [18]. Therefore, this article sets out a method for optimizing the production of chitin and chitosan by *Cunninghamella elegans* UCP/WFCC 0542 grown by submersed fermentation and uses two agroindustrial wastes, namely corn steep liquor CSL and cassava wastewater CW, which is an economic alternative for providing carbon and nitrogen at low cost. The physicochemical characteristics and cytotoxicity of the synthesized chitin and chitosan are also described.

2. Results and Discussion

2.1. Elemental analysis of Cassava Wastewater (CW) and Corn Steep Liquor

The amount of nitrogen, carbon, oxygen and sulfur presented in the composition of cassava and corn steep liquor CSL is shown in Table 1. The CSL constituted the main nitrogen source, and this waste plus the cassava wastewater CW were the carbon source. Both residues also provide other important nutrients for the metabolism of the microorganism.

Table 1. Percentage of nitrogen, carbon, hydrogen, and sulfur present in the composition of cassava and corn steep liquor.

Sample	Nitrogen (%)	Carbon (%)	Hydrogen (%)	Sulfur (%)
Corn Steep Liquor (CSL)	6.49	37.77	6.74	0
Cassava wastewater (CW)	2.04	33.35	6.74	0

2.2. Influence of the Culture Media on the Biomass, Chitin and Chitosan Production by C. elegans

Table 2 present the values of biomass production, pH, chitin and chitosan yields obtained in each trial as a response for the 2^2 factorial design using different CW and CSL concentrations. The responses recorded were biomass production, chitin and chitosan yield by *C.elegans.* An estimate of pure experimental error was calculated from four replicates run corresponding to a central point of the complete factorial (trial 5). These results indicated a higher biomass production (9.93 g/L) during 96 h of fermentation of *Cunninghamella elegans* using conditions 3 (5% CW, 8% CSL). This suggests a culture medium with a greater concentration of CSL in relation to cassava wastewater CW concentrations could favor growth by *C. elegans*. CSL consists of amino acids and carbohydrates which influence the growth of Mucoralean fungi [19]. Similar results were reported by Berger *et al.* [6] and Cardoso *et al.* [20] also showed the positive influence in biomass production of the same concentrations of CSL (8%) used in this study. Probably, the considerably higher carbon and nitrogen contents of CSL compared with cassava wastewater CW, as shown in Table 1, favored this result. Other studies also suggest using CSL as a source of carbon and nitrogen in the composition of culture media instead of glucose and yeast extract, for example by using this substrate to produce succinic acid by *Actinobacillus succinogenes* [21]; and to produce a biosurfactant by the yeast *Candida sphaerica* [22].

Table 2. Design matrix for the factorial experiments used to study the influence of factors, cassava wastewater (CW) and corn steep liquor (CSL) concentrations, varied symmetrically around the central point according to the 2^2 levels.

Assays	Cassava wastewater (CW) % (*v/v*)	Corn steep liquor (CSL) % (*v/v*)	pH	Biomass (g/L)	Chitin (mg/g)	Chitosan (mg/g)
1	5.00	4.00	6.98	6.93	69.57	50.12
2	10.0	4.00	6.98	5.67	89.39	57.82
3	5.00	8.00	6.18	9.93	50.09	44.51
4	10.0	8.00	6.20	8.57	60.71	34.47
5	7.50	6.00	6.50	7.16	70.03	47.37
6	7.50	6.00	6.35	8.00	72.40	48.93
7	7.50	6.00	6.23	7.70	76.64	46.00
8	7.50	6.00	6.48	7.31	70.27	45.81

On the other hand, contrary result was observed with condition 2 (10% CW, 4% CSL) which gave the best chitin (89.39 mg/g) and chitosan (57.82 mg/g) yields. Similar result was obtained by Lins *et al.* [19] who obtained the highest chitosan production by *Rhizopus arrhizus* also using 4% of CSL. There was an increase in the pH range from 5.6 (start of fermentation) to 6.98–6.18. A similar result was observed by Cardoso *et al.* [20]. However, the lower pH in Table 2 (6.18 and 6.20) resulted in the higher yields of biomass. Probably this acidic condition is better for the *C. elegans* growth. The influence of the culture media pH in the biomass yield was also observed by Nwe *et al.* [23] and Nwe and Stevens [14] who observed that the slightly acidic pH values are more favorable to the fungal growth.

Therefore a greater CW concentration and a lower CSL concentration favored these response variables. The addition of CSL in culture media improved chitosan production by *Syncephalastrum racemosum*,

but at a 2% concentration [15] which is lower than the concentration shown by trial 2, used in this study. Santos *et al.* [16] also showed the positive effect of this agroindustrial waste as a nutritional source for chitosan production by *Cunninghamella elegans* but using a 0.45% concentration of CSL, which is much less than the 4% concentration used in this study. From these results, considering the high nutritional value of corn steep liquor, it can be suggested that the choice of this waste at lower concentrations could be sufficient to increase chitosan production by *Cunninghamella elegans*.

White *et al.* [24] suggest that the composition of fungal and bacterial cell walls can be attained by genetic manipulation or alteration of culture conditions and consequently, these changes could result in a potential for altering cell wall synthesis in fungi to improve chitosan productivity. The positive influence of high and low concentrations of CW and CSL, respectively, in the production of chitin and chitosan. On the other hand, the growth of the fungus was stimulated in the presence of low concentrations of CW and high concentrations of CSL, a source of nitrogen and carbon.

The best results for biomass, chitin and chitosan production obtained in this study (Table 2) are compared with the literature in Table 3. The different results presented in Table 3 for the chitin and chitosan yields of each microorganism prove that the chitosan content of fungi depends on the fungal strains, the age of the mycelia age, the cultivation medium and the growth conditions and chitin and chitosan extraction method used [2,4,24,25].

Table 3. Biomass, chitin and chitosan production by *C. elegans* grown on agroindustrial waste compared with results obtained by the literature.

Microorganism	Substrate	Biomass ($g\ L^{-1}$)	Chitin ($mg\ g^{-1}$)	Chitosan ($mg\ g^{-1}$)	Reference
Cunninghamella elegans	CSL and CW	9.93	89.39	57.82	This study
Rhizopus arrhizus	CSL and CW	8.80	54.38	20.51	[6]
Rhizopus arrhizus	Synthetic medium for Mucorales	-	92	13	[8]
Rhizopus arrhizus	Modified synthetic medium for Mucorales	-	94	14	[8]
Rhizopus arrhizus	CSL and honey	20.6	-	29.3	[20]
Rhizopus arrhizus	CSL 4%	13.00	30.40	12.85	[19]
Mucor circinelloides	Yam bean	20.70	500	64	[4]
Cunninghamella elegans	Yam bean	24.30	440	66	[2]
Aspergillus niger	Potato dextrose broth	9.00	-	107	[10]
Lentinus edodes	Potato dextrose broth	1.4	-	33	[10]

Table 3. *Cont*.

Microorganism	Substrate	Biomass (g L^{-1})	Chitin (mg g^{-1})	Chitosan (mg g^{-1})	Reference
Zygosaccharomyces rouxii	Yeast malt extract broth	4.4	-	36	[10]
Candida albicans	Yeast malt extract broth	1.8	-	44	[10]
Mucor rouxii	YPD medium	9–14	-	40–80	[24]
Mucor racemosus	YPD medium	15.0	-	35.1	[25]
Cunninghamella elegans	YPD medium	25.0	-	20.5	[25]
Gongronella butleri	Sweet potato pieces and mineral solution and urea	56.3	-	127	[23]

- Data not shown.

Figure 1 presents the influence of CW (1), CSL (2) and the interaction between these factors (1 × 2) in the biomass production by *C. elegans,* using a factorial design, with statistical significance ($p < 0.05$). The increase of CSL percentage provided a higher yield of biomass and the opposite was observed when the percentage of CW was increased. The interaction between these factors had a negative effect on biomass production, but not a statistically significant one. These results suggest that, in future research, the biomass production of *C. elegans* could be increased in a culture media with higher CSL and low CW concentrations.

Figure 1. Pareto graph showing the effect of independent variables on the production of biomass by *Cunninghamella elegans* strain UCP/WFCC 0542. Statistically significant ($p < 0.05$).

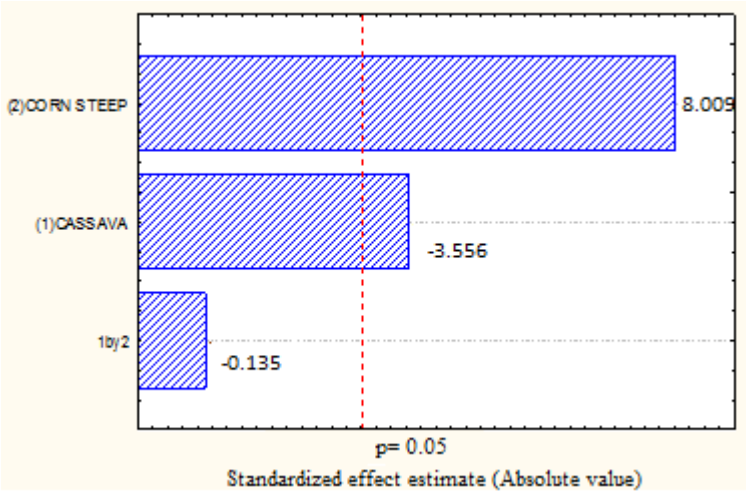


Figure 2 shows contrary results compared to Figure 1 for the influence of CSL and CW in chitin production. The Pareto chart presents the positive and negative influence of CW and CSL, respectively

for chitin production by *Cunninghamella elegans*, with statistical significance ($p < 0.05$). As in Figure 1, the interaction between these two variables did not favor (at least not in a statistically significant way), the production of chitin. These results confirm the data obtained in trial 2 (10% CW, 4% CSL, Table 2).

Figure 2. Pareto graph showing the effect of independent variables on the chitin yield by *Cunninghamella elegans* strain UCP/WFCC 0542. Statistically significant ($p < 0.05$).

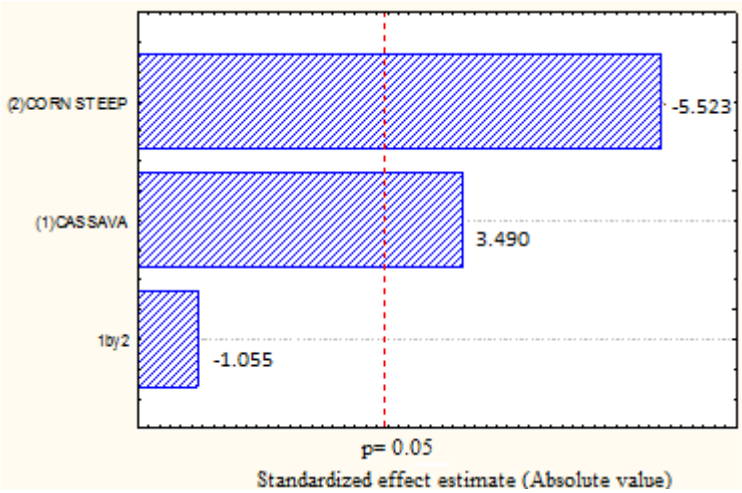
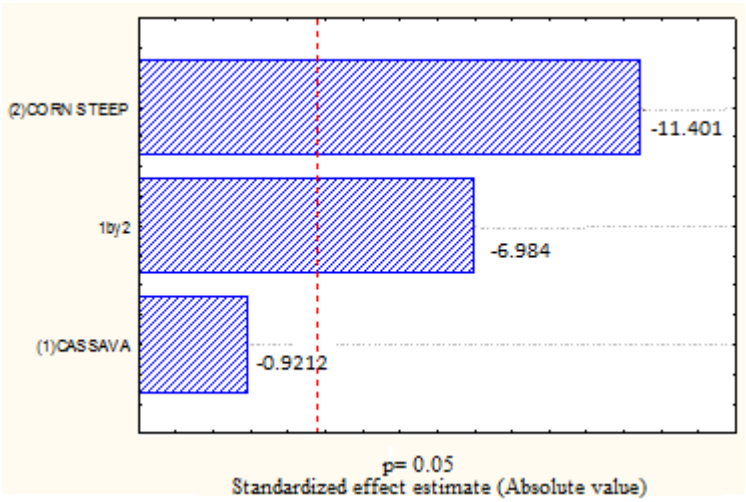


Figure 3 presents the negative influence of CW and CSL, and the interaction between these variables, respectively, for chitosan production by *C. elegans.* An increase in the percentage of the two substrates in the culture medium will not favor the production of chitosan by this fungus, which was statistically significant for CSL and interaction of the two wastes ($p < 0.05$) and of no statistical significance for CW ($p < 0.05$). This suggests the use of these wastes in smaller concentrations in the culture medium to achieve better yields of chitosan.

Figure 3. Pareto graph showing the effect of independent variables on the chitosan yield by *Cunninghamella elegans* strain UCP/WFCC 0542. Statistically significant ($p < 0.05$).



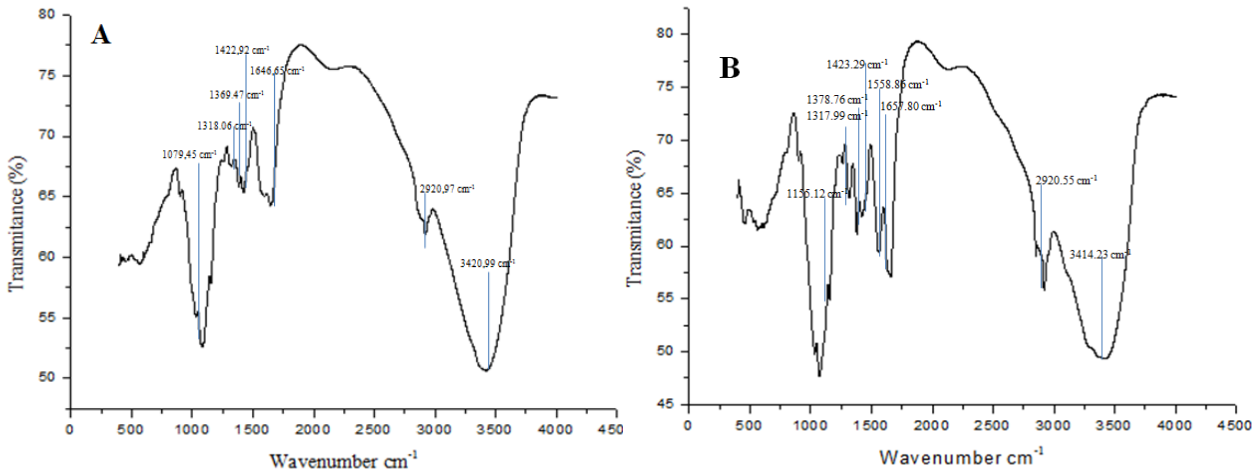
2.3. Characterization of Chitosan Extracted from the C. elegans Strain (UCP/WFCC 0542)

2.3.1. Infrared Spectroscopy (Deacetylation Degree—DD%)

The infrared spectra of the biopolymers obtained from *Cunninghamella elegans* are presented in Figure 4. The infrared spectrum of fungal chitin showed the presence of characteristic bands such as 1,317.99 cm^{-1}, 1,558.86 cm^{-1} and 1,657.80 cm^{-1}, corresponding to CN bond stretching plus CH_3 wagging; N–H deformation in the CONH plane, including amide II; and carbonyl group stretching, C=O (amide I). In a similar way, chitin shows specific bands at 1,155.12 cm^{-1} in the amide II region; 1,378.76 cm^{-1}, corresponding the C-O stretching of the -CH_2-OH group (amide II region); 1,423.29 cm^{-1}, corresponding to the axial deformation of the amide C-N; 2,920.55 cm^{-1}, assigned to C-H stretching; and 3,414.23 cm^{-1}, corresponding to the axial deformation of OH, which appears as overlapping the axial NH deformation band. These results are in agreement with Arbia *et al.* [5]; Chatterjee *et al.* [26]; Ebrahimzadeh *et al.* [27]; Cardoso *et al.* [20]; and Santos *et al.* [28]. The presence of two types of amide group (amide I and II) in the chitin structure is similar to what was observed by Stamford *et al.* [2] and Fai *et al.* [4].

Similar to the infrared spectrum of fungal chitin, the fungal chitosan also presented amide bands as 1,318.06 cm^{-1}, 1,422.92 cm^{-1}, 1,576.13 cm^{-1} and 1,646.65 cm^{-1}, but these peaks are less intense than the in fungal chitin, particularly the peak of 1,646.65 cm^{-1}. Fungal chitosan also presented bands at 2,920.97 cm^{-1} and 3,420.99 cm^{-1}, as observed in the spectrum of fungal chitin. Ebrahimzadeh *et al.* [27] related that during chitosan production, acetyl is eliminated after hydrolysis and, consequently, the carbonyl band gets eliminated in chitosan. However, the infrared spectrum of fungal chitosan with these amide bands, i.e, the presence of an acetyl group on the amino group (stretching, C=O, amide I) shows that the fungal chitosan is not completely deacetylated. The same results were observed by Mario *et al.* [11] who related that, as expected, *N*-deacetylation is associated with a progressive weakening of the band occurring at 1,655 cm^{-1}(amide I vibrational mode) and the disappearance of the band at 1,550 cm^{-1} (amide II vibrational mode).

Figure 4. Infrared absorption spectra of microbiological polymers obtained from *Cunninghamella elegans* (**A**) Chitosan; (**B**) Chitin.



The infrared spectrum of chitin and chitosan is one of the analytical methods used to define the Deacetylation Degree (DD%). The DD% is an important parameter that determines the physicochemical properties of the chitosan because it is linked to the cationic properties of chitosan [4]. The DD% value is directly proportional to the positive charge density on the molecule which confers unique and greater ability to the chitosan for specific industrial, medical or pharmaceutical applications, such as its use as a

coagulation agent in physical and chemical waste-treatment systems and as an antimicrobial agent [27,29,30]. The infrared spectra of the chitin and chitosan obtained by *C. elegans* in trial 2 of the 2^2 factorial experimental design and commercial chitin and chitosan were used to determine the degree of deacetylation. The DD% calculated for fungal chitin and chitosan were 40.98% and 88.24%, respectively. The fungal chitosan value is comparable with the literature, as verified in Table 4.

2.3.2. Viscosity and Molecular Mass

The viscosity of fungal chitosan from *Cunninghamella elegans* (UCP/WFCC 0542) was 3.34 centipoises (cP), considerably lower than the viscosity of crab chitosan with medium (MMW) and mow (LMW) molecular weight (29.7 and 287.2 cP, respectively).

These results were similar to those reported by Pochanavanich and Suntornsuck [10] who stated that the viscosity of fungal chitosan between 3.1 cP to 6.2 cP and commercial crab shell chitosan (Sigma) was 372.7 cP. Khalaf [29] obtained a fungal chitosan with 2.7–6.8 cP and crab shell chitosan (Sigma) with 316.2 cP. These results are presented in Table 4.

Table 4. Properties of fungal and crab chitosan.

Chitosan sample	DD * (%)	Viscosity (cP)	MW ** (Da)	Reference
Cunninghamella elegans UCP/WFCC 0542	82.24 ± 2.0	3.34	5.00×10^3	This study
Crab shell (Sigma)	97.9 ± 0.9	372.7	9.4×10^5	[10]
Aspergillus niger TISTR3245	90.9 ± 2.1	6.2	1.4×10^5	[10]
Rhizopus oryzae TISTR3189	87.9 ± 2.1	3.5	6.9×10^4	[10]
Candida albicans TISTR5239	83.8 ± 0.8	3.1	1.1×10^5	[10]
Crab shell (Sigma)	96.8	316.2	-	[29]
Aspergillus niger	84.2	5.9	-	[29]
Penicillium citrinum	78.5	4.6	-	[29]
Fusarium oxysporum	73.4	2.7	-	[29]
Rhizopus oryzae	90.2	6.8	-	[29]
Penicillium waksamanii	65.1 ± 3.1	11.3 ± 0.6	-	[29]
Penicillium citrinum	62.4 ± 2.7	10.2 ± 0.4	-	[29]
Penicillium viridicatum	47.5 ± 1.9	8.9 ± 0.2	-	[29]
Penicillium aurantiogriseum	47.3 ± 2.3	9.4 ± 0.2	-	[29]
Mucor rouxii	27.3	-	-	[24]
Rhizopus oryzae	91.5	7.2	-	[30]
Aspergillus niger	89.6	6.4	-	[30]
Penicillium expansum	80.2	4.8	-	[30]
Fusarium moniliforme	75.3	3.6	-	[30]
Crab shell (Sigma Aldrich)	96.8	316.2	-	[30]
Gongronella butleri	92.0	-	3×10^4	[23]
Mucor rouxii	82.8–89.8	-	2.48×10^4–5.59×10^4	[26]

- Data not shown, ***** DD (%) = Degree of deacetylation (%), ****** MW = Molecular Weight

The viscosity of the chitosan is directly related with the molecular weight of this biopolymer [30]. High molecular weight chitosan has a higher viscosity than low molecular weight chitosan. Probably, this means that the molecular weight of fungal chitosan may be lower than that of crab chitosan.

This was confirmed with the result obtained for the average viscosimetric molecular weight (M_V) of chitosan from *Cunninghamella elegans* obtained in this study, which was 5.2×10^3 g/mol, or low molecular weight. The result is also in agreement with the literature, which reports molar weights ranging between 1.0×10^3 to 9.0×10^5 g/mol [2,6].

Thus fungal chitosan could have potential food, medicinal and agricultural applications as an antimicrobial and preservative agent [10,29]. Omogbai and Ikenebomeh [30] related that the vicosity of chitosan is an important factor which determines its commercial applications and significantly affects its antimicrobial activities. Furthermore, some applications of chitosan are limited by its high molecular weight and viscosity, resulting in low solubility in aqueous solutions such as medical applications which require a low molecular weight chitosan with a high solubility and low viscosity in water at physiologically acceptable pH values [31].

The chitosan of low molecular weight and high DD has a large charge density and a high solubility and is useful in pharmaceuticals, biomedicals and food [23]. In addition, chitosan with a low molecular weight was reported to reduce the tensile strength and elongation of the chitosan membrane, but to increase its permeability. This characteristic can be promising for specific applications [32]. The chitosan with a low viscosity was also reported to have more antimicrobial activity [33]. Ebrahimzadeh *et al.* [27] on relating the results of their study showed that the viscosity of extracted chitosans increased with the increase in DD, but there were some exceptions.

2.3.3. Elementary Analysis

Table 5 shows the carbon, nitrogen and hydrogen percentages of Sigma chitin and chitosan from crustacea, used as standard, and that extracted from *Cunninghamella elegans* under condition 2, which showed the highest yield of these biopolymers.

Table 5. Percentage of carbon, nitrogen, hydrogen, carbon/nitrogen and degree of deacetylation of chitin and chitosan samples of crustacean (standard) and extracted from *Cunninghamella elegans*.

Sample	% N	% C	% H	C/N
Crustacean Chitin (Sigma)	5.30	40.25	7.11	7.59
Crustacean Chitosan (Sigma)	7.03	37.88	6.59	5.39
Fungal Chitosan	5.59	30.52	7.66	5.46
Fungal Chitin	5.27	40.69	9.13	7.53

The fungal chitosan 2 showed lower nitrogen (5.59%) and carbon (30.52%) content than the Sigma crustacean chitosan (7.03% of N, 37.88% of C) and the crustacean chitosan (41.2% to 44.5% carbon and 7.0% to 8.5% nitrogen) obtained by Santos *et al.* [16]. However, the C/N ratio of the fungal chitosan (5.46) is similar to the calculated values of C/N obtained by Santos *et al.* [28].

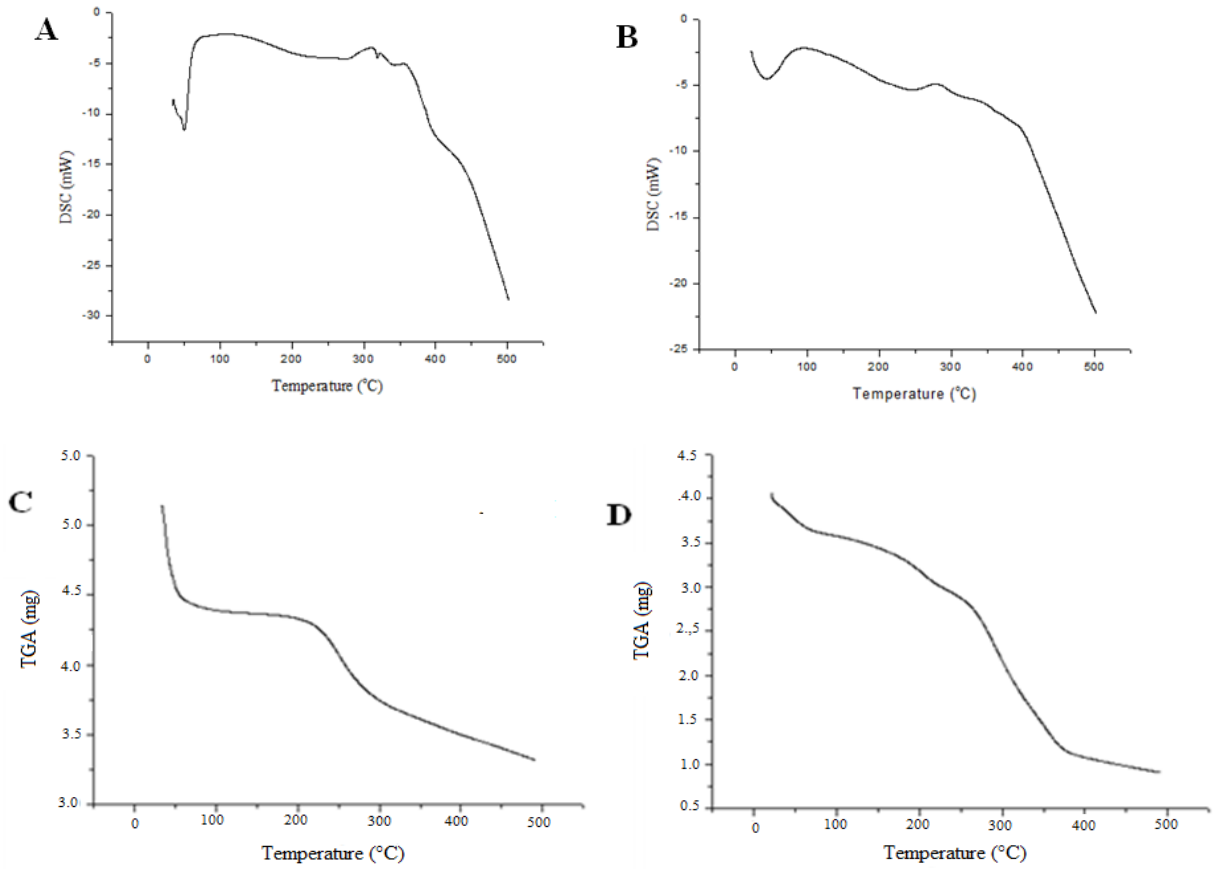
The nitrogen content of chitosan increases with a longer deacetylation reaction and a more efficient deacetylation of chitin with a higher degree of deacetylation of chitosan. This was also observed by Yen *et al.* [34] who treated the crustacean chitin with 30 ml of 40% sodium hydroxide solution at 105 °C for 60, 90 and 120 min to obtain the chitosan denominated: C60, C90 and C120, respectively. Chitosan C120 showed the higher nitrogen content (9.5% ± 0.2%) and degree of deacetylation

(93.3% ± 0.4%). Probably, in this study, the degree of deacetylation (82%) and nitrogen content (5.59%) of the fungal chitosan 2 were consequences of the low sodium hydroxide solution concentration (1M or 4% NaOH) and of the short period (15 min) of deacetylation used, when compared with the methodology by Yen *et al.* [34]. In addition, the carbon content of chitosan is lower than that of chitin due to the loss of acetamido groups during the deacetylation reaction, and the lower the carbon content of chitosan, the higher the degree of deacetylation. A similar result is observed in fungal chitosan 2 with a lower carbon content (25.10%) than fungal chitin 2 (39.69%).

2.3.4. Thermal Analysis

The thermal properties of chitin and chitosan samples obtained from TGA–DSC over a temperature range of 0–500 °C are illustrated in Figure 5.

Figure 5. Microbiological biopolymers from *Cunninghamella elegans* UCP/WFCC 0542: DSC curves for chitosan (**A**), chitin (**B**) and TGA thermograms for chitosan (**C**) and chitin (**D**), under continuous flow of dry nitrogen gas (50 mL min^{-1}), at a heating rate of (10 °C min^{-1}).



The thermograms were characterized by the peak temperature of endotherms corresponding to water evaporation, which depends on the sample drying process, and the peak temperature of exotherms representing the decomposition of amine units in the polymer as related by Sreenivasan [35] and Santos *et al.* [28]. The first registered thermal event was an endothermic peak at 51.38 °C and 42 °C for

chitosan and chitin, respectively; and these peaks are expected to reflect physical and molecular changes during N-deacetylation and carboxymethylation [4]. Each endo- or exothermic peak temperature and area changed as a function of primary and higher order structures of the macromolecule [36]. These biopolymers showed a similar trend in DSC and TGA but the chitin had higher thermal stability than the corresponding chitosan. Consequently, the endothermic peak area was higher for chitosan, 310.25 °C (Figure 5A) than for chitin, 280.57 °C (Figure 5B), *i.e.*, the endothermic peak area increased with the increase in N-deacetylation and carboxymethylation, indicating that a definite correlation exists between the water holding capacity and chemical and supramolecular structure of these polymers, as observed by Zhang *et al.* [37] and Yen and Mau [38]. The DSC was used as an effective technique to correlate the heat of the reaction to the degree of deacetylation (DD%) and carboxymethylation [36].

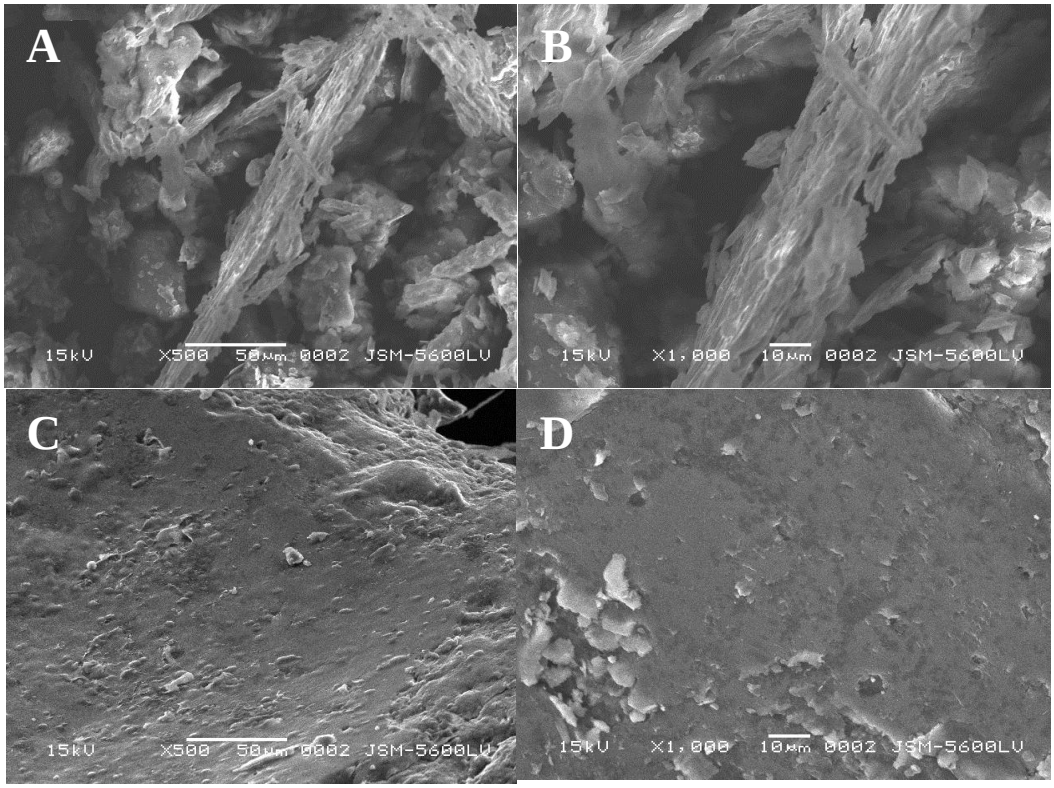
The two DSC processes are consistent with the trend observed in the TGA curves. The TGA measurements indicate that chitosan and chitin samples lost water at a relatively low temperature (below 100 °C, Figures 5C,D). This means that this water is physically adsorbed and/or weakly hydrogen-bonded to chitosan molecules (stage 1), as shown by Zawadzki and Kaczmarek [39]. The second stage, which began at 172 °C for chitosan and at 109 °C for chitin, is due to the decomposition temperature of these polymers with a carbonized residue formation [4]. Most probably in this stage, the hydrogen-bonded water is released [39]. The third weight loss point was above 300 °C which represents the start of carbonized material consumption, a result similar to that found by Fai *et al.* [4] and Liu *et al.* [40].

2.3.5. Scanning Electron Microscopy

The chitin and chitosan produced by *Cunninghamella elegans* growth in trial 2 were selected for examination by scanning electron microscopy (SEM, Figure 6). The chitin showed a prominent arranged microfibrillar crystalline structure (Figure 6A,B) which was absent in the chitosan (Figure 6 C,D). Similar results were observed in crustacean chitin by Yen *et al.* [34], and Arbia *et al.* [5], and in fungal chitin by Chan, Chen, and Yuan [41]. Also, fungal chitosans did not show the microfibrillar structure in SEM [38]. Yen *et al.* [34] related that the crystalline structure observed between fungal and crab chitins might also be attributed to their different intersheet or intrasheet hydrogen-bonding systems. However, the preview of this microfibrilar structure in the chitin produced by *C. elegans* may have been caused by the process for extracting this biopolymer *i.e.* the deproteinization step.

The fungal chitosan also exhibited a structure that was more compact and dense than the fungal chitin nor was this chitosan porous. This biopolymer also showed crumbling layers of flakes as observed in crustacean chitosan by Yen *et al.* [34] and few aggregated flakes with a dense and firm structure, without porosity, as related by Yen and Mau [38] for fungal chitosan. These authors compared different chitosans by SEM after a longer N-deacetylation step that used a sodium hydroxide solution at 105 °C, for 60, 90 and 120 min, and they stated that it seems that the longer the deacetylation, the more clearly the cloudy and fibrillar crystalline structure of chitosans was observed. Probably, the same structure could be observed in the fungal chitosan obtained in this study if deacetyation were to be more prolonged. The fungal chitosan obtained in this study also showed rough surfaces, devoid of a recognizable irregular spatial pattern.

Figure 6. Microbiological biopolymers from *Cunninghamella elegans* UCP/WFCC 0542: SEM electromicrographies of (**A**) chitin at 500 × magnification, (B) chitin at 1000 × magnification, (C) chitosan at 500× magnification and (D) chitosan at 1000 × magnification. The measurement bar = 50 µm.



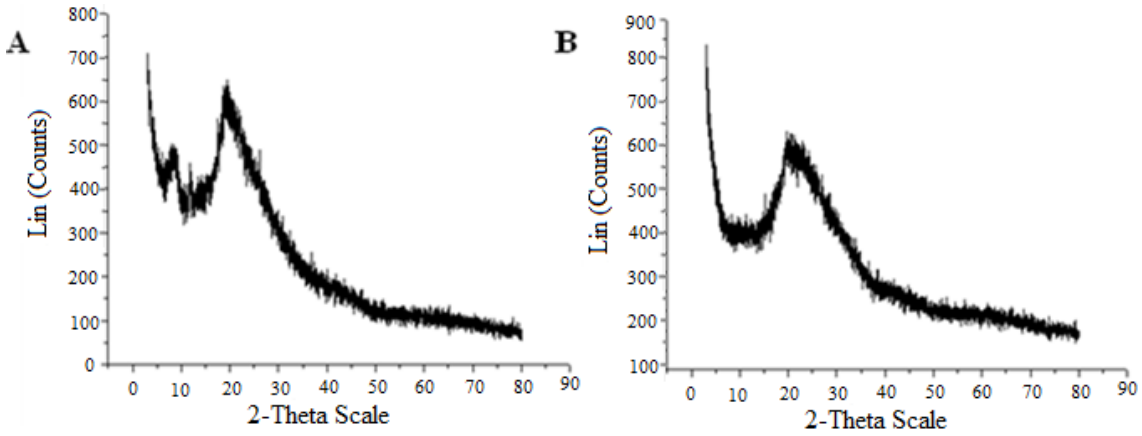
2.3.6. X-ray Diffraction

The specifics in the polymorphic form and crystalline structures of chitin and chitosan were determined by X-ray diffraction. These patterns are indicative of different spacing in the crystal planes, and a polymorphic structure. Diffraction provides accurate measurements of the crystalline content, which greatly affects physical and biological properties of the polymer [4,16,26]. The powder diffraction pattern of chitosan (Figure 7A) from *Cunninghamella elegans* grown in trial 2 (Table 3) showed strong Bragg refractions at an angle of 20.0° 2θ and 9.0° which are two characteristic peaks of chitosan, as observed by Wang *et al.*[42]. However, one peak at about 9.00° disappeared in the fungal chitin. Probably a purification process would be necessary to obtain a satisfactory biopolymer, and a similar result was described by Zhang *et al.* [37].

The crystallinity indices of chitin and chitosan grown from *C. elegans* in the best trial 2 of the factorial design and of commercial chitin and chitosan (Sigma) were determined from the scattering intensity at two angles, one at 2θ= 9–10° and the other at 2θ =19–20° (Figure 7). The results are supported by the literature [26,37], in which the crystallinity indices of chitin obtained from *Cunninghamella elegans* biomass in trial 2 (35.80%) and of commercial chitin (45.63%) were higher than the chitosan from *C. elegans* biomass in trial 2 (21.17%) and commercial chitosan (23.82%). The higher crystalline index of chitin reflected its higher degree of crystallinity and a more ordered structure [43]. This crystallinity is reinforced by the prominent arranged microfibrillar crystalline structure

of chitin in SEM (Figure 6A,B). The lower crystallinity of chitosan indicates disruption of intra- and inter- molecular hydrogen bonds, and the lower crystallinity of fungal chitin and chitosan produced by *C. elegans* might indicate their improved water solubility in comparison with commercial chitin and chitosan, probably due to the more severe extraction conditions during thermochemical extraction, as related by Zhang *et al.* [37]. This lower crystallinity of fungal chitosan in relation commercial chitosan to is supported by the intensity of its peak which was also less than the peak of commercial chitosan around 20°. With these results, and based on the literature, it can be concluded that fungal chitosan displays an organized reticular structure and its crystallinity is related to the DD% function [4,26].

Figure 7. X-ray diffractograms of chitosan (**A**) and chitin (**B**) obtained from *Cunninghamella elegans*.



2.4. Cytotoxicity test using chorioallantoic membrane (HET-CAM test)

The cytotoxicity of chitin and chitosan were verified using the HET-CAM test. Cytotoxicity was evaluated for development of irritation symptoms, such as haemorrhage, coagulation (intra- and extravascular protein denaturation) and vasoconstriction, when the test substances were added to the membrane and left in contact for 5 min (Table 6). The polymers were shown to be non-irritating (IS = 0.0) because they did not prompt vasoconstriction, haemorrhage or coagulation in the CAM within 5 min.

The CAM has been proposed as a model for a living membrane because it has a functional vasculature. The acute effects induced by a test substance on the small blood vessels and proteins of this soft tissue membrane are proposed to be similar to those of the rabbit eye test, while offering the advantages of being more universally acceptable as it is a non-animal test and is completed more rapidly [44]. Several studies have been conducted to evaluate the feasibility of using HET-CAM as a complete replacement for the *in vivo* rabbit ocular test. This test has several advantages, including due to its being simple, rapid, sensitive, easily performed and relatively cheap [45,46].

In Germany and France, HET-CAM has been officially accepted as a valid *in vitro* assay, at least for predicting several irritating substances [47]. Current laws regulating animal experimentation allow protocols that use chick embryos without authorisation from animal experimentation committees;

however, in the UK, the British Animal Welfare Act (1986) states that an embryo egg up to 10 days of gestational age can be used as a non-animal test [44,45].

Table 6. Cytotoxicity of chitin and chitosan evaluated for the development of irritant endpoints: vasoconstriction, haemorrhage and coagulation.

Assays	Chitin	Chitosan	1% SLS
Vasoconstriction	0.0	0.0	6.0 ±1.0
Haemorrhage	0.0	0.0	48 ±3.0
Coagulation	0.0	0.0	63 ±3.0
Irritation potential	0.0	0.0	17.74 ± 0.4

Non-irritating: 0–0.9; slightly irritating: 1–4.9; Irritating: 5–8.9 and severely irritating: 9–21; Positive control: 1% sodium lauryl sulfate (SLS); mean values (%) in quintuplicate

3. Experimental

3.1. Microorganism and Maintenance

A *Cunninghamella elegans* strain UCP/WFCC 0542 was isolated from mangrove sediments situated in Rio Formoso, Pernambuco State of Brazil, and belongs to the Culture Collection of the "Universidade Católica de Pernambuco" (UCP), located in the Nucleus of Research in Environmental Sciences, Catholic University of Pernambuco, Brazil —NPCIAMB/UNICAP. The Culture Collection is registered in the World Federation for Culture Collection (WFCC). The fungus is maintained on potato dextrose agar (PDA) medium at 5 °C. The culture is transferred to a new medium every four months.

3.2. Chemicals, Cassava wastewater (CW) and Corn Steep Liquor

All reagents used were of analytical grade. The acetic acid and NaOH were obtained from Vetec (São Paulo, Brazil), and the crustacean chitin and chitosan from Sigma Aldrich (St. Louis, MO, USA). The tropical residue cassava wastewater (CW) was kindly provided from local industry and corn steep liquor (CSL), which is a byproduct of the corn manufacturing industry (kindly donated by Corn Products do Brasil, Cabo de Santo Agostinho, PE, Brazil) was used as the soluble substrate. These agro-industrial wastes were used as the carbon and nitrogen source as per the 2^2 factorial designs.

3.3. Elementary analysis *of Cassava wastewater (CW) and Corn Steep Liquor*

The elementary analysis of CW and CSL was carried out on a model EA 1110 Carlo Erba Instruments (city, country) elemental analyzer.

3.4. Biomass Production by C. elegans

Cunninghamella elegans was grown in Petri dishes (9 cm in diameter), containing PDA medium at 28 °C for 8 days. A suspension was prepared and counted to 10^7 sporangioles/mL, using a hematocytometer. Petri dishes with PDA were inoculated with 1 mL of the sporangiole suspension and maintained for 18 h at 28 °C. At the end of the desired incubation period a total of 20 discs of PDA medium (1 cm diameter) with the mycelium of *C. elegans* were inoculated in Erlenmeyer flasks

containing 200 mL of the alternative medium, pH 5.6, with varying levels of corn steep liquor (CSL) and cassava wastewater (CW) concentrations. These parameters were varied symmetrically around the central point according to the 2^2 factorial design (Tables 1 and 2). The flasks were incubated at 28 °C in an orbital shaker at 150 rpm, for 24 h. Thereafter, the culture used as pre-inoculum was transferred to the Erlenmeyer flasks with 150 mL of the alternative medium, pH 5.6 and incubated at 28 °C in an orbital shaker at 150 rpm for 72 h. The mycelia were harvested, washed twice in distilled water by filtration, using a nylon membrane silkscreen (120 F), and underwent lyophilization. Afterwards, the biomass was maintained in a vacuum dissector until constant weight.

3.5. Factorial design

A 2^2 full factorial design was carried out to analyze the main effects and interactions of cassava wastewater (CW) (5%–10%) and corn steep liquor (CSL) (4%–8%) on the response variable of biomass, chitin and chitosan yield by *C. elegans* and to select the best condition for the production of the mycelia and biopolymers as per the variables established (Table 7). The Pareto diagrams were compiled to validate the influence between these agro-industrial wastewaters (independent variables) and the response variables. An estimate of pure experimental error was calculated from four replicates run corresponding to a central point of the complete factorial. The data obtained from the experiments were subjected to statistical analysis by STATISTICA software version 7.0 (StatSoft Inc., Tulsa, OK, USA) and the significance of the results was tested at $p < 0.05$ level.

Table 7. Design matrix for the factorial experiments used to evaluate the influence of 2 factors (cassava wastewater (CW) and corn steep liqu**o**r) on biomass, chitin and chitosan production by *Cunninghamella elegans* UCP/WFCC 0542, with experimental conditions set at the mean of two extreme levels (−1 and +1) and a central point (0).

Independent Variable	Factor levels		
	−1	**0**	**+1**
Cassava wastewater (CW) % (v/v)	5.0	7.50	10.00
Corn steep liquor (CSL) % (v/v)	4.00	6.00	8.00

3.6. Determination of pH

After culture, the pH of the cell-free metabolic liquid was determined by potentiometry. All experiments were performed in triplicate.

3.7. Chitin and Chitosan Extraction

The extraction of chitin and chitosan was carried out using dry biomass of *Cunninghamella elegans* following the methodology of Hu *et al.* [48]. After drying the biomass, it was treated with 1 M NaOH solution (1:30 *w/v,* 121 °C, 15 min). Alkali-insoluble material was obtained by centrifugation (4,000 g, 20 °C, 10 min), and extracted using 2% of acetic acid (1:30 *w/v*, 100 °C, 15 min) followed by centrifugation at 4,000 g, 20 °C, 15 min. The supernatant was obtained and the pH was adjusted to 10, and maintained overnight at 5 °C, until the chitosan fraction was precipitated. The chitosan was

obtained by centrifugation at 4,000 g, and washed with distilled water four times, freeze-dried, and kept in a dissecator until constant weight.

3.8. Characterization of chitin and chitosan

3.8.1. Infrared Spectroscopy (Deacetylation Degree–DD%)

The degree of deacetylation (DD%) for microbial chitin and chitosan were determined using infrared spectroscopy as per Baxter *et al.* [49], using the absorbance ratio A1655/A3450 and calculated as shown in Equation (1):

$$DD\ (\%) = 100 - [(A1655/A3450) \times 115] \quad (1)$$

Two milligram samples of fungal chitin and chitosan, which had been dried overnight at 60 °C under reduced pressure, were thoroughly blended with 100 mg of KBr, to produce 0.5 mm thick disks. The disks were dried for 24 h at 110 °C under reduced pressure. Infrared spectrometry was undertaken with a Bruker 66 Spectrometer (Bruker Optics, Ettlingen, German), using 100 mg KBr disks for reference.

3.8.2. Viscosity

The viscosity of 1% chitosan in 1% acetic acid solution was determined using a Brookfield digital rheometer (Model DV-II, Brook Engineering Laboratories, Inc., Stoughton, MA, USA) at 25 °C, Spindle CPE-40, 0.5 mL sample volume.

3.8.3. Molecular Weights of Chitosan

The molecular weights of chitosan were determined by viscosity, using the procedure described by Fai *et al.* [4]. The viscosity of chitosan was determined using an AVS-350 viscometer (Schott-Geräte, Mainz, German), type/capillary: Cannon-Fenske d_{inside}= 1.01mm, at 25 °C. After obtaining the intrinsic viscosity from tables, K and a, were obtained for HAc/NaAc. K = 0.076, a = 0.76. The flow time was determined in seconds. Using the Mark-Houwinks equation, the average viscosimetric molecular weight was expressed in g/mol.

3.8.4. Thermal Analysis

Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC) were carried out using Shimadzu model 50WS and Shimadzu model DSC-50WS thermal analysis instruments, respectively (Shimadzu, Kyoto, Japan). An accurately weighed (10 mg) chitosan sample was placed in an aluminum cup and sealed. The experiment consisted of heating the samples from 0 to 400 °C under the continuous flow of dry nitrogen gas (50 mL·min^{-1}), at a heating rate of 10 °C·min^{-1}.

3.8.5. Elemental Analysis

The elemental analysis was carried out on the elemental analyzer model EA 1110 Carlo Erba Instruments equipment and approximately 3 mg of chitin and chitosan was used. The elemental analysis was also used to determine the percentage of carbon, hydrogen and nitrogen.

3.8.6. Scanning Electron Microscopy

The dried sample was ground under vacuum using a sputter coater and its surface was observed in a scanning electron microscope, Series XL 30 (Umax) Environmental Scanning Electron Microscopy (ESEM) equipped with a tungsten filament using a 20 kV accelerating voltage**.**

3.8.7. Crystallinity Index

The X-ray diffractograms of chitin and chitosan were obtained in the X-Ray Laboratory of the Physics Department—Federal University of Pernambuco—UFPE. The measurement was taken using Siemens Model 5000 D X-ray equipment (Siemens, Munich , Germany), Cu Kα radiation with λ = 1.542 Å, in a scanning range between 4° and 50° with a rate of 0.02 min^{-1}. The interplanar distance was determined by the width of the half peak height of greatest intensity (IC). The crystallinity index (ICR) was determined using the following equation:

$$\text{Crystallinity index (\%)} = 100\{[I(\theta c) - I(\theta a)] / I(\theta c)\} \quad (2)$$

where I (θc) is the relative intensity of the crystalline (2θ = 20°) and I (θa) corresponds to amorphous regions (2θ = 12°) for chitosan.

3.9. Cytotoxicity Test Using Chorioallantoic Membrane (HET-CAM Test)

To evaluate the cytotoxicity and biocompatibility of chitin and chitosan, Hen's Egg Tests (HETs) were performed on the chorioallantoic membrane (HET-CAM) as per the methodology described by Steiling *et al.* [47]. All assays were repeated five times. Membranes were observed for 5 min for signs of vasoconstriction, haemorrhage and coagulation. The time (in seconds) at which the indicated processes began were applied in Equation (1) [50]:

$$\frac{(301 - hemorrhage)5}{300} + \frac{(301 - lysis)7}{300} + \frac{(301 - coagulation)9}{300} \quad (3)$$

After application of the formula above, it was possible to quantify the observed potential for irritation (irritation score-IS) and to obtain means and standard deviations for the analysis as follows: 0–0.9 no irritation, 1–4.9 slight irritation, 5–8.9 moderate irritation and 9–21 severe irritation [50].

4. Conclusions

Considering the results obtained with corn steep liquor (CSL) and cassava wastewater (CW) these renewable substrates may be used as alternative substrate sources for chitin and chitosan production by *Cunninghamella elegans*. The use of tropical residues as nutrients for the large-scale production of these biopolymers is viable, economic and environmentally friendly. The microbiological chitin and chitosan obtained are not irritating, are biocompatible and have chemical properties that enable it to be used for biotechnological applications. The fundamental data obtained contributes to our understanding of the abilities and potential of *C. elegans*, and effectively reduced the costs of chitin and chitosan production.

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Conflicts of Interest

The authors declare no conflict of interest.

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QUARTO ARTIGO

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Article

Green Conversion of agroindustrial wastes into Chitin and Chitosan by *Rhizopus arrhizus* and *Cunninghamella elegans* Strains

Lúcia Raquel Ramos Berger[1,2]; Thayza Christina Montenegro Stamford[3]; Thatiana Montenegro Stamford-Arnaud[3]; Sergio Roberto Cabral de Alcântara[4]; Antonio Cardoso da Silva[2]; Adamares Marques da Silva[1,2] ; Aline Elesbão do Nascimento[2]; Galba Maria de Campos-Takaki[2,*]

[1] Post-Graduation Program in Biological Sciences, Federal University of Pernambuco. 50670-420, Recife, PE, Brazil, E-mail: quelberger@hotmail.com (L.R.R.B.); adamaresmarques@hotmail.com (A.M.S.)

[2] Nucleus of Research in Environmental Science and Biotechnology (NPCIAMB), Catholic University of Pernambuco. 50050-590, Recife, PE, Brazil; E-mail: antoniocardoso2000@yahoo.com.br (A.C.S.); E-mail: galba_takaki@yahoo.com.br (G.M.C.T.)

[3] Department of Tropical Medicine, Center of Health Sciences, Federal University of Pernambuco. 50670-420, Recife, PE, Brazil E-mail: thatianaarnaud@hotmail.com (T.S.A.), thayzastamford@yahoo.com.br (T.C.M.S.)

[4] Post-Graduation Program in Development and Environment, Federal University of Paraíba, Campus 1. 58051-900, João Pessoa, PB, Brazil; E-mail: pohlux@gmail.com (S.R.C.A.)

† These authors contributed equally to this work.

***** Author to whom correspondence should be addressed; E-Mail: galba_takaki@yahoo.com.br; Tel.: +55-81-2119-4017; Fax: +55-81-2119-4043.

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Abstract: The present study aims to produce chitin and chitosan by *Cunninghamella elegans* and *Rhizopus arrhizus* strains using green metabolic conversion of agroindustrial wastes (corn steep liquor and molasses). The physicochemical characteristics of the biopolymers and antimicrobial activity were described. Chitin and chitosan were extracted by alkali-acid treatment, and characterized by infrared spectroscopy, viscosity and X-ray diffraction. The effectiveness of chitosan from *C.elegans* and *R. arrhizus* in inhibiting the growth of *Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Escherichia coli* and *Yersinia enterocolitica* were evaluated by determining the minimum inhibitory (MIC) and bactericidal concentrations (MBC). A

higher production of biomass (24.60 g/L), chitin (83.20 mg/g) and chitosan (49.31 mg/g) was obtained by *R. arrhizus*. Chitin and chitosan of both fungi showed similar degree of deacetylation, respectively of 25% and 82%, crystallinity indexes of 33.80% and 32.8% to the chitin, and 20.30% and 17.8% to the chitosan. Both chitosan presented similar viscosimetry of 3.79-3.40cP and low molecular weight of 5.08 $_{x}$ 10^3 g/mol and 4.68 $_{x}$ 10^3 g/mol. The both chitosan showed identical MIC and MBC for all bacteria assayed. These results suggest an environmentally friendly conversion of agroindustrial wastes and economic chitin and chitosan production and the antimicrobial potential of chitosan against pathogenic bacteria.

Keywords: Zygomicetes; polymer; agroindustrial waste; antibacterial activity,

1. Introduction

Chitosan is a natural co-polymers of chitin, composed by units of 2-amino-2-desoxi-Dglycopyranose and of 2-acetamide-2-desoxi-D-glycopyranose interconnected by glycosidic bonds β-1,4 in variable proportions. The first type of units is frequently present in chitosan [1]. Chitin is present as structural element in the exoskeleton of crustaceans, mollusks, annelids, coelenterates, insects, and is also a major component of the fungal cell wall, particularly of Zygomycetes [2,3]. Chitosan is a cationic and linear polymer, naturally found in the cell wall of fungi, mainly in the Mucorales order. Although the main commercial source of chitosan is crustacean shells, some studies have purposed that cultivation of selected fungi could provide an effective source of chitosan for industrial applications [1,2,4].

Recent advances in fermentation technology to the fungal production of chitin and chitosan has received worldwide attention and it's suggested that many of these problems found in the traditional biopolymers extraction can be overcome [5,6]. The use of chitin and chitosan from fungi biomass has great advantages, such as independence from seasonal factors, wide scale production, simultaneous extraction of the polymers, and the fact that the process of chitosan extraction is simple and cheap, resulting in reduced time and cost. Moreover, this strategy avoids protein contamination, particularly from proteins that could cause allergic reactions in individuals with shellfish allergies [4, 7,8].

The biowaste or some industrial by-products such as molasses, corn steep liquor and cassava wastewater can be used as very economic nutritional sources to grow fungi. This alternative favor the obtaining of a byproduct with high value added and decrease the total production costs [9,10]. The molasses cane sugar, a byproduct of the sugar industry, has constituted a large amount of fermentable sugars and is considered a waste of easy handling, low cost, with great potential and many applications at industrial level. By virtue of its composition, the molasses is used mainly as a source of carbon and energy necessary supplement it with nitrogen and some minerals, especially phosphorus and magnesium [11]. The corn steep liquor, a residue from the corn processing industry, has a large amount of amino acids, vitamins and mineral necessary to the growth of microorganisms [2]. Thus the substrates molasses and corn steep liquor could be considered low-cost alternatives to meet the nutrient and energy (carbon, hydrogen, oxygen and nitrogen) requirements for growth of any microorganism.

Chitin and chitosan has emerged as one of promissory functional materials of choice for various modern bio-based industrial applications such as cosmetics, waste water treatment, pharmaceuticals, food industrial, agricultural and environmental sectors. Their characteristics such as non-toxicity, biodegradability, biocompatibility, antimicrobial activity, environmentally safe and easy to obtain favor their current and future applicability [12,13].

Antimicrobial activity of chitosan has been pointed out as one of its most promising properties and this activity depends on its molecular weight, deacetylation degree and methods used to obtain the polymers [14,15]. The antimicrobial activity of chitosan has been pointed out as one of the most interesting properties of chitosan [16]. Several researchers demonstrated that this polysaccharide has antimicrobial action in a great variety of microorganisms, included gram-positive bacteria and various species of yeast [1, 17, 18]. Moreover, chitosan has numerous advantages over other chemical disinfectants since it possesses a stronger antimicrobial activity, a broader range of activity, a higher antibacterial activity even at low concentrations, and a lower toxicity towards mammalian cells [12].

In this point of view, the present study aims to optimization the produce chitin and chitosan by *Cunninghamella elegans* and *Rhizopus arrhizus* using two agroindustrial waste, corn steep liquor and molasses, as alternative low cost source of carbon and nitrogen. The physicochemical characteristics and antimicrobial activity of the chitin and chitosan synthesized are also described.

2. Results and Discussion

4.1. Comparative Influence of the Molasses and Corn Steep Liquor on the Biomass, Chitin and Chitosan Production by C. elegans and R.arrhizus.

The influence of different molasses and corn steep liquor concentrations in biomass, chitin and chitosan yields by *C.elegans* and *R. arrhizus* was observed in this study. Table 1 present the comparative analysis of the results obtained in each assay of the 2^2 factorial design. The molasses and corn steep liquor in the highest concentrations presented directly proportional effect on the increase of biomass production for both fungi. The best yields of biomass by *C.elegans*, 16.00 and 14.47 $g.L^{-1}$, and *R. arrhizus*, 24.60 and 21.00 $g.L^{-1}$, were obtained in the assays 4 (4.00% molasses, 8.00% corn steep liquor) and 6 (2.50 % molasses, 5.00 % corn steep liquor), respectively, which shows higher concentrations of molasses and corn steep liquor. On the other hand, the assay 2 (4% molasses, 2% corn steep liquor), which the molasses concentration is twice higher than corn steep liquor concentration, provided the best yields of chitin by *C elegans* (72.29 mg. g^{-1}) and *R. arrhizus* (83.20 $mg.g^{-1}$).

The greats yields of chitosan, 26.29 $mg.g^{-1}$ and 33.13 $mg.g^{-1}$ from *C.elegans*, and 49.31 $mg.g^{-1}$ and 40.67 $mg.g^{-1}$ from *R. arrhizus* were presented in the culture media 1, consisting of the lower molasses (1%) and corn steep liquor (2%) concentrations, and in the central point with intermediate concentrations (2.5% molasses, 5% corn steep liquor) of these substrates, respectively. Therefore, *R arrhizus* presented biomass and chitosan yields higher than 30% when compared to those obtained by *C. elegans*. The production of chitin by *R. arrhizus* was also 13% higher than the yield obtained by *C. elegans.*

Table 1. Biomass, chitin and chitosan produced by *Cunninghamella elegans* and *Rhizopus arrhizus* on each assays with different concentrations of molasses and corn steep liquor varied symmetrically around the central point according to the 2^2 factorial design. An estimate of pure experimental error was calculated from four replicates run corresponding to a central point of the complete factorial (assays 5-8).

Assays	Biomass ($g.L^{-1}$)		Chitin ($mg.g^{-1}$)		Chitosan ($mg.g^{-1}$)	
	C.elegans	*R.arrhizus*	*C.elegans*	*R.arrhizus*	*C.elegans*	*R.arrhizus*
1	7.41	8.25	64.96	45.72	26.29	49.31
2	9.53	13.15	72.29	83.20	21.40	31.83
3	7.93	17.50	70.40	57.40	25.63	20.14
4	16.00	24.60	50.00	56.46	17.40	25.87
5	14.47	18.00	59.09	73.29	28.33	37.67
6	13.61	21.00	63.00	70.48	26.84	40.67
7	13.58	20.60	69.11	70.53	33.13	39.00
8	13.90	19.30	60.40	69.23	29.43	38.15

There was an decrease in the pH range from 5.6 to 4.41, similar result was observed by Santos et al.[10]. The lower pH resulted in the higher yields of biomass, probably this acidic condition is better for the *C. elegans* and *R. arrhizus* growth. The influence of the culture media pH in the biomass yield was also observed by Nwe et al. [19] and Nwe et Stevens [20] who observed that the slightly acidic pH values is more favorable to the fungal growth. The low pH is optimal for the activity of chitin deacetylase and consequently favors the enzymatic deacetylation of chitin into chitosan, increasing the yield of this biopolymer [21].

The bioprocesses are dependent of culture media and under appropriate and favorable conditions to the maintenance of microorganisms to express their biotechnology potential [22]. Therefore, specific concentrations of molasses and corn steep provided the conditions to obtain satisfactory yields of biomass, chitin and chitosan for *C elegans* and *R. arrhizus* when compared to the values presented in the literature (Table 2). The results obtained in this study compared with the yields of biomass, chitin and chitosan by others microorganisms proves that these biopolymers content depends of the fungal strains, mycelia age, cultivation medium, growth conditions and chitin and chitosan extraction method used [23, 24].

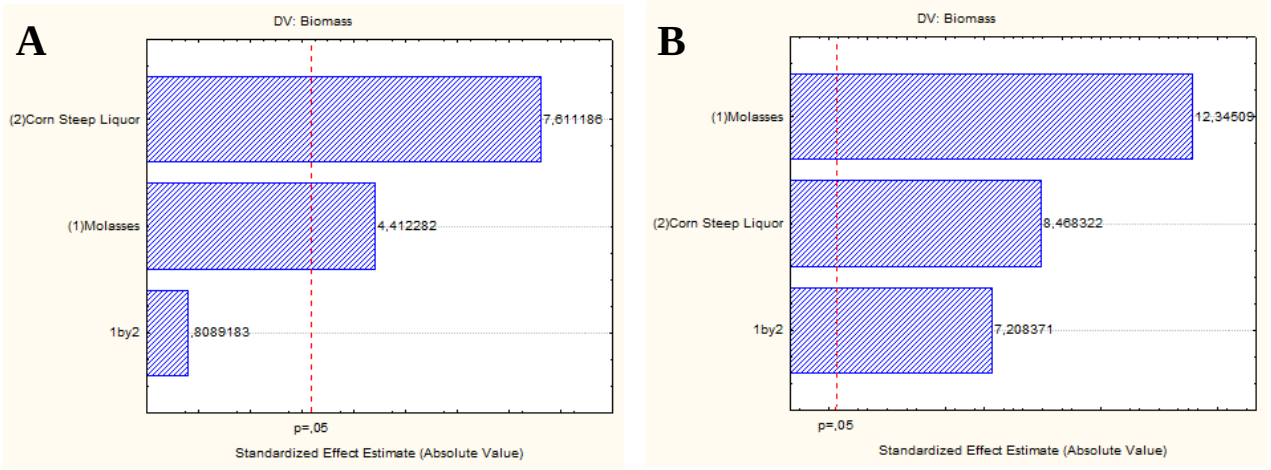
138 **Table 2.** Biomass, chitin and chitosan production by *C.elegans* and *R. arrhizus* grown on
139 Agroindustrial waste compared results obtained by the literature.

Microorganism	Substrate	Biomass ($g.L^{-1}$)	Chitin ($mg.g^{-1}$)	Chitosan ($mg.g^{-1}$)	Reference
Cunninghamella elegans	Corn steep liquor and molasses	16.00	72.29	26.29	This study
Rhizopus arrhizus	Corn steep liquor and molasses	24.60	83.20	49.31	This study
Rhizopus arrhizus	Corn steep liquor and cassava wastewater	8.80	54.38	20.51	[2]
Cunninghamella elegans	Coconut water	2,19	389	129	[2]
Rhizopus arrhizus	Corn steep liquor 4%	13.00	30.40	12.85	[25]
Mucor circinelloides	Yam bean	20.70	500	64	[4]
Cunninghamella elegans	Yam bean	24.30	440	66	[8]
Absidia corymbifera	Candy effluent, corn steep liquor	12.68	12.89 (%) 128.9 mg/g	52.71(%) 520mg/g	[22]

140 - Data not shown

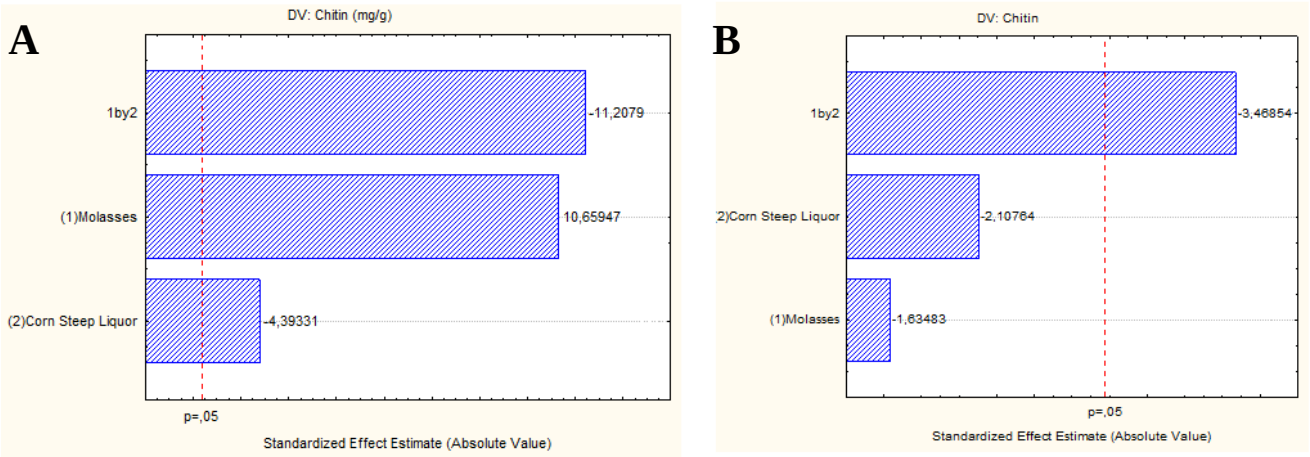
141 The alteration of culture conditions can increase the production of chitin deacetylase, enzyme
142 responsible for the bio-conversion of chitin to chitosan; and consequently it can influence the cell wall
143 synthesis in fungus to improve chitosan productivity [26].
144 The Pareto graph was used to show the effect of the independent variables, molasses and corn steep
145 liquor, on biomass, chitin and chitosan productions by *C. elegans* and *R. arrhizus*. This statistical
146 analysis has proved effective in assessing the influence of independent variables during the search for
147 an optimization of the specific result [5].
148 The Pareto graphics (Figure 1 A and B) shows the positive effect of the molasses and corn steep
149 liquor, including the interaction of these two independent variables on the biomass production by both
150 fungi, as also observed in Table 1, assay 4 (4.00% molasses, 8.00% corn steep liquor) with the higher
151 concentrations of these variables and biomass production.
152

Figure 1. Pareto graphics showing the effect of the independent variables, corn steep liquor and molasses, on the biomass production by *Rhizopus arrhizus* (A) and *Cunninghamella elegans* (B).



In the figure 2A, the Pareto graphics also confirms the positive influence of molasses and the negative influences of corn steep liquor and the interaction between these variables in the production of chitin by *Rhizopus arrhizus*. The same result was showed in the assay 2 (4% molasses, 2% corn steep liquor), which provided the best yields of chitin with only the molasses in the higher concentrations. The Pareto chart, figure 2 (B) shows that lower concentrations of molasses and corn steep liquor favors the increase of chitin produced by *C. elegans*. The same result occurred in the assays 2 and 3 of the factorial design which presented the highest chitin yields for this fungus grown in low concentrations of corn steep liquor (assay 2) or molasses (assay 3).

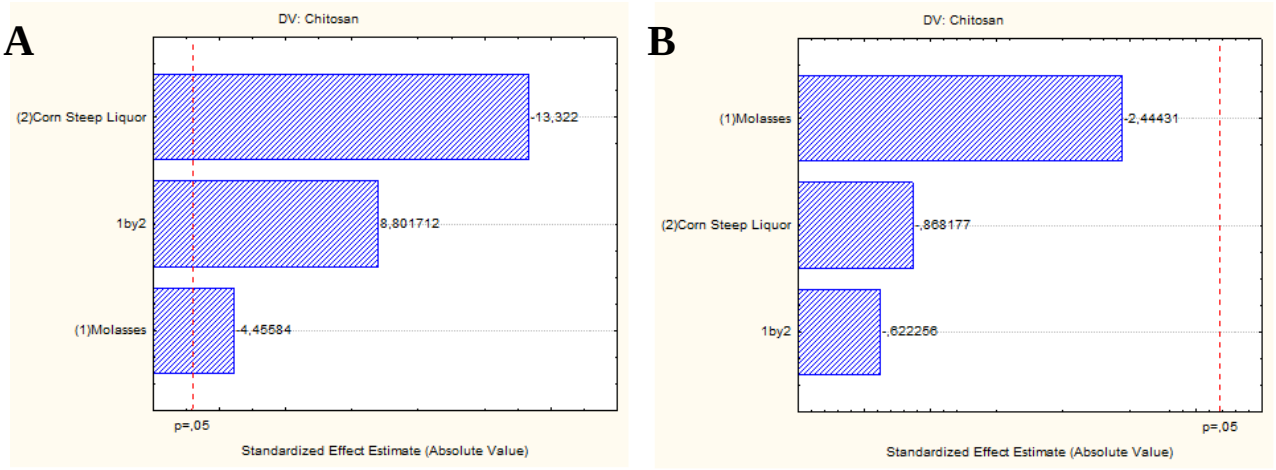
Figure 2. Pareto graphics showing the effect of the independent variables, corn steep liquor and molasses, on the chitin yield by *Rhizopus arrhizus* (A) and *Cunninghamella elegans* (B).



The chitosan results showed in Table 1 were also corroborated by Figure 3, which shows the negative effect in the chitosan production by *C.elegans* with the increase of these substrates. The

decrease of the molasses or corn steep liquor concentrations, or the increase of these two substrates simultaneously in the culture medium promote higher production of chitosan by *R. arrhizus*. However, higher concentrations of corn steep liquor and molasses than those used in the central point shown not to favor the chitosan production (assay 4, Table 1).

Figure 3. Pareto graphics showing the effect of the independent variables, corn steep liquor and molasses, on the chitosan yield by *Rhizopus arrhizus* (A) and *Cunninghamella elegans* (B).



The analysis of these results suggests an economic culture medium, with specific concentrations of molasses and corn steep liquor as sources of carbon and nitrogen, to obtain better yields of biomass, chitin and chitosan by *C. elegans* and *R. arrhizus*. Thus, probably to obtain better biomass production by these fungi would be necessary to increase the concentration of molasses and corn steep liquor. From these fungi, the great chitin yields could be obtained using a culture medium with higher concentrations of molasses and lower concentrations of corn steep liquor. Furthermore, the increase of chitosan production could be achieved using values from molasses or corn steep liquor below to the lower levels of these substrates tested or intermediate concentrations. These great influence of culture conditions and nutritional sources for obtaining satisfactory yields of microbial chitosan were also observed by Pochanavanich and Suntornsuk [24] and Nadarajah et al [27].

Other studies also show increased production of biomass, chitin and chitosan by Mucoralean fungi such *Absidia corymbifera* [25], *Rhizopus arrhizus* [2, 25,28], *C. elegans* [10] and *Syncephalastrum racemosum* [9] in culture medium with specific concentrations of corn steep liquor. Santos et al [10] reported the increase of biomass production by *Cunninghamella elegans* is provided due to the presence of considerable amounts of amino acids (alanine, arginine, histidine, leucine, lysine, tyrosine, phenylalanine) and vitamins (biotin, choline, inositol, niacin, pyridoxine, thiamine) that are essential for the growth of the microorganism. Edwinoliver et al [29] also suggest that the use of corn steep liquor for lipase production from *Aspergillus niger* makes the process green, because this substrate is renewable and economically viable at an industrial scale.

Amorim et al [11] also showed that molasses is an inexpensive carbon sources for the growth and chitosan production by *Cunninghamella bertholletiae* and the authors suggest the cultivation of this fungus by using only these carbon sources without nitrogen supplements resulting in satisfactory

growth and chitosan production. However these authors have also shown that high concentrations of molasses inhibit the production of chitosan, as observed in this study. Amorim et al [11] suggested that the concentration of sugar was not the only factor responsible for the decrease of chitosan in molasses medium. Probably this could be explained by the presence of other substances generated during their different production processes which inhibited the enzyme that produces chitosan, as shown from the highest quantities of inorganic impurities detected by thermogravimetric analyses in chitosan preparations obtained from the *C. bertholletiae* cell growth in molasses.

The controlled use of low cost substrates for replacing or supplementing the culture media, generally used with commercial nutrients, can decrease the final value of byproducts, mainly in large scale production, and in addition this alternative promotes recovery of environments contaminated by agribusiness.

4.2. Characterization of Chitin and Chitosan Extracted from the Cunninghamella elegans and Rhizopus arrhizus

4.2.1. Infrared Spectroscopy (Deacetylation degree–DD%)

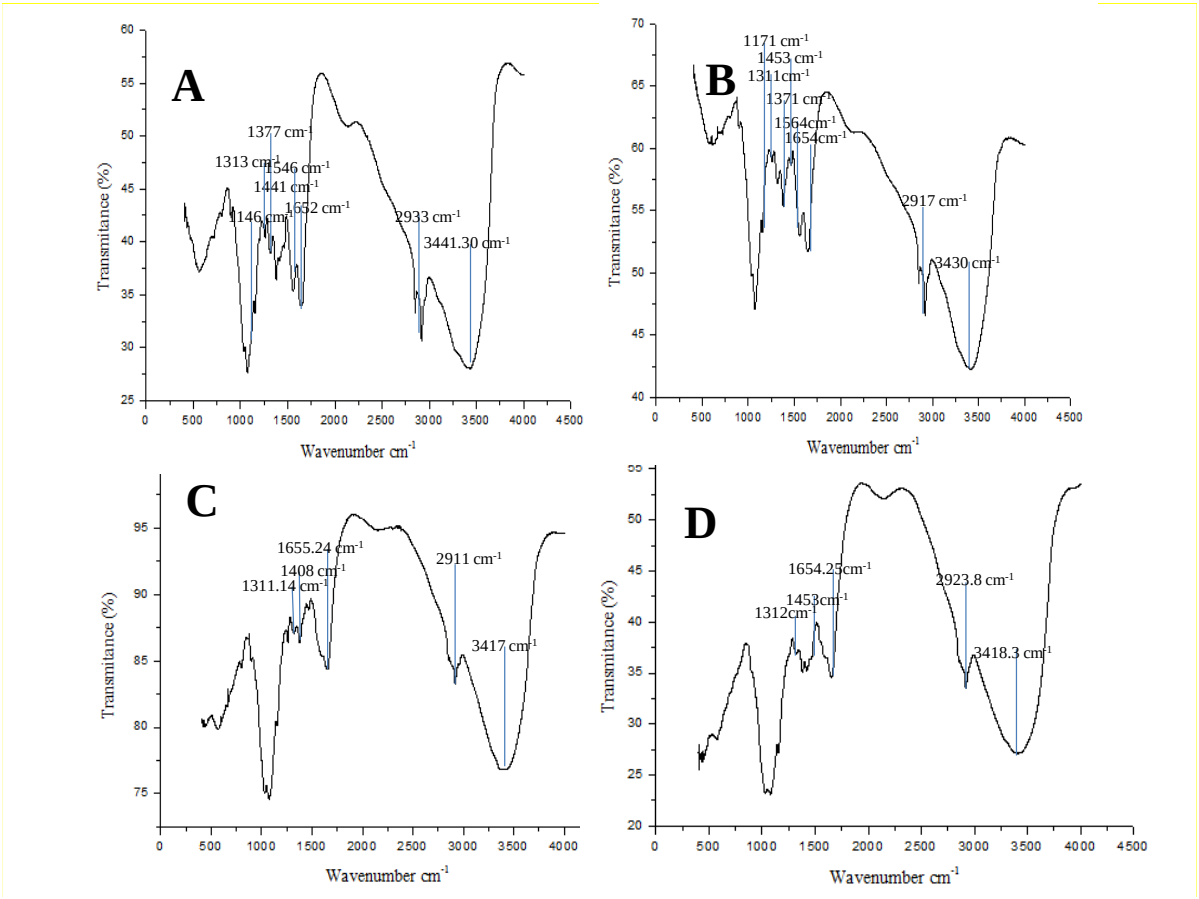
The infrared spectrum of chitin and chitosan from *Cunninghamella elegans* and *Rhizopus arrhizus* (Figure 4) growth in the culture medium of the assay 2 and 1, respectively, were similar to those reported in the literature [5,8, 30]. The chitin showed the presence of two types of amide group (amide I and II). The most significant parts of both chitin was the characteristics bands related to the CN bond stretching plus CH_3 wagging (1311 and 1313 $cm^{-1)}$; the N–H deformation in the CONH plane, including amide II to (1546 and 1564 $cm^{-1)}$; and the carbonyl groups stretching, C=O (amide I) (1652 and 1654 $cm^{-1)}$. In a similar way, chitin shows the specific bands in the amide II region: 1146 cm^{-1} and 1171 cm^{-1}; 1371 and 1377 cm^{-1} (C-O stretching of the -CH_2-OH group); 1441 cm^{-1} and 1453 cm^{-1}, the axial deformation of the amide C-N; 2933 cm^{-1} and 2917 cm^{-1}, assigned to the C-H stretching; and 3441 cm^{-1} and 3430 cm^{-1}, corresponding the axial deformation of OH, which appears overlapping the band of axial NH deformation.

The infrared spectrum of fungal chitosan also presented the most significant amide bands as 1312-1311 cm^{-1}, 1453-1408 cm^{-1}, and 1654-1655 cm^{-1}, but these peaks are less intense than in Fungal Chitin, mainly the peak 1654-1655 cm^{-1}. When compared with the infrared spectrum of chitin, the chitosan spectrum showed the disappearing of the band at 1550 cm^{-1} (amide II vibrational mode) and the progressive weakening of the band 1655 cm^{-1}(amide I vibrational mode) as consequence of the *N*-deacetylation process. Fungal Chitosan also presented the bands 2920.97 cm^{-1} and 3420.99 cm^{-1}, as observed in the spectrum of Fungal Chitin. Ebrahimzadeh et al. [30] related that during the chitosan production, acetyl is eliminated after hydrolysis and, consequently, the carbonyl band gets eliminated in chitosan. However, the infrared spectrum of Fungal Chitosan with these amide bands, i.e, the acetyl in the amino group (stretching, C=O, amide I) shows that the Fungal Chitosan is not completely deacetylated.

Deacetylation Degree (DD%) is an important parameter associated with the physico-chemical properties of the chitosan, because it is linked to the chitosan cationic properties [4, 8, 24]. In the

present study, chitin presented 24.99% and 40% of DD and chitosan showed 80% and 82% DD from *C. elegans* and *R. arrhizus*, respectively which are similar to the reported by the literature [11,23,31].

Figure 4. Infrared absorption spectra (**A**) Chitosan produced by microbiological *Cunninghamella elegans* assay **2**; (**B**) Chitin produced by microbiological *Cunninghamella elegans* assay **2** (C) Commercial chitosan (Sigma).



4.2.2. Viscosity and molecular weight

The viscosity and molecular weight of fungal chitosan were 3.40 centipoises and 4.96 x 10^3 g.mol^{-1} from *Rhizopus arrhizus* and 3.79 (cP) and 5.08 x 10^3 g.mol^{-1} from *Cunninghamella elegans* respectively. The result is in agreement with the literature, which reports molar weights ranging between 1,0 x 10^3 to 9,0 x 10^5 g.mol^{-1} [2, 11]. The method for chitin and chitosan extraction used in this study with high temperatures and NaOH solution may have influenced the breaking of the polymer resulting in a lower molecular weight chitosan. These results are considerably lower than the viscosity of crab chitosan and similar to others fungal chitosan [24]. Some authors related the viscosity of fungal chitosan between 2.7 to 11.3 cP [30,32] and the crab shell chitosan between 316.2 and 372.7 cP [24].

The viscosity of chitosan is directly proportional its molar mass and the results presented in this study corroborate with this statement. The isolate chitosan showed low viscosity and also exhibited lower molar mass, suggesting the molecular weight of fungal chitosan may be lower than that crab chitosan. These characteristics provides improved solubility in water at physiologically acceptable pH values which facilitates some applications in food, medical, agricultural and an antimicrobial and

preservative agent according the literature [24,32,33]. Tayela et al [15] showed that most bioactive chitosan type, to inhibit *Candida albicans* growth, showed the lowest molecular weight (32 kDa) and the highest deacetylation degree (94%).

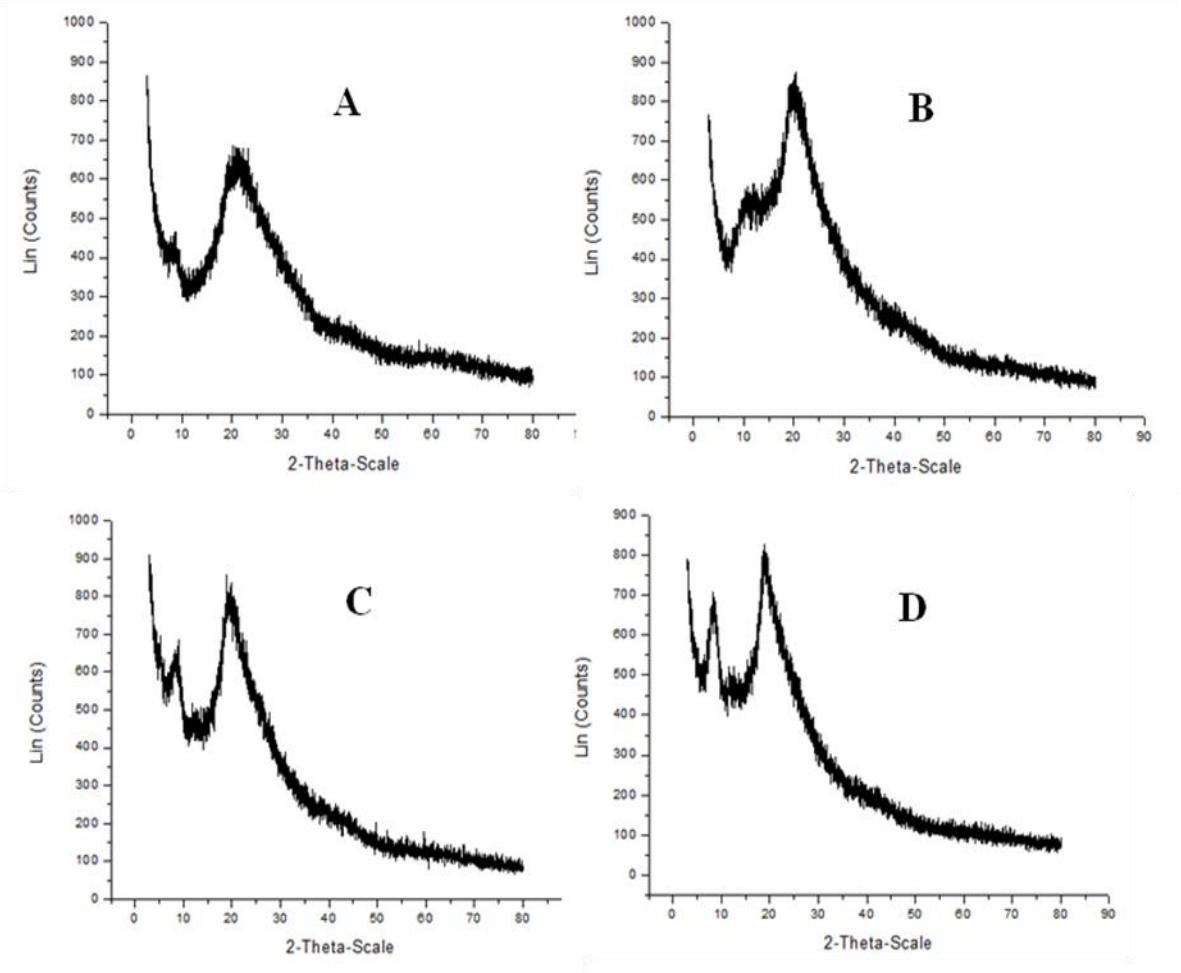
4.2.3. X-ray diffraction

X-ray diffraction is commonly used to determine the polymorphic forms of a compound having different crystalline structures for which distinct powered X-ray diffraction patterns are obtained [4,31]. Based on these patterns it is possible to observe differences in the spacing of crystal planes and polymorphic structure, and provide accurate measurements of the crystalline content, which influences the physical and biological properties of the polymer [4,13,31].

The Figure 5 shows the X-ray diffractograms of chitin and chitosan from *C. elegans* and *R. arrhizus* growth in the assay 2 and 1 of the factorial design, respectively. The crystallinity indexes of these biopolymers were determined from the scattering intensity at two angles, one at 2θ= 9-10º, representing the diffraction intensity of amorphous regions and another at 2θ =19-20º, the diffraction intensity of the crystalline regions. This peak at about 9.00° disappeared in both fungal chitin, probably this suggests a biopolymer with more crystalline structure. This could also indicate the need of a purification process to obtain a satisfactory biopolymer [34]. The diffraction pattern of chitosan (Figure 5 C,D) from *Cunninghamella elegans* grown in the Assay 2 (Table 3) showed strong Bragg refractions at an angle 20.0° and 9.0° which are two characteristic peaks of chitosan, similar to the observed by the literature [4,7] .

The crystallinity indexes results were 33.80% and 32.8% to the chitin, and 20.30% and 17.8% to the chitosan obtained by *Cunninghamella elegans* and *Rhizopus arrhizus* in Assay 2, respectively. Overall, the crystallinity index of chitin was higher than that of chitosan, in which a lower crystallinity of polysaccharides indicates disruption of intra- and inter-molecular hydrogen bonds. The higher crystalline index of chitin reflected its higher degree of crystallinity and a more ordered structure and the lower cristallinity of chitosan indicates disruption of intra- and inter- molecular hydrogen bonds [35]. These results were also lower than those obtained for chitin (45.60%) and chitosan (23.82%) from crustacean standard (data not shown). The results are supported with the literature [4,31]. Tolaimate et al [36] suggested that these lower crystallinity indexes in chitin and chitosan produced by microbial fermentation might indicate their improved water solubility in comparison with chitin and chitosan prepared from the chemical extraction method, probably due to the more severe extraction conditions during the chemical extraction. Some authors also related that the cristallinity index of chitosan is related to its deacetylation degree function [4,31].

Figure 5. X-ray diffractograms of chitin (**A,B**) and chitosan (**C,D**) obtained from *Cunninghamella elegans* and *Rhizopus arrhizus* biomass, respectively.



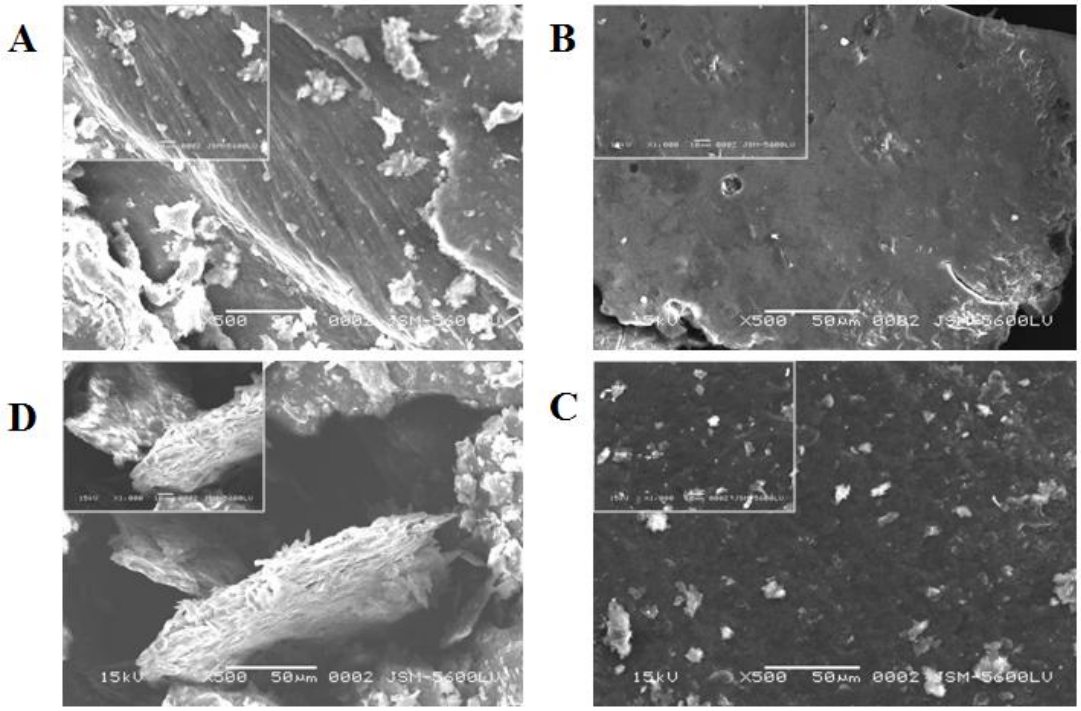
4.2.4. Scanning electron microscopy

The chitin and chitosan produced by *Cunninghamella elegans* and *Rhizopus arrhizus* growth in the assay 1 and 2 were selected to the scanning electron microscopy examination (Figure 6).

The chitin showed prominent arranged microfibrillar crystalline structure in SEM (Fig. 6A and D) which was absent in the chitosan (Fig. 6B and C), as observed by Ming-Tsung et al [37], Arbia et al. [5]; and Chan, Chen, and Yuan [38] ,Yen; Mau [39]. The crystallinity of fungal chitin observed is reinforced with the prominent arranged microfibrillar crystalline structure of this biopolymer in SEM (Figure 5A, D).

Ming-Tsung et al [37] related that the observed crystallinity structure between fungal and crab chitins might also be attributed to their different intersheet or intrasheet hydrogen-bonding systems. However, the preview of this microfibrilar structure in the chitin may have been provided by the extraction process of this biopolymer, i.e, the deproteinization step. The fungal chitosan exhibited a structure more compact, dense, with layers of crumbling flake without porosity as observed in crustacean chitosan by Ming-Tsung et al [37] and Yen; Mau [39] to the fungal chitosan.

Figure 6. SEM photographs of chitin (A) and chitosan (B) produced by *Rhizopus arrhizus* and chitosan (C) and chitin (D) produced by *Cunninghamella elegans*, in the Assay 2 and 1, at 500x and 1000x magnification. The measurement bar = 50 µm.



4.3. Antimicrobial activity

The effectiveness of chitosan from *C. elegans* and *R. arrhizus* in inhibiting the growth of *L. monocytogenes*, *S. aureus*, *P. aeruginosa*, *S. enterica*, *E. coli*, and *Y. enterocolitica* by determining the MIC and MBC is shown in Table 3. The both chitosan showed identical MIC and MBC for all bacteria assayed. These results are in agreement with those reported in literature [40, 41]. The chitosan samples tested were more effective to inhibit Gram negative bacteria compared to Gram positive, except for *Listeria*, which showed higher MIC and MBC.

The antimicrobial activity of chitosan is well documented against a number of pathogenic microorganisms, with MIC varying from 0.01% to 1% [41,42]. The antibacterial activity of chitosan *in vitro* depends on the physicochemical characteristics of chitosan and the specie, or even the strain of the bacteria tested [14,43].

Chung et al [14] investigated the relation between antimicrobial activity of the chitosan and the characteristics of the cellular wall of bacteria. They verified that the chitosan is antibacterial agent more efficient to Gram-negative bacteria due the composition of phospholipids and carboxylic acids of the bacterial cellular wall. These results suggest that the effects of the chitosan are distinct in the two types of bacteria: in the case of the gram-positive, the hypothesis is that chitosan of low molecular mass penetrates more easily in gram-negative bacteria, causing riots in the metabolism of these microorganisms. In addition the authors demonstrated that although the cell wall hydrophilicity is similar among Gram-negative bacteria, the distribution of negative charge on their cell surfaces can be quite different. Most negatively charged cell surfaces have a greater interaction with chitosan. This

could explain why the susceptibility of *Listeria*, *S. enterica* and *Y. enterocolitica* to both microbial chitosan in this study were different from the other Gram-negative bacteria tested (Table 3).

Table 3. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of chitosan from *C. elegans* and *R. arrhizus* against food pathogenic and spoilage bacteria.

Microorganism	*C. elegans*		*R. arrhizus*	
	MIC	MBC	MIC	MBC
S. aureus	300 µg. mL^{-1}	500 µg. mL^{-1}	300 µg. mL^{-1}	500 µg. mL^{-1}
E. faecalis	400 µg. mL^{-1}	600 µg. mL^{-1}	400 µg. mL^{-1}	600 µg. mL^{-1}
E. coli	200 µg. mL^{-1}	400 µg. mL^{-1}	200 µg. mL^{-1}	400 µg. mL^{-1}
P. aeruginosa	200 µg. mL^{-1}	400 µg. mL^{-1}	200 µg. mL^{-1}	400 µg. mL^{-1}
L. monocytogenes	500 µg. mL^{-1}	1000 µg. mL^{-1}	500 µg. mL^{-1}	1000 µg. mL^{-1}
Y. enterocolítica	300 µg. mL^{-1}	600 µg. mL^{-1}	300 µg. mL^{-1}	600 µg .mL^{-1}
S. enterica	300 µg. mL^{-1}	500 µg. mL^{-1}	300 µg. mL^{-1}	500 µg .mL^{-1}

*All bacterias growth in presence of acetic acid 1%

Chitosan exhibited antibacterial activity only in an acidic medium, which was usually attributed to the poor solubility of chitosan above pH 6.5 and more positively charged polycationic molecules with stronger affinity for cells [44,45]. Therefore, for the antimicrobial activity chitosan was dispersed in a solution of 1% acetic acid. As acetic acid itself has antibacterial activity, a positive control, where chitosan was replaced with sterile distilled water and 1% acetic acid, was used for each microorganism, to assure that the antimicrobial activity evidenced is attributed to chitosan. Microbial growth was observed in all positive controls. In addition, the viability of the bacterial strains was confirmed by verifying their growth in BHI agar without adding chitosan.

Studies with electronic micrographs demonstrate that in Gram positive bacteria the chitosan weakens or even though breaks up the bacterial cellular wall, while in the Gram negative the cytoplasm is concentrated and the cell interstice, extended. The Gram-negative bacteria possess the external membrane of the cellular wall composed by lipopolysaccharides (LPS) that provide a hydrophilic surface to the bacterium. The LPS also have anionic groups (phosphate, carboxyl), which contribute for the stability of the LPS through divalent electrostatic interactions with cations). The removal of these cátions for chelant agents result in the run down and molecule release of the LPS. On the other hand, the cellular wall of the Gram-positive bacteria is composed mainly for peptidoglican (PG) and teichoic acid -TA (polymer polyanion), which are linked covalently to the acid Nacetylmuramic of the PG or anchored in direction to the cytoplasmic membrane, via glycolipid (lipoteichoic acid -LTA), which provides a binding site with the chitosan, causing functional riots in the membrane [1,18].

3. Experimental Section

3.1. Materials

All reagents used were of analytical grade. The acetic acid and sodium hydroxide were obtained from Vetec (São Paulo, Brazil). The molasses from sugar cane was procured from local market and corn steep liquor, a byproduct of corn manufacturing industry, was kindly donated by Corn Products do Brasil, Cabo de Santo Agostinho, PE, Brazil. These agroindustrial wastes were used as soluble substrates and carbon and nitrogen source in the 2^2 factorial design.

3.2. Microorganisms and Biomass Production to Obtain Chitin and Chitosan

Cunninghamella elegans strain UCP/WFCC 0542 and *Rhizopus arrhizus* UCP/WFCC 0402 isolated from mangrove sediments situated in Rio Formoso, PE, Brazil. These strains were deposited in the Culture Collection of Catholic University of Pernambuco, Brazil, registered in the World Federation for Culture Collection (WFCC). The fungus were maintained on Potato Dextrose Agar (PDA) medium at 5 °C and transferred to a new medium every four months.

To biomass production, both fungi were grown in Petri dishes containing PDA medium at 28 ºC for 8 days until sporulation. Petri dishes containig PDA were inoculated with 1 mL of the sporangioles suspension (10^7spores. mL^{-1}) of *C. elegans* and *R. arrhizus* and maintained for 18 hours at 28 °C. After the incubation time, 20 discs (1 cm diameter) with mycelium of both fungi were cut from these Petri dishes and inoculated in Erlenmeyer flask containing 200 mL of the alternative medium with molasses and corn steep liquor, pH 5.6. The flasks were incubated at 28ºC in an orbital shaker at 150 rpm, during 96 hours. The mycelia were harvested, washed twice in destilled water by filtration, utilizing a silkscreen nylon membrane (120 F). After lyophilization the biomass were maintained in a vacuum dissecator until constant weight.

3.3. Factorial Design

A 2^2 full factorial design was carried out to analyze the main effects and interactions of molasses (1-4%) and corn steep liquor (2-8%) on the response variable of biomass, chitin and chitosan yield by *C. elegans* and *R. arrhizus* to select the best condition for mycelia and biopolymers productions in accordance with the variables established (Table 4).The Pareto diagrams were undertaken to validate the influence between these agroindustrial wastewaters (independent variables) and the response variables. An estimate of pure experimental error was calculated from four replicates run corresponding to a central point of the complete factorial. The data obtained from the experiments were subjected to statistical analysis by STATISTICA software version 7.0 (StatSoft Inc., city, OK, USA) and significance of the results was tested at the $p<0.05$ level.

Table 4. Design matrix for the factorial experiments used to evaluate the influence of 2 factors (molasses and corn steep liquor) on biomass, chitin and chitosan production by *Cunninghamella elegans* UCP/WFCC 0542 and *Rhizopus arrhizus* UCP 402/WFCC, with experimental conditions set in the average of two extreme levels.

Assays	Factor levels	
	Molasses[1]	Corn steep liquor[2]
1	-1	-1
2	+1	-1
3	-1	+1
4	+1	+1
5	0	0

[1] Concentration of Molasses (%, v/v): 1.00 at level -1; 2.50 at level 0; 4.00 at level +1

[2] Concentration of corn steep liquor (%, v/v): 2.00 at level -1; 5.00 at level 0; 8.00 at level +1

3.5. Chitin and Chitosan Extraction

Chitin and chitosan were extracted using dry biomass of *C. elegans* and *R. arrhizus* as described by Jin Hu et al [46]. The lyophilized biomass was deproteinized with 1M NaOH solution (1:30 w/v, 121°C, 15 min). The alkali-insoluble fraction was separated by centrifugation (4000g, 20 °C, 10 min), and treated using 2% of acetic acid (1:30 w/v, 100°C, 15 min) followed by centrifugation at 4000 g, 20°C, 15 min. The acid insoluble material was considered the chitin, and the supernatant was alkalized to pH 10, maintained overnight at 5°C and centrifuged (4000g, 20 °C, 10 min) for chitosan precipitation The chitin and chitosan was washed with distilled water four times, freeze-dried, and kept in a dissecator until constant weight.

3.6. Characterization of Chitin and Chitosan from the Cunninghamella elegans and Rhizopus arrhizus

3.6.1. Infrared Spectroscopy (Deacetylation degree–DD%)

The degree of deacetylation (DD%) for microbial chitin and chitosan were determined using the infrared spectroscopy according to Baxter et al [47], using the absorbance ratio A1655/A3450 and calculated according to equation 1:

$$DD\ (\%) = 100-[(A1655/A3450) \times 115] \quad \textbf{Equation 1}$$

Two milligrams sample of fungal chitin and chitosan, which had been dried overnight at 60ºC under reduced pressure were thoroughly blended with 100 mg of KBr, to produce 0.5 mm thick disks. The disks were dried for 24 hrs at 110ºC under reduced pressure. Infrared spectrometer was recorded with a Bruker 66 Spectrometer, using a 100 mg KBr disks for reference.

421 3.6.2. Viscosity and Molecular Weights of Chitosan

422 The viscosity of 1% chitosan in 1% acetic acid solution was determined using a Brookfield digital
423 Rheometer (Model DV-II, Brook Engineering laboratories, Inc., Stoughton, MA) at 25°C, Spindle
424 CPE-40, 0.5 mL sample volume.
425 The molecular weight of chitosan was determined using an AVS-350 viscometer (Schott-Geräte),
426 type/capillary: Cannon-Fenske d_{inside}= 1.01mm, at 25ºC. After obtaining the intrinsic viscosity from
427 tables, K and a, were obtained for HAc/NaAc. K = 0.076, a = 0.76. The flow time was determined in
428 seconds. Using the Mark-Houwinks equation, the average viscosimetric molecular weight was
429 expressed in $g.mol^{-1}$.

430 3.6.3. Crystallinity Index

431 The X-ray diffractograms of chitin and chitosan were obtained in the X-Ray Laboratory of the
432 Physics Department – Federal University of Pernambuco—UFPE. The measurement was taken using
433 SIEMENS Model 5000 D X-ray equipment, Cu Kα radiation with λ = 1.542 A0, in a scanning range
434 between 4° and 50° with a rate of 0.02 min−1. The interplanar distance was determined by the half
435 height width of the peak of greatest intensity (IC). The crystallinity index (ICR) was determined with
436 the following equation:
437 Crystallinity index (%) = 100{[I(θc) − I(θa)]/I(θc)} **Equation 2**
438
439 Where I (θc) is the relative intensity of the crystalline (2θ = 20°) and I (θa) corresponds to amorphous
440 regions (2θ = 9°) for chitosan.

441 3.6.2. Scanning Electron Microscopy

442 The dried sample was ground under vacuum using a sputter coater and its surface was observed in a
443 scanning electron microscope Series XL 30 (Umax) ESEM (Env.Scan. Electron Micros) with tungsten
444 filament, using a 20 kV accelerating voltage.

445 *3.7 Bacterial Strains and Culture Conditions to Antimicrobial Assay*

446 *Listeria monocytogenes* ATCC 7664, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella enterica*
447 ATCC 6017, and *Yersinia enterocolítica* ATCC 9610 for antimicrobial assay were provided by
448 FIOCRUZ, Rio de Janeiro, Brazil. *Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis* ATCC
449 29212 and *Escherichia coli* ATCC 8739 were donated by the Antibiotics Institute, UFPE (Recife,
450 Brazil). Stock cultures were kept on Muller Hinton agar slants with blood added (5% v/v) at 4ºC.
451 Inoculum used in the experimental assays were obtained from overnight cultures grown (18 hours) in
452 Brain Heart Infusion broth (DIFCO Laboratories, Detroit, MI, USA) at 37°C. After incubation, the
453 bacterial cells were separated from the growth medium by centrifugation at 10.000 xg for 15 minutes
454 at 4°C, washed thrice in buffer KCl (0.05M KCl, 1 mM KH_2PO_4, 1 Mm $CaCl_2$, 0.1 mM$MgCl_2$, pH
455 6.0), and resuspended in buffer KCl. Suspensions were adjusted so that the optical density (OD) at 660

nm was 1.5, which provided bacterial inoculum of approximately 5 x 10^8 Colony Forming Unity per mL (CFU.mL^{-1}).

3.8 Chitosan Solution Preparation

Chitosan from C. elegans and R. arrhizus were solubilized in a solution of acetic acid 1% at concentration to 20 mg/mL (v/v). The pH of the solutions were adjusted to pH 5.8 using HCl and NaOH.

3.9 Antimicrobial Activity

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of chitosan on the assayed bacteria were carried out using the broth dilution method (Heilman test) as described by Chambrevil;Marmonier [48]. For this, a 0.1 mL aliquot of bacterial inoculum was inoculated into screw-capped 13 x 130 mm sterile tubes containing 0.9 mL of Brain Heart Infusion (BHI) broth containing the desired chitosan concentration (5000 µg. mL^{-1} to 50 µg. mL^{-1}) followed by shaking using Vortex for 30 seconds. The system was incubated at 37°C for 24 hours and the MIC was defined as the lowest chitosan concentration providing no visible growth (turbidity) and the MBC was the lowest chitosan concentration able to cause a 99.9 % kill rate of the initial inoculum. MBC was found by inoculating a 25-µL aliquot of the chitosan-treated assay into sterile Muller Hinton agar Petri dishes and was followed by incubation at 37ºC for 48 hours. For positive control, chitosan was replaced with sterile distilled water and 1% acetic acid. The assays were made in triplicate and the results expressed as average values. Also, the viability of the bacterial strains was assessed by verifying their capacity to grow in a Muller-Hinton agar without adding chitosan.

4. Conclusions

These results suggest a new economic culture medium to improve the chitin and chitosan production from mycelial biomass by *C. elegans* and *R. arrhizus* with low molecular weight and degree of deacetylation of approximately 80%. The results confirm the antimicrobial potential of chitosan against pathogenic bacteria. Therefore chitosan shows great promise as an alternative natural antimicrobial against Gram positive and Gram negative bacteria. However, further study on the mechanism of chitosan against bacteria pathogens at the molecular level is needed.

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Conflicts of Interest

The authors declare no conflict of interest

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CAPÍTULO IV

Aplicação de quitosana microbiológica e comercial na remoção de Cd(II) em solução aquosa

QUINTO ARTIGO

Chitosan membranes of low and medium molecular weight as promising adsorbents of Cd (II) from aqueous solution

Trabalho aceito para publicação como capítulo do livro: “Industrial, Medical, and Environmental Applications of Microorganisms: Current Status and Trends".

Chitosan membranes of low and medium molecular weight as promising adsorbents of Cd (II) from aqueous solution

Lúcia Raquel Ramos Berger[*1,3]; Camilo Enrique La Rotta Hernández[3]; Thayza Christina Montenegro Stamford[2,3]; Patrícia Mendes de Souza[3]; Luciana de Oliveira Franco[4] and Galba Maria de Campos-Takaki[3]

[1]Center of Biological Sciences, Federal University of Pernambuco, 50670-420, Recife, PE, Brazil;
[2]Center of Health Sciences, University Federal of Pernambuco, 50670-420, Recife, PE, Brazil;
[3]Nucleus of Research of Environmental Sciences and Biotechnology, Catholic University of Pernambuco, 50050-900, Recife, PE, Brazil;
[4]Department of Microbiology, Center for Biology Sciences, Federal Rural University of Pernambuco, 52171-900, Recife, PE – Brazil.
*Corresponding author: e-mail: quelberger@hotmail.com

ABSTRACT: Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine linked to N-acetyl-D-glucosamine moieties. Its cationic character confers special features and this is closely related to the metal removal availability. The aim of this study was to development chitosan membranes applied to the removal of Cd (II) from aqueous solutions. Suspensions of chitosan with low and medium molecular masses containing 1.0, 2.5 and 4.0 % (w/v) were prepared in acetic acid 1% (v/v) by continuous overnight stirring at 25º C. The obtained hydro-gels were casted using silicone molds and dried at 50°C for 18 hours. Chitosan membranes were put in contact with 20 ppm $CdCl_2$ solutions during 24 h at 25ºC. The Cd quantification was performed from calibration curves of Cd^{2+} in HCl 1.0 mol L^{-1} ranging 1.0 and 10.0 ppm throughout Anodic Stripping Voltammetry Analysis (ASVA) and square wave voltammetry (SWV). Removal values clearly showed a direct relationship between the increase of chitosan concentration and the molecular mass. Best cadmium removal values of 80% and 96% were obtained with membranes using low MW chitosan at 1.0% and medium MW chitosan at 4.0%, respectively.

Keywords Crustacean; chitosan; Cadmium removal; Membranes.

1.Introduction

The pollution caused by heavy metals has increased rapidly since the industrial revolution. In recent years, metal contamination has become a growing environmental concerns because the toxicity at relatively low concentration and tendency to bioaccumulation. Cadmium (Cd) is a heavy metal widely used in industrial processes such as: anticorrosive agent, stabilizer in plastic products, dying and pigments, neutron-absorber in nuclear plants, in batteries and also fertilizers. Although some products that containing Cd can be recycled, the highest pollution caused by this metal is due to dumping and incineration of the cadmium-polluted wastes. In general, Cd intoxication is related to renal, respiratory, reproductive and skeletal systems damages in superior organisms, including man [1,2].

Extensive studies on Cd removal from waste-waters have been accomplished including reverse osmosis, electrodeposition, ion exchange, filtration and chemical precipitation. Some of these methods remain inefficient for the removal of low concentration and their high maintenance and operation costs. Chelating polymers are an interesting alternative for metal sorption because they can be reusable and renewable, allow easy separation and have higher adsorption capacities and selectivity [3,4]. One example of biopolymer used as promising alternative, as chelating agent is the chitosan [5-8].

Commercial chitosan is derived from chitin using thermochemical deacetylation with strong alkali. Chitin corresponds to a waste product from crustacean exoskeletons obtained after the industrial processing of seafood [9].The crustacean processing by seafood industry generates nearly 40% of shells wastes [10], with a world production near to 1.44 million metric tons of dry weight per year [11,12]. This renewable raw material has a slow biodegradation, however can be used to obtain more valuable and biologically sustainable materials, such as chitin and chitosan since their production is economically feasible and environmentally compatible [13].

Chitin is a biopolymer of N-acetyl-D-glucosamine (GlcNAc) and D-glucosamine residues linked by β-(1-4) glycosides bonds, and actually the second most abundant organic compounds on earth, after cellulose [14]. Chitosan is a cationic amino polysaccharide; essentially composed by β-1,4-D-glucosamine (GlcNAc) linked to N-acetyl-D-glucosamine residua. This biopolymer has been extensively studied due to its unique optical and structural features such biodegradability, biocompatibility and biological activity. Chitosan has the ability to form hydro-gel films that can be applied in metal removal [14]. Due to this, chitosan have drawn the attention of multidisciplinary research groups in medical applications, food, pharmaceutical, cosmetics, agricultural, textile, waste water treatment, and other industries [15,16]. The characteristics of chitosan hydro-gels depend on its

deacetylation degree (DD), the distribution of acetyl groups, the chain length, the molecular weight distribution, and the solubility [17].

Previous studies have shown that the binding of metallic cations to chitosan occurs between the void orbitals of the metal and both, the hydroxyl groups and the free electron pairs at the nitrogen in amine groups present in the chitosan. In contrast to chitin, chitosan also has superior adsorption ability for heavy metals due to its higher content of amino moieties [8]. Based on these properties observed for chitosan we were encouraged to the development of chitosan based membranes able to be applied to the removal Cd^{2+} ions from aqueous solutions.

2.METHODOLOGY

2.1. Material and reagents

All chemicals had analytical grade. Acetic acid, hydrochloric acid and Cadmium (II) chloride ($CdCl_2$) were purchased from Vetec, Brazil. All solutions were prepared in deionized water Milli-Q. Two types of crustacean chitosans of low and medium molecular mass (LMM and MMM) were purchased from Sigma-Aldrich®, Bangalore, India.

2.2. Apparatus

For infrared spectra an Infrared spectrometer Bruker 66 was used. The viscosity of chitosan was determined using an AVS-350 viscometer (Schott-Geräte), type/capillary: Cannon-Fenske d_{inside}= 1.01mm, at 25ºC using a flow time determined in seconds. For electrochemical analyses a PalmSens® LabSolutions potentiostat with data acquisition software PS-Trace® 4.2 were used.

2.3. Chitosan characterization

Infrared Spectroscopy (Deacetylation degree–DD%)

The degree of deacetylation (DD, %) for microbial chitin and chitosan were determined using the infrared spectroscopy according to Domszy & Roberts [18]. The absorbance ratio A1655/A3450 and calculated according to **equation 1**:

$$DD\ (\%) = 100-[(A1655/A3450) \times 100 / 1.33] \qquad \textbf{Equation 1}$$

Two milligrams sample of chitin and chitosan, which had been dried overnight at 60ºC under reduced pressure and then compressed with 100 mg of KBr, to produce disks (0.5 mm of thickness). The disks were dried for 24 hrs at 110ºC under reduced pressure. As reference disks KBr were used. The molecular weights of chitosan were determined by viscosity, using the procedure described by Santos *et al.*[19]. Using the expression in **Equation 2** from Mark-Houwinks that gives a relation between intrinsic viscosity [η] and molecular mass M

$$[\eta] = KM^a \qquad \textbf{Equation 2}$$

From this equation the molecular weight of a polymer can be determined from data on the intrinsic viscosity and vice versa. The **Mark–Houwink** parameters, **a** and **K**, depend on the particular polymer-solvent system. For HAc/NaAc: K = 0.076, a = 0.76.

2.4.Chitosan membranes production

Chitosan hydro-gels of 1.0, 2.5 and 4.0 % (w/v) were prepared in 1% (v/v) acetic acid aqueous solution. These gels were obtained by continuous overnight stirring at 25º C and 1h of ultrasonication to avoid the presence of bubbles. Chitosan membranes were casted using (3.0 x 3.0 x 0.2 cm) silicone molds and dried at 50°C for 18 hours.

2.5. Removal of Cd (II)

Removal experiments were performed immersing each membrane of 1 cm^2 into 10 mL of aqueous solutions containing 20 ppm of Cd^{2+} as $CdCl_2$ in 0.02 mol L^{-1} HCl during 24 hours at 25ºC with no stirring. After this period of time each solution containing residual Cd were analysed by Anodic Stripping Voltammetric Analysis (ASVA) and square wave voltammetry (SWV). Removal percentage was determined using **equation 3:**

$$\text{Removal } \% = (C_o - C_f)/ C_o)*100 \qquad \textbf{Equation 3}$$

Where C_o corresponds to the initial concentration of Cd(II) and Cf the final concentration after the sorption process.

2.6.Electrochemical Analysis

All electrochemical analyses were performed using the same cyclic voltammetry technique and apparatus. The electrochemical system was by a single electrochemical cell of 15 mL composed, a 0.5 cm diameter glassy carbon working electrode, a Pt wire of 1.0 cm as counter-electrode and $Ag|AgCl_2$ in saturated KCl as reference. As support electrolytes 100 mmol L^{-1} H_3PO_4 or KCl solutions. The Cd quantification was performed from calibration curves of Cd^{2+} as $CdCl_2$ in HCl 1.0 mol L^{-1} ranging 1.0 and 10.0 ppm throughout ASVA and SWV according to the following parameters: Ec = 0.6 V, Tc = 60 s, Ed = – 1,4 V and td= 60 s. And square wave parameters for stripping step of: F= 25Hz; E0 = -1.4 V, Ef = 0.0, Estep = 0.005 and Amplitude of 0.025 mV.

3.Results and discussion

3.1.Chitosan Characterization

Despite to have used the same volume of hydrog-gels per casting and similar fabrication procedures, when some dried membranes were removed from the castings, a more susceptibility to tear down was observed when lower concentrations of chitosan and lower molecular masses were used. From more fragile to the strongest membranes can be classified as follows: 1% LMM > 1% MMM > 2.5 % LMM > 2.5% MMM > 4.0% LMM. In the case of chitosan membranes of 4.0% MMM, its rupture was related with its rigidity.

Deacetylation degree (DD%) is an important parameter associated with the physical–chemical properties of chitosan, since it is directly related to its cationic behaviour in water dispersions [20]. **Table 1** showed the results obtained for the intrinsic viscosity determination for LMM and MMM chitosan. It can be seen values of 83% (±2,0) and 85% (±2,0) of DD, respectively. These results were in accordance with previous observations made by Santos *et al.* [19] who reported similar DD values ranging 80 to 90%. The average molecular masses for LMM and MMM chitosans were 2.25×10^4 and 5.6×10^5 g/mol, respectively. These results were in accordance with previous works that showed values of 1.0×10^4 to 9.0×10^5 g/mol, for LMM and MMM, respectively [19,21,22]. LMM chitosan was reported to reduce the tensile strength and elongation in chitosan membranes but to increase its permeability.

Table 1. Values of Intrinsic viscosity, Molecular weight and Deacetylation degree of crustacean chitosan of low and medium molecular weight.

Characterization	Chitosan	
	MMM	LMM
Intrinsic viscosity [η] (mL/g)	0.4005	0.0088
Molecular weight (MW)	5.6×10^5 g/mol	2.25×10^4 g/mol
Deacetylation degree (DD)	85% (±2,0)	83% (±2,0)

3.2.Electrochemical analyses

The Anodic Stripping Voltammetric Analysis (ASVA) and square wave voltammetry is an electrochemical technique used for the determination of metals at trace levels in aqueous solutions, replacing the spectroscopic and titrimetric methods [23,24]. Anodic stripping voltammetry is a voltammetric method for quantitative determination of specific ionic species. The analyte of interest is electroplated on the working electrode during a deposition step, and oxidized from the electrode during the stripping step. The current is measured during the stripping step. The oxidation of species is registered as a peak in the current signal at the potential at which the species begins to be oxidized. **Figure 1**, shows the ASVA voltammograms (left) and the calibration curve (right) obtained for the studied ion. As can be seen a current peak was observed at -600 mV and its intensity was proportional to the increase in Cd(II) concentration. The method showed high reproducibility and good sensibility for the concentration range studied.

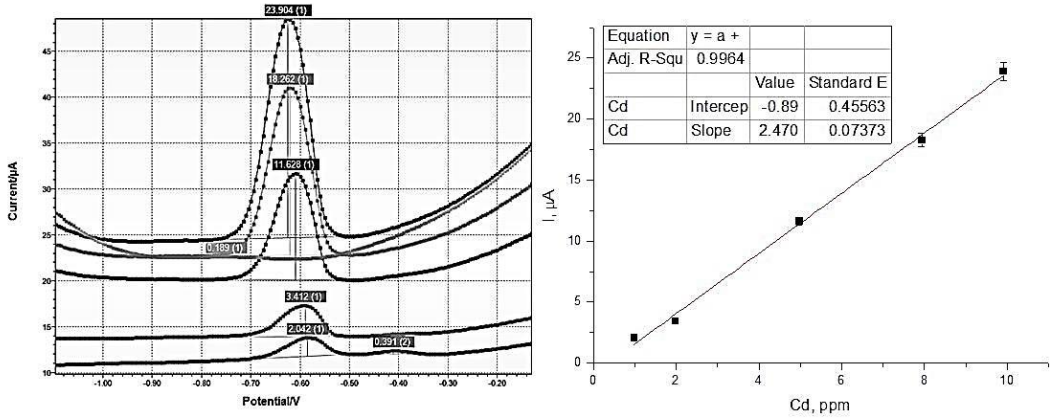

Fig 1. Cyclic voltammetries responses obtained for Cd^{2+} as $CdCl_2$ in HCl 1.0 mol L^{-1} ranging 1.0 and 10.0 ppm absorbed in glassy carbon-paste electrodes throughout ASVA and SWV (left) and calibration curve obtained (right).

Table 2 shows that all the values of current in the Cd (II) electrolyte solutions decreased (8.982 to 1.71 µA) when compared with the current (48.5046 µA) from the initial concentration **(**20 ppm of Cd^{2+}) after exposure to chitosan membranes for 24 hours. This was due to the lower concentration of the metal which was redissolved in aqueous solution, since most of these metal ions retained in the chitosan membranes between 80.03 to 95.60%.

The efficiency of cadmium adsorption was directly proportional to the increase of the concentration and molecular weight of the chitosan gels employed. The application of a membrane with low MW and the lowest concentration of chitosan of 1.0%, showed good Cd adsortion of at least 80%, even when this value was lower compared with the obtained for gels containing higher chitosan concentrations of 4.0%, where the Cd adsorption reached 96%.

Table 2. Values of Current (µA) and Concentration (ppm) for Cd(II) at the initial time (T0) and after 24 hours (T24h) for chitosan membranes.

Samples No.	Chitosan (%)	Chitosan (g mol^{-1})	Cd(II) Initial Concentration (ppm)	Current µA	Cd(II) Final Concentration (ppm)	Current µA	Removal (%)
			T 0h			T 24h	
1	1	Low MW	20	48.5046	3.08168	6.715	84.592
2	1	Low MW	20	48.5046	3.99947	8.982	80.03
3	2.5	Low MW	20	48.5046	2.52745	5.346	87.363
4	2.5	Low MW	20	48.5046	2.72299	5.829	86.385
5	4	Low MW	20	48.5046	0.99592	1.563	95.02
6	4	Low MW	20	48.5046	0.99551	1.562	95.02
7	1	Medium MW	20	48.5046	1.24814	2.186	93.76
8	1	Medium MW	20	48.5046	1.4372	2.653	92.81
9	2.5	Medium MW	20	48.5046	1.79225	3.53	91.04
10	2.5	Medium MW	20	48.5046	1.28134	2.268	93.59
11	4	Medium MW	20	48.5046	0.88054	1.278	95.60
12	4	Medium MW	20	48.5046	1.05543	1.71	94.72

Liao *et al*.[25] related that the binding of metal cations to chitosan occurs between the hydroxyl groups and the free electron pairs of the nitrogen in the amine group with the void orbitals of the metal. In contrast to chitin, chitosan has superior adsorption ability for heavy metals due to its higher content of amino groups [26]. So, these results showed that probably the largest concentration and molecular mass of chitosan in the membranes provides more amine and hydroxyl groups available to interact and adsorb Cd^{2+} ions. The removal of nearly 100% of metal ion by a low cost biopolymer membrane-shaped suggests a future alternative to supplement or

even replace procedures with high cost and low efficiency for decontaminating industrial waste by heavy metals. Since our results have shown good removal levels at higher concentration is to be expected that can be applied to the removal of very low concentration as the ones described by the rule.

According to the Brazilian legislation on water pollution, the minimum acceptable concentration for Cd is 0.01 ppm. Since our results have shown good removal levels at higher concentration is to be expected that can be applied to the removal of very low concentration as the ones described by the rule.

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SEXTO ARTIGO

Produção de quitina e quitosana por *Cunninghamella elegans* e *Rhizopus arrhizus* e aplicação de membranas de quitosana para remoção de Cd(II) em soluções aquosas

Trabalho a ser submetido ao periódico:

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Produção de quitina e quitosana por *Cunninghamella elegans* e *Rhizopus arrhizus* e aplicação de membranas de quitosana para remoção de Cd(II) em soluções aquosas

Lúcia Raquel Ramos Berger[*1,2]; Camilo Enrique La Rotta Hernández[2]; Thayza Christina Montenegro Stamford [3] e Galba Maria de Campos-Takaki[2]

[1] Programa de Pós Graduação em Ciências da Biológicas, Universidade Federal de Pernambuco, 50670-420, Recife, PE, Brasil;
[2]Núcleo de Pesquisas em Ciências Ambientais, Universidade Católica de Pernambuco, 50050-900, Recife, PE, Brasil;
[3]Centro de Ciências da Saúde, Universidade Federal de Pernambuco, 50670-420, Recife, PE, Brasil;
*Corresponding author: e-mail: quelberger@hotmail.com

Nos últimos anos, a poluição do meio ambiente por metais pesados tem despertado a atenção internacional, principalmente devido às atividades industriais. Esses poluentes são altamente tóxicos em baixas concentrações nos ecossistemas, não biodegradáveis, e sua tendência à bioacumulação na cadeia alimentar pode resultar em sérios problemas à saúde dos organismos (HEIDARI *et al.*, 2013; PAPAGEORGIOU *et al.*, 2009).

O cádmio (Cd^{2+}) é um metal pesado muito utilizado em processos indústrias como revestimento anticorrosivo; na fabricação de baterias níquel-cádmio; em pigmentos para tintas e plásticos; em vários tipos de ligas e revestimentos anticorrosivos; na produção de fertilizantes; na queima de combustíveis fóssies; durante a obtenção de zinco, ferro e aço e na produção de cimento. A presença desse metal dissolvido em água, mesmo em baixas concentrações, é extremamente prejudicial para o ambiente aquático e à saúde humana, uma vez que pode provocar cãibras musculares, problemas pulmonares crônicos, degradação renal, proteinúria, deformidade óssea, atrofia testicular (GODT *et al.*, 2006; MADALA *et al.*, 2013; ZAINI *et al.*, 2009). A Organização Mundial da Saúde (OMS) definiu a concentração máxima de 0,003 mg/L de Cd^{2+} na água potável (WHO, 2008) e no Brasil o Conselho Nacional de Meio-Ambiente (CONAMA) estabeleceu uma menor tolerância ambiental para este íon de 0,01 mg/L (JANEGITZ *et al.*, 2007).

Vários métodos são utilizados para a remoção de metais pesados em águas residuais como por exemplo precipitação química, eletrodiálise, eletrodeposição, troca iônica, osmose inversa, filtração, precipitação química e processos de extração com solvente (ALIABADI *et al.*, 2013; SALIM *et* MUNEKAGE, 2009). No entanto, algumas dessas técnicas muitas vezes são ineficazes para baixas concentrações de metal, demandam muito tempo de aplicação e consumo de energia, resultando em custo elevado de manutenção e operação. Desse modo, o método de adsorção pode ser considerado processo eficaz e amplamente utilizado devido à sua simplicidade, as condições operacionais moderadas e a viabilidade económica (IRANI *et al*., 2011; SALIM *et* MUNEKAGE, 2009).

Os biopolímeros, com destaque para a quitosana, podem ser considerados alternativas promissoras para a adsorção de metais pesados, uma vez que eles podem ser reutilizados, são renováveis, biodergradáveis, apresentam fácil separação e elevada capacidade adsortiva e seletividade (BARCICKI *et al*., 1980;TABAKCI *et al*., 2008; VANDENBOSSCHE *et al*., 2013; WANG *et al*., 2001).

A quitosana é derivada da desacetilação da quitina, considerada o segundo mais abundante polímero na natureza, sendo composta por unidades monoméricas de β-1,4 *N*-acetilglucosamina (NWE *et al*., 2010). A quitina é encontrada como elemento estrutural no exoesqueleto de crustáceos, moluscos, anelídeos, celenterados, insetos e parede celular dos fungos, especialmente de Zygomycetes (FRANCO *et al*., 2004). A quitosana é um polímero catiônico e linear, composta essencialmente de (β-1,4 D-glicosamina, ligado resíduos N-acetil-D-glicosamina (ANDRADE *et al*., 2000; CAMPOS-TAKAKI *et al*., 2005; CHATTERJEE *et al*., 2005). A quitina e a quitosana tem sido muito estudadas devido as suas propriedades únicas, com destaque para: biodegradabilidade, biocompatibilidade, atividades biológicas, aplicações químicas (STAMFORD *et al*., 2007), capacidade para formar membranas e adsorção de íons metálicos. Entretanto, a quitina é extremamente insolúvel o que dificulta sua aplicabilidade em várias áreas. Em contraste, maior aplicação pode ser encontrada para quitosana devido à sua solubilidade em soluções ácidas , grupos amino reativos e elevada densidade de carga (ZAMANI *et al*., 2007).

A quitina e a quitosana são obtidas comercialmente a partir da carapaça de custáceos, matéria prima abundante na indústria pesqueira, representando produção mundial de aproximadamente 1,44 milhões de toneladas de matéria seca por ano (KNORR *et al*., 1991; RØDDE *et al*., 2008). Porém, entre as principais limitações

apresentadas pelo uso desta fonte destaca-se a obtenção de quitosana com propriedades físico-químicas heterogêneas e inconsistentes. Desse modo, a produção de quitina e quitosana por fungos da ordem Mucorales, classe Zygomycetes torna-se uma alternativa promissora porque estes polímeros podem ser obtidos simultaneamente, com propriedades físico-químicas homogêneas e mais apropriadas para determinada aplicação, e o cultivo destes fungos em substratos nutricionais de baixo custo pode reduzir em até 73 % as despessas com a produção Além disso, as características e os rendimentos de quitina e quitosana podem ser otimizados, controlando parâmetros do processo de fermentação tais como: o pH, a concentração de nutrientes no meio de fermentação e o tempo de incubação (BATISTA *et al*., 2013; BERGER *et al.*, 2011; STAMFORD *et al*., 2007; CARDOSO *et al*., 2010).

A milhocina, resíduo do processamento industrial do milho (CARDOSO, 2007), e a casca de mamão, grupo Formosa (*Carica papaya* L.) podem ser considerados fontes nutricionais ricas e de baixo custo com significantes quantidades de aminoácidos, vitaminas e minerais, que são essenciais para o crescimento dos microrganismos. Além disso, o mamão é produzido em todas as regiões do Brasil, e consumido por grande número de pessoas, mas sua casca rica em nutrientes é descartada pela indústria de processamento de alimentos (RINALDI et al., 2010).

As características da quitosana, entre elas o grau de desacetilação (GD%), distribuição de grupos acetil, comprimento da cadeia, massa molecular e solubilidade interferem na eficiência das membranas deste polímero no processo de adsorção dos metais pesados (BATISTA, 2011). Desse modo o uso de quitosana microbiológica, com características mais homogeneas que o polímero comercial, para produção de membranas a serem aplicadas na remoção de cádmio pode ser considerada uma alternativa promissora do ponto de vista economico e ambiental.

O crescimento de fungos e a produção de quitina e quitosana podem ser otimizados através da utilização da abordagem multivariada, onde todas as variáveis independentes são considerados simultaneamente. O presente estudo tem como objetivo comparar o crescimento e a produção de quitina e quitosana por *R. arrhizus* e *C. elegans* usando diferentes quantidades de milhocina e suco de casca de mamão Formosa em delineamento central composto rotacional de 2^2 (DCCR). Outro objetivo foi comparar a aplicação dessa quitosana fúngica e da quitosana comercial na remoção de cádmio em água.

2. Materiais e Métodos

2.1. Microrganismos

No presente estudo foram utilizados os fungos da ordem Mucorales, *Cunninghamella elegans* (WFCC / UCP 542) e *Rhizopus arrhizus* (WFCC / UCP 402), isolados de sedimento de mangue em Rio Formoso, Pernambuco, Brasil e depositados na Coleção de Culturas UCP (Universidade Católica de Pernambuco), Núcleo de Pesquisas em Ciências Ambientais da Universidade Católica de Pernambuco, Brasil. A coleção está registrada na "World Federation for Culture Collection – WFCC". As estirpes foram mantidas em meio BDA (Oxoid, Kansas City, EUA) em 5°C e transferidas para um novo meio a cada quatro meses.

2.2. Reagentes, Milhocina e Suco da Casca de Mamão (SCM)

Todos os reagentes usados foram de grau analítico. O ácido acético, o hidróxido de sódio, o ácido clorídrico e o cloreto de cádmio ($CdCl_2$) foram obtidos daVetec (São Paulo, Brasil). A milhocina, subproduto da indústria de processamento de milho, foi gentilmente cedido por "Corn Products do Brasil", localizada em Cabo de Santo Agostinho, Pernambuco, Brasil, e foi usado como substrato solúvel. A casca de mamão (*Carica papaya* L.), do grupo Formosa, foi gentilmente cedido por uma indústria local. O suco foi produzido com casca fresca triturada com água destilada obtendo as concentrações finais de 17,82, 25,00, 42,50, 60,00 e 67,18% (v/v). A milhocina e o suco de casca de mamão (SCM) foram utilizados como fonte de carbono e nitrogênio no meio de cultura, de acordo com delineamento composto central rotacional de 2^2 (CCRD).

As análises elementares da milhocina e do suco de casca de mamão foram realizadas no equipamento elementar analisador modelo EA 1110 Carlo Erba Instruments.

Todas as soluções usadas nos ensaios para remoção de cádmio com membranas de quitosana foram preparadas com água Mili-Q. Nesses experimentos com as membranas foram utilizados dois tipos de quitosanas de crustáceos de baixa (BMM) e média massa molecular (MMM) como padrões, da Sigma-Aldrich®, Bangalore, Índia.

2.3. Condições de cultivo e produção de biomassa

Cunninghamella elegans e *Rhizopus arrhizus* foram cultivados em placas de Petri com BDA, pH 5.6, a 28 °C durante sete dias. Após esse período, as placas forma utilizadas para obtenção de suspensão com 10^7 esporangíolos/mL. Placas de Petri com BDA foram inoculados com 1 ml dessa suspensão e mantidas durante 18 horas a 28 °C. Em seguida, a partir dessas placas foram cortados o total de 20 discos com 1 cm de diâmetro contendo cada um micélio jovem desses fungos crescidos em BDA. Os 20 discos foram utilizados para inocular Erlenmeyers de 500 mL, com 200 mL de cada meio de produção, como apresentado pelo Delineamento Central Composto Rotacional de 2^2 (Tabelas 1). Esses frascos foram incubados em agitador orbital a 150 rpm, 28 ° C durante 96 horas. No final do período de incubação, as biomassas foram filtradas, lavadas duas vezes com água destilada, submetidas ao processo de liofilização e mantidas em dessecador a vácuo.

2.4. Extração de quitina e quitosana

No presente trabalho foram utilizadas diferentes metodologias para extração de quitina e quitosana a partir da biomassa liofilizada de *C. elegans* e *R. arrhizus* para obtenção de polímeros com diferentes massas molares, uma vez que os processos de extração influenciam nesta e em outras características físico-químicas.

As biomassas obtidas por cada condição do Delineamento Central Composto Rotacional de 2^2 (Tabela 1 e 2) foram submetidas ao processo de extração de quitina e quitosana proposto por HU *et* al, (1999). As etapas desse processo foram a desproteinização da biomassa liofilizada com NaOH a 1M na proporção de 1:40 (p/v), seguida de autoclavagem (121 ºC, 15 minutos), centrifugação (4000 rpm, 15 minutos). O sobrenadante foi descartado e o precipitado submetido à hidrólise ácida usando ácido acético 2% (v/v), autoclavagem (100 ºC, 10 minutos) e centrifugação (4000 rpm, 15 minutos). O precipitado correspondeu à quitina, que foi lavada com água destilada gelada até pH próximo a neutralidade, e com o sobrenadante foi realizada a correção do pH para 10-12 ocorrendo a precipitação da quitosana. Em seguida, esse biopolímero foi submetido a lavagens sucessivas com água destilada gelada até pH próximo a neutralidade e seco a temperatura ambiente.

O processo de extração de quitina e quitosana proposto por FRANCO *et al.*, (2004) foi utilizado a partir da biomassa obtida do ensaio do DCCR que mostrou melhor rendimento de quitosana. Nessa metodologia a biomassa foi desproteinizada com solução de NaOH 2 % p/v (30:1 v/p, 90^{o}C, 2 h); seguida de centrifugação (4000 rpm, 15 min), para separação da fração alcalina insolúvel. O material álcali insolúvel foi hidrolisado com ácido acético 10 % (40:1 v/p, 60^{o}C, 6 h) e centrifugado (4000 rpm, 15 min), para separar o precipitada (quitina), da quitosana presente no sobrenadante. A quitosana foi obtida por precipitação após o ajuste do pH do sobrenadante para 9, usando solução de NaOH a 10 N. A quitina e a quitosana foram lavadas com água destilada gelada e secadas a temperatura ambiente.

2.5. Delineamento Central Composto Rotacional de 2^2 e análise estatística

Os fungos *Cunninghamella elegans* e *Rhizopus arrhizus* foram cultivados em Delineamento Central Composto Rotacional de 2^2 com o objetivo de analisar os principais efeitos e interações do suco de casca de mamão nas concentrações finais de 17,82; 25,00; 42,50; 60,00 e 67,18% (v / v), e da milhocina em concentrações finais de 0,32; 0,50; 2,50; 4,00 e 4,62,% (v / v).

As variáveis resposta foram os rendimentos de biomassa (g/L), quitina (mg/g) e quitosana (mg/g) obtidos em cada condição de cultivo testada para anbos os fungos. Essas concentrações de milhocina e suco de casa de mamão foram definidas com base em experimentos preliminares. A estimativa do erro experimental foi calculada a partir de cinco repetições correspondentes ao ponto central do DCCR. As análises dos dados e os gráficos foram feitos com o software STATISTICA versão 7.0 (StatSoft Inc., OK, EUA) e a significância dos resultados foi calculada no nível $p < 0,05$.

Tabela 1. Matriz do Delineamento Central Composto Rotacional (DCCR) de 2^2 para estudo da influência das variáveis independentes (suco da casca de mamão, SCM, e milhocina) na produção de biomassa, quitina e quitosana por *Cunninghamella elegans* UCP 542 e *Rhizopus arrhizus* UCP 402. Condição 9 a 12 são os pontos centrais. A estimativa do erro puro experimental foi calculado a partir de quatro repetições do ponto central (9-12) do DCCR.

Condição	Milhocina[a]	Suco de Mamão[b]
1	-1	-1
2	-1	+1
3	+1	-1
4	+1	+1
5	-1,41	0
6	+1,41	0
7	0	-1,41
8	0	+1,41
9	0	0
10	0	0
11	0	0
12	0	0

[a] Concentração de milhocina (%, v/v): 0,32 (-1,41); 0,5 (-1); 2,5 (0); 4,0 (+1); 4,62 (+1,41)

[b] Concentração de suco de mamão (%, v/v): 17,82 (-1,41); 25(-1); 42,5 (0); 60 (+1); 67,18 (+1,41)

2.6. Caracterização da quitina e quitosana

Nesse estudo foram selecionadas para caracterização físico-química as quitinas e quitosanas obtidas nos ensaios do DCCR de 2^2 que apresentaram os melhores rendimentos destes polímeros para *Cunninghamella elegans* e *Rhizopus arrhizus.* As análises realizadas foram a espectroscopia vibracional na região do infravermelho, difração de raio x, viscosidade, e massa molar. Essas caracterizações foram realizadas na Central Analítica, Departamento de Química Fundamental da UFPE.

2.6.1. Espectroscopia vibracional na região do infravermelho (Grau de desacetilação– GD%)

O grau de desacetilação (GD%) da quitina e quitosana de *C. elegans* e *R. arrhizus* foram determinados através da espectroscopia vibracional na região do infravermelho de acordo com BAXTER *et al.* (1992), utilizando a razão das absorbancias A1655/A3450 e a equação 1:

GD (%) = 100-[(A1655/A3450) x 115] **Equação 1**

Duas miligramas de amostra de quitina e quitosana secas durante a noite a 60 º C, sob pressão reduzida, foram bem misturados com 100 mg de KBr para produzir discos espessos de 0,5 mm. Os discos foram secos durante 24 horas a 110 º C sob pressão reduzida. O Espectrôfotometro de infravermelho utilizado foi o Bruker 66 Spectrometer, usando discos de KBr de 100 mg como referência.

2.6.2. Determinação da Viscosidade e Massa Molar da Quitosana

A massa molar foi determinado por viscosidade, segundo a metodologia proposta por Santos et al. As medidas de viscosidade foram feitas utilizando um capilar de vidro tipo Cannon-Fenske (diâmetro interno=1,01mm) termostatizado a (25± 0,01)ºC, em um viscosímetro AVS-350 da Schott-Geräte. Para a determinação da viscosidade intrínseca, [η], foram preparadas soluções de quitosana (utilizando tampão de ácido acético como solvente) com concentrações variando de 9,0 x 10-4 a 3,0 x 10-3g.mL^{-1}. Os tempos de escoamento foram determinados em segundos. As amostras foram feitas em quatro replicatas e a média das medidas foi calculada.

2.6.3. Difração de Raio-X

Os difratogramas de raios-X das quitinas e quitosanas foram obtidos em aparelho de difração de raios-X modelo SIEMENS D5000, radiação de Cu-Kα sendo λ = 1,542Aº, em uma faixa de varredura entre 4º e 50º com taxa de 0,02º.min-1. O índice de cristalinidade (ICR) foi determinado através da equação 2:

%ICR = (IC . IA/ IC) x 100 **Equação 2**

sendo: Ic e IA as intensidades dos sinais das regiões cristalinas (2θ=20°) e amorfas (2θ=12°), respectivamente.

2.6. Produção de membranas de quitosana

Os Hidro-géis de quitosana de 2,5% (p/v) foram preparados em 1% (v/v) de solução aquosa de ácido acético. Estes géis foram submetidos a agitação magnética até completa dissolução da quitosana, a 25 °C e em seguida sonicados por 1 hora em ultra-som para evitar a presença de bolhas. O volume total de 3 mL de cada hidro-gel foi despejado em moldes de silicone (3,0 x 3,0 x 0,2 centímetros) e secos a 50 ° C durante 18 horas para obtenção das membranas de quitosana.

Foram produzidas oito tipos de membranas classificadas como: QCr(MMM) = quitosana de crustáceo de média massa molecular; QCr(BMM) = quitosana de crustáceo de baixa massa molecular; 1QF:2QCr(MMM) = quitosana de fungo obtida pelo método de extração (A) proposto por FRANCO *et al*., (2004) e quitosana de crustáceo de média massa molecular na proporção de 1:2 (v/v); 1QF:1QCr(BMM) = quitosana de fungo obtida pelo método de extração (B) proposto por HU *et al*., (1999) e quitosana de crustáceo de baixa massa molecular na proporção de 1:1 (v/v); QF(MW) = quitosana de fungo obtida pelo método de extração A; QCr(MMM)+NH_4OH = quitosana de crustáceo de média massa molecular tratadas com hidróxido de amônia; QCr(BMM)+NH_4OH = quitosana de crustáceo de baixa massa molecular tratadas com hidróxido de amônia; QF(MMM)+NH_4OH = quitosana de fungo obtida pelo método de extração (A) tratadas com hidróxido de amônia.

O tratamento das membranas submetidas ao hidróxido de amônia foi realizado colocando-se as membranas secas em dessecador saturado com NH_4OH por 1 hora e depois mantidas a temperatura ambiente, dentro da capela química com exaustor ligado por 1 hora.

2.7. Análises eletroquímicas

No presente estudo foram escolhidos os métodos de Voltametria por Redissolução Anódica (VRA) e de Voltametria de Onda Quadrada (VOQ), que são técnicas eletroquímicas utilizadas para determinação quantitativa de baixas concentrações de metais pesados em soluções aquosas, como substituintes dos métodos

espectroscópicos e de titulação. Através das análises de VRA, a determinação quantitativa da espécie iônica que se pretende analisar, neste caso os íons de Cd^{2+}, é obtida. Para essa determinação ocorrem duas etapas, sendo primeiro a etapa de deposição ou pré-concentração, quando o analito de interesse (Cd^{2+}) é reduzido ao seu estado elementar e é consequentemente eletrodepositado na superfície do eletrodo de trabalho, neste caso o eletrodo de carbono vítreo, devido a aplicação do potencial redutor. Em seguida, ocorre a segunda etapa com a aplicação de um potencial de varredura anódico de forma pulsada por onda quadrada que causa a oxidação do cádmio (Cd^{2+}), redissolvendo-o e resultando em uma corrente anódica com relação linear à concentração do metal. A corrente é medida durante a fase de extração. A oxidação das espécies é registado como um pico no sinal de corrente para o potencial que a espécie começa a ser oxidada(SANNA *et al*., 2000; OLIVEIRA *et al*., 2004) .

Todas as análises eletroquímicas foram realizadas utilizando o mesmo aparelho e a mesma técnica de voltametria cíclica. O sistema eletroquímico foi composto por uma única célula eletroquímica de 15 mL de capacidade com eletrodo de carbono vítreo de 0,5 cm de diâmetro, como eletrodo de trabalho; um fio de platina de 1,0 cm como contra-eletrodo e um eletrodo de Ag|AgCl2 em KCl saturado como eletrodo de referência. Como eletrólitos de suporte foram utilizadas soluções 100 mmol L^{-1} H_3PO_4 ou KCl. A quantificação Cd foi realizada a partir de curvas de calibração de Cd^{2+} como $CdCl_2$ em HCl 0,02 N variando 1,0 a 20,0 ppm. Todas as leituras das amostras foram realizadas através de análise por voltamétrica de redissolução anódica (VRA) e voltametria de onda quadrada (VOQ) de acordo com os seguintes parâmetros: Ec = 0,6 V, Tc = 60 s, Ed = - 1,4 V e td = 60 s. E parâmetros de onda quadrada para a etapa de redissolução forma de: F = 25Hz; E0 = -1,4 V, Ef = 0,0, Estep = 0,005 e amplitude de 0,025 mV.

2.7. Remoção de Cd(II) por membranas de quitosana

Os experimentos de remoção foram realizados imergindo cada membrana de 1 cm^2 (0,0083 g de quitosana) em 10 mL de solução aquosa contendo as concentrações iniciais (C_o) de 20, 200 e 2000 ppm de Cd^{2+} como $CdCl_2$ em 0,02 mol L^{-1} de HCl, sem ajuste do pH, durante 24 horas, a 25 ºC, sem agitação. Após este período de tempo cada solução contendo Cd residual foi analisada por voltamétrica de redissolução anódica

(VRA) e voltametria de onda quadrada (VOQ). A remoção percentual foi determinada através da equação 3:

Remoção (%) = $(C_o-C_f/C_o)*100$ **Equação 3**

Onde C_o corresponde à concentração inicial de Cd (II) e C_f a concentração final, após o processo de sorção.

A capacidade de adsorção de Cd^{2+}, q, foi calculada de acordo com a equação 4:

$qt = V/m*(C_o-Cf)$ **Equação 4**

Onde q_t, C_o, C_f, V e m são as quantidades de soluto adsorvido por unidade de massa do adsorvente (mg/g) em t (min), a concentração inicial do íon metálico, a concentração final do íon metálico (mg/L), volume de solução (L) e massa seca do adsorvente (g), respectivamente.

Para o estudo do equilíbrio da adsorção de Cd(II) pelas membranas de quitosana foi realizado o cálculo de q_e através da equação 5:

$q_e= V/m (C_o-C_e)$ **Equação 5**

Nessa equação, q_e e C_e são a quantidade de soluto adsorvido por unidade de massa de adsorvente (mg/g) no equilíbrio e a concentração de equilíbrio de cádmio (mg/L), respectivamente.

2.8. Isoterma de adsorção de Langmuir

Os dados experimentais foram analisados utilizando a equação 6 da isoterma de adsorção de Langmuir na forma linear:

$1/q_e = (1/q_{max}.K_L) . 1/C_e + 1/q_{max}$ **Equação 6**

Onde q_{max} é a máxima capacidade de adsorção (mg/g) onde K_L e q_{max} podem ser calculados pelos coeficiente angular e intercepto da equação linear do gráfico de $1/ q_e$ versos $1/C_e$. Todas as equações 3, 4, 5, 6 foram utilizadas neste estudo de acordo com HYDARI *et al*., (2012).

3. Resultados e Discussão

3.1. Análise comparativa do efeito das concentrações de milhocina e suco de casca de mamão na produção de biomassa, quitina e quitosana por *Cunninghamella elegans* e *Rhizopus arrhizus*

Através do delineamento central composto rotacional (DCCR) foi possível avaliar a relação entre as diferentes concentrações de milhocina e suco de casca de mamão aplicados no meio de cultivo como fontes de C, N e outros nutrientes, e as produções de biomassa, quitina e quitosana por *Cunninghamella elegans* e *Rhizopus arrhizus*. Esses fungos mostraram requisitos nutritivos específicos para aumentar a resposta de uma variável particular, como apresentado na Tabela 2. A maior produção de biomassa foi obtida na presença dos níveis mais elevados das fontes de C e N, como mostra os ensaios 8, 4 e 6, com valores de 10,62, 11,00 e 12,00 g/L para *C. elegans* e 11,90, 14,20 e 12,75 g/L para *R. arrhizus*. Por outro lado, os ensaios com as menores concentrações de milhocina e suco de casca de mamão apresentaram os melhores rendimentos de quitina de 126 mg/g, e quitosana, 77,78 mg/g para *C. elegans* e 137,14 mg/g para *R. arrhizus.* A presença de menores concentrações de suco de casca de mamão e das maiores concentrações de milhocina proporcionaram maiores rendimentos de quitosana por *R. arrhizus.*

Tabela 2. Resultados para produção de biomassa (g/L), quitina (mg/g) e quitosana (mg/g) por *C. elegans* e *R. arrhizus* obtidas no Delineamento Central Composto Rotacional 2^2 (DCCR) para estudo da influência de concentrações de milhocina e suco de casca de mamão. A estimativa do puro erro experimental foi calculado a partir de quatro repetições do ponto central (9-12) do DCCR.

	Biomassa(g/L)		Quitina (mg/g)		Quitosana (mg/g)	
Condição	*C. elegans*	*R. arrhizus*	*C. elegans*	*R. arrhizus*	*C. elegans*	*R. arrhizus*
1	4,11	3,75	**126,0**	**137,14**	**77,78**	47,50
2	5,19	7,23	74,53	99,90	69,00	70,18
3	10,2	10,55	99,90	101,58	45,35	**77,76**
4	**11,0**	**14,20**	76,50	97,96	50,00	64,67
5	5,04	5,26	92,36	123,67	67,85	43,98
6	12,0	12,75	100,48	77,01	49,37	72,37
7	7,69	6,64	98,58	109,77	55,22	70,90
8	10,62	11,90	94,04	97,39	68,11	72,80
9	9,69	9,38	112,64	110,57	53,54	71,75
10	9,28	9,42	120,10	101,89	57,88	73,74
11	9,43	9,94	119,27	108,25	52,73	74,44
12	9,47	9,61	120,47	111,82	53,35	75,00
13	9,62	9,61	116,19	111,82	51,34	75,00

Uma das primeiras aplicações da milhocina na microbiologia foi realizada por Moyer e Coghill em 1986 que observaram o aumento do rendimento de penicilina com a adição desta fonte nutritiva para modificar a solução de sais minerais Czapek-Dox. Em 1984, a milhocina já foi considerada fonte nutritiva viável economicamente quando comparada com susbtâncias mais caras como o extrato de levedura e a peptona; e utilizada com sucesso como suplemento para substituir extratos, ou como a principal fonte de nitrogênio e carbono para todos os microrganismos (LIGGETT *et* KOFFLER, 1984).

Outros estudos também relatam semelhante influência positiva da milhocina no crescimento e na produção de quitina e quitosana por fungos da ordem Mucorales (BERGER *et al.*, 2011;LINS *et al.*, 2010; CARDOSO *et al.*, 2012; BATISTA *et al.*,

2013; SANTOS *et al*., 2013), mas nenhum estudo foi encontrado, até o momento, sobre a utilização da casca de mamão para este objetivo. Os resultados obtidos neste estudo são comparáveis aos obtidos pela literatura (tabela 3).

Tabela 3. Substratos, biomassa, quitina, quitosana e grau de desacetilação obtidos a partir de vários trabalhos.

Microrganismo	**Substrato**	**Biomassa**	**Quitina**	**Quitosana**	**GD**	**Referência**
Cunninghamella elegans	Hesseltine et Anderson (1957) modificado	10,41 g/L	288 mg/g (28,8 %)	NA	NA	ANDRADE *et al.* 2000
Mucor javanicus	Hesseltine et Anderson (1957) modificado	8,71 g/L	23,9% (239 mg/g)	NA	NA	ANDRADE *et al.*, 2003
Cunninghamella elegans	Meio a base de jacatupé (*Pachyrhizus erosus* L. Urban)	24,3 g/L	440mg/g	66mg/g	6,2% (quitina), 85% (quitosana)	STAMFORD *et al.* 2007
Rhizopus arrhizus	Meio Hesseltine et Anderson (1957)	NA	92mg/g	13mg/g	78 %	CARDOSO *et al.* (2010)
Rhizopus arrhizus	Meio Andrade *et al.*(2000)	NA	94mg/g	14mg/g	95%	CARDOSO *et al.* (2010)
Rhizopus arrhizus	Milhocina 4%	13,1 g/L	30,4 mg/g	12,85 mg/g	95%	LINS *et al.* (2010)
Rhizomucor miehei	Sabouraod dextrose (SDB)	4,1 (g^{-1})	NA	13,67% (136,7 mg/g)	98,6%	TAJDINI *et al.* (2010)
Mucor racemosus	Sabouraod dextrose (SDB)	3,8 (g^{-1})	NA	11,72% 117 mg/g)	97,1%	TAJDINI *et al.* (2010)
Rhizopus arrhizus	Milhocina e manipueira	8,80 g/L	54,38 mg/g	20,51 mg/g	82% (quitosana), 25% (quitina)	BERGER *et al.* (2011)
Mucor circinelloides	Yam bean (jacatupé)	20,7 g/L	500 mg/g	64 mg/g	83% DD	FAI *et al.*, (2011)
Rhizopus arrhizus	Milhocina e mel de abelha	20,6 g/L	NA	29,3 mg/g	86%	CARDOSO *et al.* (2012)

A milhocina apresenta quantidade de nitrogênio de 3,85 a 4,1% , sendo grande parte aminoácidos e polipeptídios, e também 11% de açúcares redutores, glicose, e vitaminas do complexo B. Entre os constiuintes desse substrato encontram-se: alanina, arginina, ácido aspártico, cistina, ácido glutâmico, histidina, isoleucina, leucina, lisina, metionina, fenilalanina, prolina, treonina, tirosina, e valina (Ligget e Koffler, 1984). A Tabela 4 mostra que a milhocina utilizada nestes estudo apresentou quantidades significativas de nitrogênio e carbono, e a casca de mamão também apresentou similar porcentagem de carbono e hidrogênio.

Tabela 4. Porcentagem de nitrogênio, carbono, hidrogênio e enxofre presentes na composição de milhocina e casca de mamão.

Amostra	Nitrogênio (%)	Carbono (%)	Hidrogênio (%)	Enxofre(%)
Milhocina	6,49	37,77	6,74	0
Casca de mamão	2,20	37,80	6,59	0

Ao contrário da milhocina, a casca de mamão é pouco explorada pelo homem, até mesmo como substrato para meios de cultivo de microrganismos. A exploração dessa fonte nutricional subutilizada na composição de meios de cultura é reforçada pela presença de maiores quantidades de nutrientes como proteínas (3%), carboidratos (1%), cálcio e ferro (7%), magnésio (8%), zinco e cobre (4%) e grandes quantidades de potássio (13%), em comparação a polpa, parte comestível deste fruto (Gondim et al., 2005). KHAN *et al*. (2009) relataram considerável quantidade de proteínas em resíduos de frutos de mamão, e sugerem a obtenção de alta produção de biomassa de *Rhizopus oligosporus* utilizando este substrato como fonte de nutrientes, em comparação com outras cascas de frutas.

Ambos os substratos também proporcionam outros nutrientes importantes para o metabolismo do microrganismo (RINALDI *et al*., 2010). SANTOS *et al.* (2013) sugerem que o aumento de biomassa é favorecido pela presença de grande quantidade de aminoácidos (leucina, isoleucina, lisina, metionina, tirosina, fenilalanina, treonina e serina) e vitaminas (biotina, colina, inositol, niacina, piridoxina, riboflavina, tiamina e ácido pantotênico) contido em milhocina que são essenciais para o desenvolvimento do microrganismo.

Os diagramas de Pareto apresentados na figura 1 confirmam a influência positiva da milhocina e do suco de casca de mamão, com significância estatística, na produção

de biomassa por *C. elegans* (A) e *R. arrhizus* (B). Semelhante resultado foi anteriormente discutido para a condição 4 do DCCR (tabela 2), que apresentou os melhores rendimentos de biomassa por ambos os fungos na presença das maiores concentrações de milhocina e suco da casca de mamão no meio de cultura.

Por outro lado, a produção de quitina por *C. elegans* (C) e *R. arrhizus* (D) mostrou ser inversamente proporcional as concentrações desses substratos, ou seja, os gráficos de Pareto (1C e 1D) evidenciam o efeito negativo do aumento de milhocina e suco de casca de mamão na obtenção de melhores rendimentos deste polímero. Esses resultados também foram observados na condição 1 do DCCR, com as melhores quantidades de quitina obtidas por esses fungos. Resposta semelhante também foi obtida para a quitosana produzida por *C. elegans*, como mostra o gráfico de Pareto (1E). Em contrapartida, maiores e menores concentrações de milhocina e casca de mamão, respectivamente, favoreceram a produção de quitosana por *R. arrhizus.*

Os maiores rendimentos de quitosana por *C. elegans* e de quitina para ambos os fungos foram obtidos no ensaio 1 do DCCR, com as menores concentrações de cada uma das variáveis independentes (milhocina 0,5%, suco de casca de mamão 25%). Desse modo, quantidades inferiores de milhocina e suco de casca de mamão podem ser suficientes para futuras obtenções de melhores rendimentos destes biopolímeros por *C. elegans* e *R. arrhizus*, o que seria interessante do ponto de vista econômico. Além disso, em adição a menor concentração de suco de casca de mamão, também foi necessária a maior concentração de milhocina para obtenção do melhor rendimento de quitosana por *R. arrhizus*, como mostrado no ensaio 3 (milhocina 4%, suco de casca de mamão 25%), tabela 2.

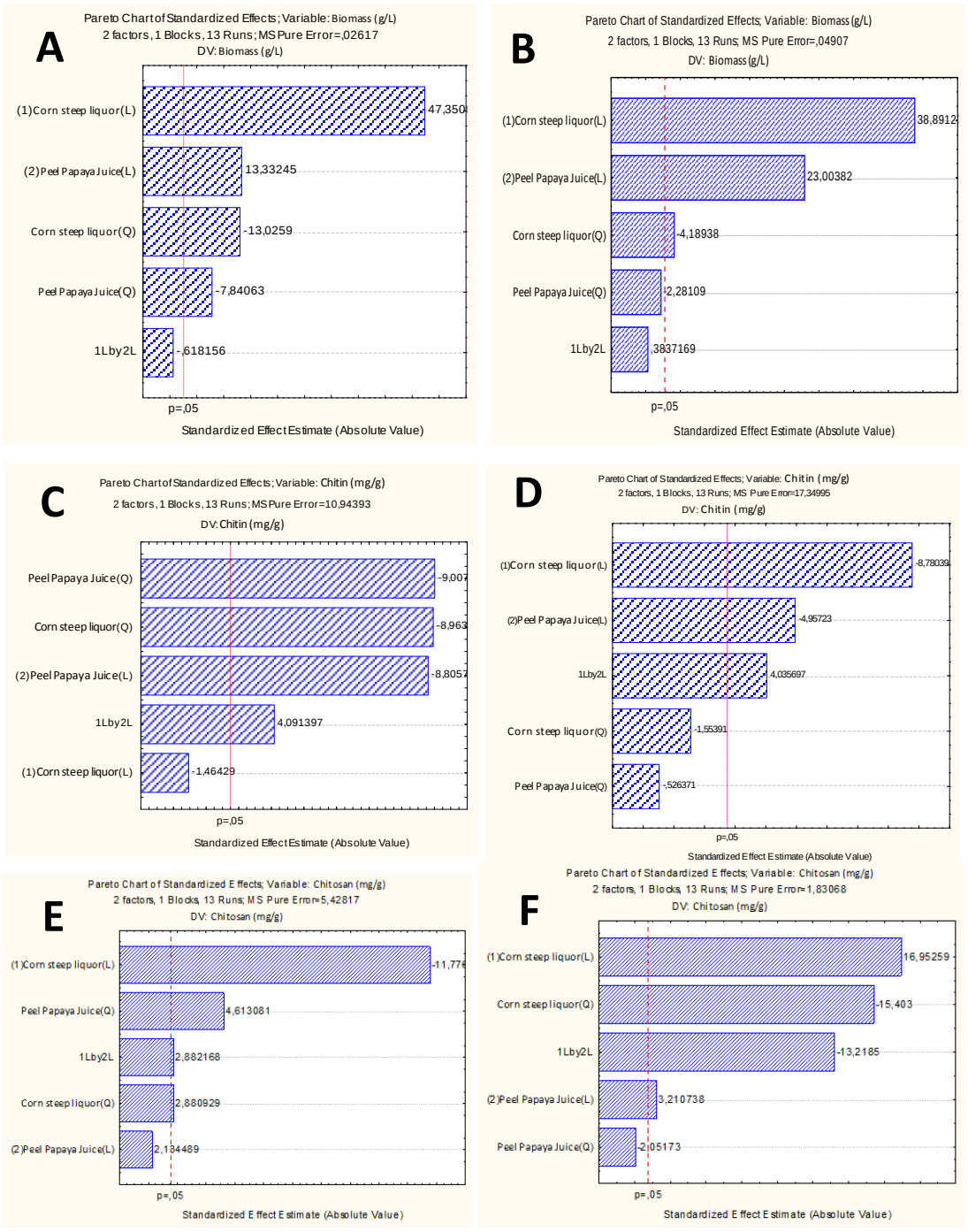

Figura 1. Diagramas de Pareto evidenciando os efeitos das concentrações de milhocina (%, v/v) e suco de casca de mamão (%, v/v) nas produções de biomassa (A), quitina(C) e quitosana (E) por *C. elegans*, e de biomassa (B), quitina (D) e quitosana (F) por *R. arrhizus* .

3.2. Caracterização físico-química da quitosana

As quitosanas extraídas das biomassas de *C. elegans* e *R. arrhizus* mostraram espectros de infravermelho (IFR), graus de desacetilação e índices de cristalinidade similares aos reportados pela literatura (STAMFORD *et al*., 2007; FAI *et al*., 2011). Os resultados de IFR desses polímeros apresentaram as bandas amida de 1657e 1660; 1558 e 1553; e 1320 e 1315 cm^{-1}; e a presença da banda 3483cm^{-1} e 3305cm^{-1}, na região da deformação axial do OH, a qual aparece sobreposta à banda de deformação axial do NH. O grau de desacetilação (% GD) é um parâmetro importante associado às propriedades físico-químicas da quitosana, porque está envolvido diretamente com as suas propriedades catiônicas. No presente estudo, as quitosana obtidas por ambos os métodos de extração utilzados para *C. elegans* e *R. arrhizus* apresentaram 83 a 85% de desacetilação, semelhantes aos observados para as quitosanas de crustáceo de baixa (83%) e média massa molar (85%).

Os difratogramas de raios-X das amostras de quitosana de crustáceo, e extraídas a partir desses dois fungos, apresentaram semelhança quanto à cristalinidade e apresentaram pico de maior intensidade próximo ao angulo de 20°. Os índices de cristalinidade foram 25,8% e 21,17% para as quitosanas obtidas por *Cunninghamella elegans* e *Rhizopus arrhizus*, também semelhantes aos obtidos pelas quitosanas comerciais, com índices de 23,8% e 25,2%.

A viscosidade e a massa molecular das quitosanas microbiológicas extraídas pelos métodos propostos por FRANCO *et al.* (2004) foram, 4,08 x 10^4 g/mol e 120 cP, resultados mais próximos aos valores apresentados pela quitosana de crustáceo de média massa (6,59x10^4 g/mol, 287 cP) . As quitosanas extraídas pelo método de HU *et al.* (1999) apresentaram menores valores de viscosidade e massa molecular de 3,71 cP e 5,05 x 10^3 g/mol que as quitosanas de crustáceo de baixa massa molar de 29,7 cP e 2,25 x 10^4 g/mol). Esses resultados eram esperados porque o método de extração influencia nas características físico-químicas desse polímero. O método sugerido por FRANCO *et al.* (2004) provavelmente resultou na quitosana com maior massa molecular porque utiliza temperaturas mais amenas (60^{o}C e 90^{o}C) durante o processo de extração, quando comparado com o método de HU *et al.* (1999), que utiliza altas temperaturas (100 ^{o}C e 120 ^{o}C) com uso de autoclave.

3.3. Produção e aplicação de membranas de quitosana para remoção de Cd(II) em soluções aquosas

Antes da leitura das correntes de cada solução de Cd(II) do grupo A (Co=2000 ppm), do grupo B (Co= 200ppm), e do grupo C (Co=20 ppm), foram determinados os valores de corrente para a curva de calibração com concentrações de 1, 2, 5, 8, 10 e 20 ppm de Cd(II).

Essa curva de calibração apresentou a equação linear (y= -8,80967+3,99362x) utilizada para converter os valores de corrente µA para ppm em cada amostra dos três grupos de experimentos. As amostras dos grupos A e B, com concentrações iniciais de Cd(II) acima dos valores dessa curva de calibração, precisaram ser dissolvidas 200x e 20x em HCl 0,002N para leitura e adequação à curva. Já as amostras do grupo C foram lidas diretamente, sem necessitar de diluição. O método mostrou alta reprodutibilidade e boa sensibilidade para a faixa de concentração estudada.

As tabelas 5, 6 e 7 mostram os valores de corrente (µA) obtidos para cada solução de Cd(II) em contato com as oito membranas de quitosana, durante a leitura após 0, 6, 12, 24, 36 e 48 horas de tempo de contato. Esses valores de corrente correspondem a média de quatro repetições por tratamento. Os resultados obtidos no tempo zero hora correspondem à amostra do branco com os valores de 38,07 µA para o grupo A e B, e 75,96 µA para o grupo C, como observado nas tabelas 5, 6 e 7.

A intensidade das correntes foi proporcional ao aumento das concentrações de Cd(II) em solução. Os menores valores de corrente foram observados após 12, 36 e 24 horas de contato das soluções iônicas dos grupos A, B e C, com cada membrana de quitosana. Além dessa diferença dos tempos de contato, cada grupo de experimento também mostrou a obtenção dos menores e maiores valores de corrente por amostras em diferentes tipos de membrana. Entre as amostras do grupo A, aquela que permaneceu em contato com a membrana QCr(BMM)+NH_4OH apresentou o menor valor de corrente 8,04 µA e as amostras em contato com a membrana QF(MMM) mostraram o maior valor de corrente de 11,34 µA. No grupo B, os menores e maiores valores de corrente foram 5,48 µA e 12,78 µA para as amostras em contato com as membranas QCr(MMM) e QCr(BMM)+NH_4OH; já as amostras do grupo C em contato com as membranas QF(MMM) e 1QF:2QCr(MMM) apresentaram o menor e o maior valor de corrente de 5,87 e 10,11 µA.

Tabela 5. Valores de corrente (µA) para Cd (II) em solução aquosa após os tempos de contatos de 0, 6, 12, 24 e 48 horas na presença de membranas de quitosana do grupo A. Concentração inicial de Cd(II) = 2000 ppm.

A (Cd^{2+}=2000 ppm)	**Corrente (µA) x Tempo de Contato (h)**				
Membranas	Tempo de contato (h)				
	0	6	12	24	48
QCr(MMM)	38,07	23,8	10,18	12,56	18,59
QCr(BMM)	38,07	21,97	9,16	10,12	19,53
1QF:2QCr(MMM)	38,07	23,95	10,76	12,56	15,19
1QF:1QCr(BMM)	38,07	19,4	10,85	12,37	14
QF(MMM)	38,07	19,29	**11,34**	14,56	16,22
QCr(MMM)+NH_4OH	38,07	16,21	9,54	12,58	12,68
QCr(BMM)+NH_4OH	38,07	15,55	**8,04**	16,75	16,54
QF(MMM)+NH_4OH	38,07	15,25	10,23	18,72	13,61

Tabela 6. Valores de corrente (µA) para Cd (II) no tempo inicial (T0) e após 6, 12, 24, 36 e 48 horas na presença de membranas de quitosana do grupo B. Concentração inicial de Cd(II) = 200 ppm.

B (Cd^{2+}=200 ppm)	**Corrente (µA) x Tempo de Contato (h)**					
Membranas	Tempo de contato (h)					
	0	6	12	24	36	48
QCr(MMM)	38,07	20,08	15,00	10,15	**5,48**	8,96
QCr(BMM)	38,07	31,24	19,77	14,64	9,37	10,45
1QF:2QCr(MMM)	38,07	30,42	18,32	15,44	10,42	12,09
1QF:1QCr(BMM)	38,07	24,86	19,33	13,76	10,62	13,23
QF(MMM)	38,07	19,96	17,81	14,07	11,24	13,18
QCr(MMM)+NH_4OH	38,07	20,91	18,38	16,47	12,38	13,33
QCr(BMM)+NH_4OH	38,07	19,77	18,01	16,66	**12,78**	13,12
QF(MMM)+NH_4OH	38,07	17,77	15,33	13,53	11,27	13,4

Tabela 7. Valores de corrente (μA) para Cd (II) no tempo inicial (T0) e após 6, 12, 24, 36 e 48 horas na presença de membranas de quitosana do grupo C. Concentração inicial de Cd(II) = 20 ppm.

C (Cd^{2+}=20 ppm)	**Corrente (μA) x Tempo de Contato (h)**					
Membranas	Tempo de contato (h)					
	0	6	12	24	36	48
QCr(MMM)	75,96	50,05	36,42	9,41	30,17	27,5
QCr(BMM)	75,96	47,47	37,01	10,4	26,4	23,28
1QF:2QCr(MMM)	75,96	44,47	16,12	**10,11**	23,85	25,67
1QF:1QCr(BMM)	75,96	46,99	9,86	6,89	19,06	24,14
QF(MMM)	75,96	45,63	17,11	**5,87**	18,38	24,6
QCr(MMM)+NH_4OH	75,96	48,05	13,09	6,75	17,34	23,22
QCr(BMM)+NH_4OH	75,96	43,49	11	9,56	16,68	24,82
QF(MMM)+NH_4OH	75,96	38,45	8,24	7,33	21,22	16,02

As maiores e menores intensidades de corrente obtidas por cada grupo de experimentos A, B e C foram diretamente proporcionais as suas respectivas concentrações de Cd^{2+} (ppm) livres em solução, ou seja, que não foram adsorvidos pelas membranas de quitosana (Figura 2).

Os gráficos da figura 2 mostram o efeito do tempo de contato das membranas na adsorção de Cd(II) com as concentrações iniciais de 2000, 200 e 20 ppm, para os grupos A, B e C, pH inicial da solução = 2,03±0,01, a 25ºC. Cada grupo de experimento alcançou o equilíbrio de adsorção, ou seja, o ponto máximo de adsorção, onde qt = qe, nos diferentes tempos de contato de 12, 36 e 24 horas para os grupos A, B e C, respectivamente. As membranas expostas à maior concentração inicial de 2000 ppm de Cd(II) mostraram a mais rápida saturação dos seus sítios de adsorção devido a maior probabilidade de contato deste íon metálico com o adsorvente, como esperado.

Após esse momento de saturação, foi observado que o processo de readsorção iniciava, como observado para cada membrana nos gráficos da figura 2.

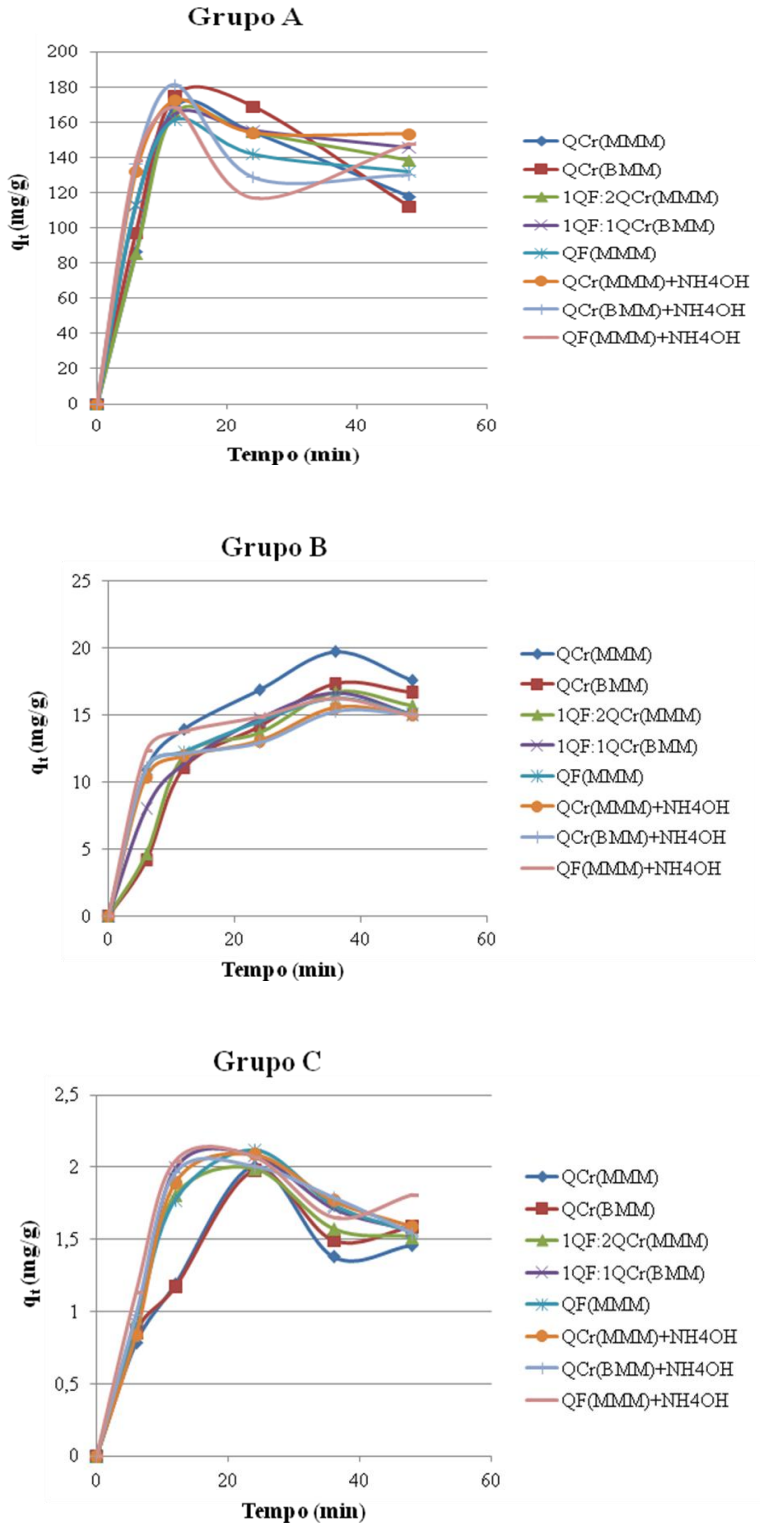

Figura 2. Influência do tempo de contato na remoção de cádmio por membranas de quitosana (pH = 2,03±0,01; 25ºC; concentração inicial de Cd^{2+} para: o grupo A= 2000 mg/L, grupo B= 200mg/L, grupo C= 20mg/L; concentração de quitosana=2,5%, ou 8,3g/L; volume da solução de Cd^{2+}=10mL).

Durante o transcorrer de cada tempo de contato das membranas de quitosana com as soluções de Cd(II), foi observado aumento do pH de 2,03±0,01 para 2,14 a 2,28 em todas as amostras. A mesma tendência de aumento do pH durante a remoção de cádmio foi observada por BENGUELLA *et* BENAISSA (2002) que observaram o aumento de 5,70 para 5,02. De acordo com os autores, isso pode ter ocorrido devido à competição entre os íons Cd^{2+} e H_3O^+ pelos sítios de ligação no adsorvente, consequentemente apenas alguns átomos de nitrogênio das glucosaminas tornam-se disponíveis para adsorção deste íon metálico.

A tabela 8 mostra os parâmetros constantes e os coeficientes de regressão obtidos durante a adequação dos resultados desse estudo no modelo de isoterma de Langmuir. Os valores de $R^2 > 0,99$ permitem concluir que o modelo de isoterma de Langmuir foi com aplicado com sucesso para o sistema de adsorção de íons de Cd^{2+} - membrana de quitosana, isto sugere que a adsorção por monocamada existiu nas condições experimentais deste estudo.

Tabela 8. Parâmetros constantes e coeficiente de regressão para o modelo de Langmuir.

Sistemas de adsorção	Modelo de Langmuir		
	q_{max} (mg/g)	K_L (L/mg)	R^2
QCr(MMM) - cádmio	87,72	0,005138	0,999
QCr(BMM) - cádmio	58,48	0,00727	0,9973
1QF:2QCr(MMM) - cádmio	51,28	0,00852	0,9965
1QF:1QCr(BMM) - cádmio	44,25	0,01253	0,9948
QF(MMM) - cádmio	40,65	0,01489	0,9937
QCr(MMM)+NH_4OH - cádmio	39,22	0,01444	0,9927
QCr(BMM)+NH_4OH- cádmio	42,37	0,01077	0,9939
QF(MMM)+NH_4OH- cádmio	43,1	0,0125	0,9943

A isoterma de Langmuir é considerada de base teórica simples e aplicável para a adsorção em superfície homogêneas com pouca interação com as moléculas adsorvidas. De acordo com essa isoterma as moléculas são adsorvidas em pontos discretos da superfície, chamados "sítios de adsorção"; a energia da espécie adsorvida é a mesma em qualquer ponto da superfície, e é independente da presença ou ausência de moléculas adsorvidas na vizinhança, isto é, a superfície é completamente uniforme sob o ponto de vista energético; a quantidade máxima possível de adsorção é a que corresponde à

monocamada; e a adsorção é localizada e ocorre por colisão de moléculas com sítios vazios (COONEY ,1999; CARVALHO, 2006).

Os valores calculados da máxima capacidade de adsorção, q_{max} (mg/g), mostram que as membranas de quitosana exibiram considerável capacidade para adsorver cádmio de soluções aquosa. O maiores valores foram observados para as membranas com quitosana de crustáceo de média massa molecular, QCr(MMM) = 87,72 e 58,48 mg/g, provavelmente devido a maior resistência física destas membranas, em comparação com as de fungo, como visualmente observado durante o transcorrer do tempo de contato. Desse modo, são necessários futuros estudos para melhorar a estabilidade física das membranas de quitosana fúngica, mas sem comprometer muito os seus sítios de adsorção.

Mesmo com os melhores resultado de q_{max} (mg/g) para as membranas de quitosana de crustáceo, as membranas com quitosana microbiológica conseguiram altos percentuais de remoção de cádmio (tabelas 9, 10 e 11). Para as membranas do grupo A (Co=2000ppm) a maior remoção de Cd^{2+} (61,71%) foi apresentada pela membrana QCR (MMM) mas, sem diferença significante em relação ao valor de 59,43% obtido pela membrana de quitosana microbiológica QF(MMM)+NH_4OH, após 12 horas de contato com solução iônica. Em concentrações inicias de Cd(II) menores de 200 e 20 ppm as membranas de quitosana de crustáceo e de fungo também mostraram semelhantes potenciais para adsorção deste metal. Os percentuais de remoção de cádmio pelas membranas do grupo B (Co=200 ppm) foram de 69,55% a 57,21%; e para o grupo C foram de 82,68 a 77,73%, não apresentando diferenças significativas entre estes resultados.

Tabela 9. Percentual de remoção de Cd(II) apresentado por cada membrana de quitosana (grupo A) durante os tempo de contato de 0, 6, 12, 24 e 48 horas, a 25ºC. Concentração inicial de Cd(II)=2000 ppm.

A (Cd^{2+}=2000 ppm)	**Remoção de Cd^{2+}(%) x Tempo de Contato (h)**				
Membranas	Tempo de contato (h)				
	0	6	12	24	48
QCr(MMM)	0	30,51	59,53	54,46	41,91
QCr(BMM)	0	34,41	**61,71**	59,66	39,61
1QF:2QCr(MMM)	0	30,19	58,30	54,46	48,86
1QF:1QCr(BMM)	0	39,88	58,10	54,86	51,39
QF(MMM)	0	40,12	57,06	50,20	46,66
QCr(MMM)+NH_4OH	0	46,68	60,90	54,42	54,20
QCr(BMM)+NH_4OH	0	48,09	64,09	45,53	45,98
QF(MMM)+NH_4OH	0	48,73	59,43	**52,22**	41,33

Tabela 10. Percentual de remoção de Cd(II) apresentado por cada membrana de quitosana (grupo B) durante os tempo de contato de 0, 6, 12, 24, 36 e 48 horas, a 25ºC. Concentração inicial de Cd(II)=200 ppm.

B (Cd^{2+}=200 ppm)	**Remoção de Cd^{2+}(%) x Tempo de Contato (h)**					
	Tempo de contato (h)					
	0	6	12	24	36	48
QCr(MMM)	0	29,91	49,26	59,6	**69,55**	62,13
QCr(BMM)	0	14,65	39,1	50,03	61,26	58,96
1QF:2QCr(MMM)	0	16,4	42,19	48,32	59,02	55,46
1QF:1QCr(BMM)	0	28,25	40,03	51,9	58,59	53,03
QF(MMM)	0	38,69	43,27	51,24	**57,27**	53,14
QCr(MMM)+NH_4OH	0	36,67	42,06	46,13	54,84	52,82
QCr(BMM)+NH_4OH	0	39,1	42,85	45,72	53,99	53,27
QF(MMM)+NH_4OH	0	43,36	48,56	52,39	57,21	52,67

Tabela 11. Percentual de remoção de Cd(II) apresentado por cada membrana de quitosana (grupo C) durante os tempo de contato de 0, 6, 12, 24, 36 e 48 horas, a 25ºC. Concentração inicial de Cd(II)=20 ppm.

C (Cd^{2+}=20 ppm)	**Remoção de Cd^{2+}(%) x Tempo de Contato (h)**					
Membranas	Tempo de contato (h)					
	0	6	12	24	36	48
QCr(MMM)	0	30,54	46,07	78,28	53,52	56,71
QCr(BMM)	0	33,59	45,93	77,73	58,45	62,13
1QF:2QCr(MMM)	0	37,13	70,58	77,67	61,46	59,31
1QF:1QCr(BMM)	0	34,16	77,97	81,47	67,11	61,12
QF(MMM)	0	35,76	69,41	**82,68**	67,92	60,58
QCr(MMM)+NH_4OH	0	32,9	74,16	81,64	69,14	62,2
QCr(BMM)+NH_4OH	0	38,29	76,62	78,32	69,92	60,32
QF(MMM)+NH_4OH	0	44,23	79,88	**80,95**	64,56	70,7

Os resultados obtidos no presente estudo confirmam a potencial da quitosana na remoção de íons metálicos. De acordo com LIAO *et al*. (2013) a ligação dos cátions metálicos na molécula de quitosana pode ocorrer com os grupos hidroxila e os pares de elétrons livres do nitrogênio do grupo amina neste polímero. Em contraste com a quitina, a quitosana apresenta maior capacidade de adsorção de metais pesados devido ao seu maior grau de grupos amina. Assim, os resultados mostraram que, provavelmente, a maior viscosidade e massa molecular da quitosana de crustáceo (QCr MMM) forneceu mais grupos amina e hidroxila disponíveis para interagir e adsorver íons de Cd^{2+}. Mesmo com essa diferença, todas as membranas testadas apresentaram considerável capacidade para remoção de altas concentrações de cádmio, superiores a concentração mínima aceitável (0,01 ppm) pelo CONAMA, podendo ser consideradas eficientes materiais para futuras aplicações em práticas de descontaminação ambiental por este poluente.

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CONCLUSÕES

- Os fungos *Cunninghamella elegans* e *Rhizopus arrhizus* apresentaram crescimento e produção de quitina e quitosana satisfatórios, com rendimentos destes biopolímeros semelhantes ou superiores aos encontrados na literatura;

- Os susbtratos sintéticos (glicose, tiamina e asparagina) e os alternativos (milhocina, manipueira, melaço e casca de mamão) podem ser aplicados como fontes de carbono e nitrogênio em meios de cultura para *C. elegans* e *R. arrhizus*, otimizando e até mesmo reduzindo os custos de produção de quitina e quitosana;

- Os maiores rendimentos de biomassa, quitina e quitosana apresentados por *C. elegans* e *R. arrhizus* foram, respectivamente, obtidos nos diferentes meios de cultura: Milhocina com Melaço, Hesseltine e Anderson (1957) modificado, e Milhocina com Suco de Casca de Mamão;

- As quitosanas extraídas de *C. elegans* e *R. arrhizus* apresentaram efeito antimicrobiano contra os microrganismos patogênicos *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Yersinia enterocolítica*, *Staphylococcus aureus* e *Escherichia coli*;

- O potencial de remoção de Cd(II) em soluções aquosas foi relativamente similar para as membranas de quitosana de crustáceo e de fungo;

- As membranas de quitosana de crustáceo e de fungo proporcionaram uma considerável remoção de Cd(II) em soluções aquosas, podendo se tornar uma alternativa de baixo custo na despoluição de ambientes contamindos por metais pesados;

ANEXOS

Produção bibliográfica durante o doutorado (2010-2013)

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1. Wager, E.; Kleinert, S. Responsible research publication: international standards for authors. A position statement developed at the 2nd World Conference on Research Integrity, Singapore, July 22-24, 2010. In Promoting Research Integrity in a Global Environment; Mayer, T., Steneck, N., eds.; Imperial College Press / World Scientific Publishing: Singapore; Chapter 50, pp. 309-16.

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