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PRODUÇÃO E CARACTERIZAÇÃO PARCIAL DA COLAGENASE DE *Candida albicans* URM3622

CAROLINA DE ALBUQUERQUE LIMA

Orientadora: Profa. Dra. Maria das Graças Carneiro da Cunha
Co-Orientadora: Profa. Dra. Ana Lúcia Figueiredo Porto

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CAROLINA DE ALBUQUERQUE LIMA

PRODUÇÃO E CARACTERIZAÇÃO PARCIAL DA COLAGENASE DE *Candida albicans* URM3622

Dissertação apresentada para o cumprimento parcial das exigências para obtenção do título de mestre em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco

Aprovado por: Profa. Dra. Maria das Graças Carneiro da Cunha
Prof. Dr. José Luiz de Lima Filho
Prof. Dr. Nelson Manuel Viana da Silva Lima
Prof. Dr. Ranilson de Souza Bezerra

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Ata da defesa de dissertação da Mestranda **Carolina de Albuquerque Lima,** realizada em 26 de fevereiro de 2008, como requisito final para obtenção do título de Mestre em Bioquímica e Fisiologia da UFPE.

Às 14:20 horas, do dia vinte e seis de fevereiro de 2008, foi aberto, no Auditório Prof. Marcionilo Lins - Depto. Bioquímica, do Centro de Ciências Biológicas, da Universidade Federal de Pernambuco, o ato de defesa de dissertação da mestranda **Carolina de Albuquerque Lima** aluna do Curso de Mestrado em Bioquímica e Fisiologia/CCB/UFPE. Iniciando os trabalhos a Profa. Dra. **Vera Lúcia de Menezes Lima** fez a apresentação da aluna, de sua orientadora Profa. Dra. Maria das Graças Carneiro da Cunha, da co-orientadora Profa. Dra. Ana Lúcia Figueiredo Porto, bem como da Banca Examinadora composta pelos professores doutores: Maria das Graças Carneiro da Cunha, na qualidade de Presidente, José Luis de Lima Filho, Ranilson de Souza Bezerra, ambos do Depto. de Bioquímica/UFPE, e Nelson Manuel Viana da Silva Lima, da Universidade do Minho. Após as apresentações, a Profa. Dra. Maria das Graças Carneiro da Cunha convidou a aluna para a apresentação de sua dissertação intitulada: "**Produção e Caracterização Parcial de Colagenase de *Candida albicans URM 3622***", e informou que de acordo com o Regimento Interno do Curso, o candidato dispõe de até 50 (cinqüenta) minutos para apresentação do trabalho e o tempo de argüição para cada examinador, juntamente com o tempo gasto pelo aluna para responder às perguntas será de 30 (trinta) minutos. A aluna procedeu à explanação e comentários acerca do tema em **40 (quarenta) minutos**. Após a apresentação da mestranda, a Sra. Presidente convidou a Banca Examinadora para ocupar seus lugares e passou a palavra ao primeiro examinador, Prof. Dr. Nelson Manuel Viana da Silva Lima que agradeceu o convite, fez alguns comentários e sugestões, e iniciou sua argüição. Ao final, o referido professor deu-se por satisfeito. Em seguida, a Sra. Presidente passou a palavra para o Prof. Dr. José Luis de Lima Filho, que agradeceu o convite, fez alguns comentários e sugestões, e iniciou sua argüição. Ao final, o referido professor deu-se por satisfeito. Logo após, a Sra. Presidente passou a palavra para o Prof. Dr. Ranilson de Souza Bezerra, que agradeceu ao convite, fez alguns comentários e sugestões, iniciando sua argüição. Ao final, o referido professor deu-se por satisfeito. Em seguida, a Sra. Presidente passou a palavra para a co-orientadora Profa. Dra. Ana Lúcia Figueiredo Porto, que fez alguns comentários sobre o trabalho da aluna Em seguida, a Sra. Presidente, na qualidade de orientadora, usou da palavra para tecer alguns comentários a respeito do trabalho da aluna, agradecer à Banca Examinadora e parabenizar a candidata.. Finalmente, a sessão foi suspensa para proceder ao julgamento pela Banca Examinadora, a qual se reuniu na Secretaria do Curso. Após alguns comentários, a Banca decidiu, por unanimidade, conceder a menção "**Aprovada com Distinção**". Nada mais havendo a tratar, lavrei a presente ata que vai assinada por mim, Secretário, e demais membros da Banca Examinadora. Recife, 26 de fevereiro de 2008.

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A Deus, presença constante em minha vida, pela força interior e necessidade da busca constante do saber.

Aos meus Pais, pelo apoio, carinho, dedicação, compreensão, sempre presentes ao meu lado, dando-me forças nas horas mais difíceis desde o início de minha vida.

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RESUMO

Colagenases são enzimas proteolíticas capazes de degradarem diferentes tipos de colágeno e apresentam alta especificidade a estas moléculas. Dentre os vários organismos capazes de produzirem estas enzimas, estão as leveduras. As colagenases têm as mais variadas aplicações. Destacam-se as aplicações na indústria de fármacos, onde são utilizadas no tratamento de cicatrizes hipertróficas, na indústria de cosméticos (dermocosméticos), usadas no tratamento de acnes e rugas, e na indústria alimentícia, no amaciamento de carne. O presente trabalho objetivou a seleção de espécies do gênero *Candida*, produtoras de colagenase extracelular, a determinação das condições de cultivo para a produção da enzima através de processo fermentativo e a caracterização bioquímica parcial da colagenase. Para isto, foram realizados experimentos de fermentação com o auxílio de planejamentos estatísticos (fracionário-2^{6-2} e completo-2^3) para a seleção das melhores condições de produção. No estudo de seleção identificou-se que a espécie *Candida albicans* URM3622 apresentou maior atividade colagenolítica qualitativa no meio ágar-gelatina (halo = 25 mm), e quantitativa (5,0 U/ml) utilizando azocoll como substrato. A análise dos dados do planejamento estatístico fracionário (2^{6-2}) demonstrou que entre as variáveis estudadas (pH, tempo de produção, velocidade de agitação, temperatura, concentrações do inóculo e do substrato), a concentração do substrato (gelatina) e a velocidade de agitação foram as variáveis mais significativas para a produção da colagenase. Com base nestes resultados foram executados dois planejamentos estatísticos completos (2^3) sucessivos nos quais foram estudados, a concentração do substrato, a velocidade de agitação e o pH. Estes planejamentos demonstraram que todas as variáveis foram significativas para a produção da enzima e que a produção máxima foi obtida utilizando o pH 7,0, a velocidade de agitação de 160 rpm e a concentração de substrato de 2%. Sob estas condições foi obtida uma atividade colagenolítica de 6,7 ± 0,2 (U/ml). No estudo da caracterização parcial da enzima observou-se que a enzima apresentou o pH 8,2 como ótimo para a sua atividade e a temperatura ótima de 45 ºC. A colagenase mostrou-se estável aos valores de pH na faixa de 7,2 a 8,2 e à temperatura na faixa de 28 °C a 45 °C. Os resultados obtidos demonstram que a *Candida albicans* (URM3622), é uma fonte viável de colagenase, sendo assim a sua produção é de interesse para as indústrias farmacêutica, cosmética e alimentícia.

Palavras-chave: Colagenase, *Candida albicans*, Planejamento Estatístico, Caracterização Parcial.

ABSTRACT

Collagenases are proteolytic enzymes with capability for degradation of different types of collagen and present high specificity for these molecules. Among the organisms that are able to produce these enzymes are the yeasts. The collagenases are used in several industrial applications pointing out in pharmaceutical industry, where they are applied in hypertrophic scars treatment, in cosmetics, in the acne and wrinkle treatment, and in the food industry as meat softening. The present work aimed the selection of *Candida* species that produce extracellular collagenase, the determination of culture conditions for enzyme production through a fermentative process and the partial biochemical characterization of collagenase. In this way, were carried out fermentation experiments using experimental statistical designs 2^{6-2} fractionary factorial and 2^3 full factorial for the selection of the best conditions of production. In the study of selection it was identified that *Candida albicans* URM- 3622 specie presented the best qualitative collagenolytic activity in agar-gelatin medium (hallo = 25 mm), and quantitative activity (5.0 U/ml) using azocoll as substrate. The analysis of 2^{6-2} fractionary statistical design data demonstrated from the studied variables pH, production time, orbital agitation speed, temperature, inoculum size and substrate concentration, the substrate concentration (gelatin) and the agitation speed were the variables more significant for collagenase production. Based on these results two 2^3 full statistical design were run successively in which were studied the substrate concentration, agitation speed and the pH. These two 2^3 full statistical design showed to be the studied variables significant for the enzyme production. The maximum production was found at pH 7.0 with an agitation speed of 160 rpm and 2 % substrate concentration, where under these conditions it was obtained a collagenolytic activity of 6.7 ± 0.2 (U/ml). In the study of partial characterization of enzyme, it was found the optimal pH of 8.2 for its activity and the optimal temperature of 45 ºC. The collagenase showed to be stable at pH range of 7.2 – 8.2 and at temperature range of 28 – 45 °C. These achieved results demonstrate that *Candida albicans* (URM-3622) can be a viable source of collagenase and could be of interest for pharmaceutical, cosmetic and food industries.

Key-words: Collagenase, *Candida albicans*, Experimental Statistical Designs, Partial Characterization.

1. INTRODUÇÃO

Colagenases são enzimas proteolíticas capazes de degradar diferentes tipos de colágeno e que apresentam alta especificidade a estas moléculas (Ravanti & Kähaäri, 2000). Estas enzimas são capazes de hidrolisar o colágeno em pH igual ou próximo ao pH fisiológico e podem ser obtidas de vários tecidos de origem animal, vegetal ou a partir de microrganismos (Said & Pietro, 2002). Após a original demonstração da atividade colagenolítica em culturas filtradas de certas espécies de *Clostridium* por Mandl (1961), um grande número de outras espécies microbianas, tais como as leveduras, têm sido utilizadas para sintetizar enzimas que podem ser classificadas como colagenases.

Leveduras são fungos, predominantemente unicelulares, que se reproduzem sexuada e assexuadamente por brotamento, fissão ou cissiparidade. Microscopicamente possuem formas variadas: pseudomicélio, blastosporos, artrosporos e micélio verdadeiro. Macroscopicamente apresentam-se como colônias brilhantes ou opacas, de textura glabra ou membranosa, consistência cremosa, com ou sem pigmentação e não formam hifas aéreas (Lacaz et al, 2002).

Dentre as leveduras que apresentam grande capacidade de produzir enzimas proteolíticas, podem ser citadas: *Bullera, Candida, Cryptococcus, Debaryomyces, Endomycopsis, Hanseniaspora, Hansenula, Kloeckera, Kluyveromyces, Metschnikowia, Nematospora, Pichia, Rhodosporidium, Rhodotorula, Saccharomyces, Saccharomycopsis, Schwanniomyces, Sporodiobolus, Torulopsis, Trichosporon, Yarrowia lipolytica.* Dentre estas, o gênero *Candida* tem sido identificado como uma fonte viável para a obtenção destas enzimas (Neves et al., 2006).

As colagenases de origem microbiana têm as mais variadas aplicações tanto no âmbito terapêutico como biotecnológico. Estas enzimas são encontradas na composição de pomadas cicatrizantes, onde são utilizadas, no tratamento de queimaduras e na remoção de cicatrizes hipertróficas (Markovich, 2008). São empregadas na indústria de cosméticos e ainda nos protocolos de trabalho de diversas áreas de pesquisa científica (Domogatsky, 2006). Ainda, através das colagenases têm-

se obtido ampla variedade de peptídeos utilizados na indústria de produtos dietéticos e agentes imunoterápicos (Ravanti & Kähaäri, 2000; Goshev et al., 2005).

Devido às inúmeras aplicações terapêuticas e biotecnológicas das colagenases, a pesquisa de novos microrganismos produtores desta enzima tem crescido nos últimos anos, pois o alto custo da colagenase comercializada dificulta a sua utilização na composição de medicamentos e na área biotecnológica. Por este motivo, torna-se necessário disponibilizar novas colagenases ao mercado de enzimas. Neste contexto, as colagenases produzidas por *Candida* representam uma fonte alternativa destas enzimas a serem utilizadas, considerando-se a possibilidade da sua obtenção a baixo custo.

2. REVISÃO DA LITERATURA

2.1. Potencial industrial das enzimas

A crescente necessidade da substituição da catálise pela biocatálise tem impulsionado um aumento significativo no consumo de enzimas a nível internacional. A tecnologia enzimática vem sendo utilizada em diversos setores industriais em processos tradicionais da indústria de alimentos, farmacêutica e da indústria de química fina. A implementação da tecnologia enzimática resulta em produtos de maior qualidade, obtidos por processos com menor consumo energético e de menor impacto ambiental. O mercado mundial de enzimas industriais é estimado em US$ 2,3 bilhões, correspondendo a aproximadamente 60% do mercado total de enzimas e tende a crescer a uma taxa de 5,7% ao ano (www.im.ufrj.br, 2008).

Neste mercado, as proteases constituem o grupo mais importante entre as enzimas industriais, sendo responsáveis por cerca de 60% do total das enzimas comercializadas em todo mundo (Sana et al., 2006) para utilização nos setores alimentícios (El-Beltagy et al., 2004), farmacêuticos (Tunga, 2003) e de detergentes (Moreira, 2002).

2.2. Importância das proteases produzidas por microrganismos

A incapacidade das enzimas de origem vegetal e animal em satisfazer a demanda mundial têm aumentado o interesse por enzimas de origem microbiana. Estes biocatalisadores são produzidos por processos de baixo custo e apresentam um recurso renovável, pois a biomassa resultante de determinados processos fermentativos pode ser utilizada como fertilizante. A utilização destes biocatalisadores tem crescido devido aos avanços no campo da microbiologia industrial, pelo fato da produção não estar condicionada às questões sazonais e geográficas e ainda pela possibilidade do uso de matérias-primas pouco dispendiosas (Rodrigues & Santánna, 2001).

Os microrganismos representam uma fonte atrativa de protease por poderem ser cultivados em grandes quantidades, em um tempo relativamente curto, por métodos estabelecidos de fermentação. Além disto, as proteases microbianas têm uma vida mais longa e podem ser armazenadas sob condições ideais por semanas, sem perda significativa da sua atividade. Em geral, as proteases microbianas são extracelulares, secretadas diretamente no meio de cultura pelo produtor, simplificando assim o processo de "dowstream" da enzima quando comparado com o processo daquelas obtidas de plantas e animais. Apesar da diversidade de microrganismos produtores de proteases, como bactérias, fungos e leveduras, somente alguns são considerados como produtores apropriados para a exploração comercial (Gupta et al*.,* 2002).

A seleção de microrganismos e a otimização da composição dos meios de cultura possibilita a obtenção de elevados rendimentos de enzimas com propriedades e especificidades bem determinadas (Rodrigues & Santánna, 2001). As fontes de carbono e nitrogênio fazem parte da composição dos meios de cultura e são nutrientes essenciais para o crescimento dos microrganismos. Cada microrganismo possui a sua própria condição especial para a produção enzimática máxima, deste modo, não existe meio estabelecido para a melhor produção de proteases de diferentes fontes microbianas (Kumar & Takagi, 2000).

2.3. Classificação das proteases

De acordo com o Comitê Internacional de Nomenclatura "União de Bioquímica e Biologia Molecular", as proteases são hidrolases classificadas no subgrupo 4, do grupo 3 (E. C. 3. 4). Estas enzimas têm classificação diversificada, não obedecem facilmente às regras da nomenclatura, devido à diversidade de ação e estrutura. Com base no ponto de clivagem na cadeia polipeptídica as proteases ou peptidases são grosseiramente subdivididas em dois grupos: 1) Exoproteases, são aquelas que clivam ligações peptídicas próximas às extremidades e; 2) Endoproteases, as que atuam nas regiões internas da cadeia polipeptídica (Monod et al. 2002).

De acordo com o grupo funcional presente no sítio catalítico, as endoproteases podem ser classificadas como: serina proteases, cisteína proteases, aspárticas

proteases e metaloproteases; enquanto que as exoproteases são divididas com base no seu mecanismo de ação em aminoproteases e carboxiproteases (Monod et al., 2002).

As aminopeptidases atuam no terminal N livre da cadeia polipeptídica podendo liberar um único resíduo de aminoácido (aminopeptidase), um dipeptídeo (dipeptídeo-peptidases) ou um tripeptídeo (tripeptídeo-peptidases). As carboxopeptidases atuam no terminal C da cadeia peptídica e liberam um único aminoácido ou um dipeptídeo. Baseado na natureza do aminoácido, no sítio ativo da enzima, as carboxipeptidases podem ser divididas em três grupos principais: serina carboxipeptidases, metalocarboxipeptidases e cisteína carboxipeptidase (Rao et al., 1998).

Ainda em relação às endopeptidases, as serina proteases caracterizam-se pela presença do grupo serina no sítio ativo. São numerosas e comumente encontradas em vírus, bactérias e eucarióticas, sugerindo que estas enzimas sejam de vital importância para os organismos (Rao et al., 1998).

As proteases aspárticas, também conhecidas como proteases ácidas, são endopeptidases que dependem do resíduo ácido aspártico para a realização da atividade catalítica (Rao et al., 1998).

As cisteína proteases são produzidas tanto por eucariotas como por procariotas, sendo reconhecidas cerca de 20 famílias. A atividade de todas as cisteínas proteases depende do centro catalítico que consistem na cisteína e histidina. Geralmente, as cisteína proteases são ativas apenas na presença de agentes redutores como HCN (Rao et al., 1998).

As metaloproteases têm os mais diversificados tipos catalíticos das proteases. São caracterizadas pelo requerimento de íons bivalentes para que ocorra atividade. Incluem enzimas de variadas origens como toxinas hemorrágicas de cobras venenosas, termolisina de bactérias e as colagenases (Rao et al., 1998).

2.4. Colagenases

Colagenases são enzimas proteolíticas capazes de degradar tanto moléculas de colágeno nativo como desnaturado (Tran & Nagano, 2002). Em geral, outras proteases

não digerem a tripla hélice do colágeno, essa degradação só é possível através da ação de enzimas específicas que requerem íons bivalentes para o desenvolvimento da sua atividade catalítica sendo, portanto, classificadas como metaloproteases (Goshev et al., 2005).

As colagenases ou metaloproteases da matriz extracelular (MMPs) são as principais proteases capazes de clivar a fibra do colágeno nativo dos tipos I, II, III, V e IX. O colágeno tipo I é clivado especificamente no sítio entre Gly^{775} – Ile^{776} da cadeia α1 e entre os resíduos Gly^{775} – Leu^{776} da cadeia α2 pela MMP-1, MMP-8 e MMP-13. Estas colagenases clivam a tripla hélice do colágeno gerando dois fragmentos, o tropocolágeno A (TCA) e o tropocolágeno B (TCB) (Duarte et al., 2005).

A atividade catalítica das colagenases frente às fibras de colágeno difere quanto à especificidade, por exemplo, MMP-1 prefere o colágeno tipo III e MMP-8 prefere o colágeno tipo I (Overall & Lopez-Otin, 2002).

Existe um grande interesse na busca de colagenases de outras fontes que não a animal, pois, esta possui a capacidade de clivar somente o colágeno nativo, isto é, são enzimas que clivam a cadeia helicoidal do colágeno resultando em dois fragmentos (Figura 1), acessíveis a poucas proteases específicas, o TCA (tropocolágeno A) e o TCB (tropocolágeno B). Diferente das anteriores, as colagenases de microrganismos possuem a capacidade de hidrolisar tanto o colágeno nativo como o colágeno desnaturado. Têm afinidade por vários sítios ao longo da cadeia, representando assim, uma fonte promissora para pesquisas e aplicações biotecnológicas (Jung & Winter, 1998).

Colagenases microbianas apresentam diferenças de acordo com o grupo de microrganismos produtores e meios utilizados. A colagenase produzida pelo *Streptomyces* sp., em meio contendo amido, apresentou pH ótimo de 7,5 e peso molecular de 116 kDa (Petrova et al., 2006), a colagenase de *Bacillus* sp. produzida em meio contendo gelatina, apresentou pH ótimo de 3,9 (Nahayama et al., 2000), enquanto que uma enzima colagenolítica obtida em meio contendo colágeno insolúvel, por *Bacillus subtilis,* apresentou sua atividade máxima a pH 9,0, temperatura de 50ºC e peso molecular de 125 kDa (Nagano & To, 1999).

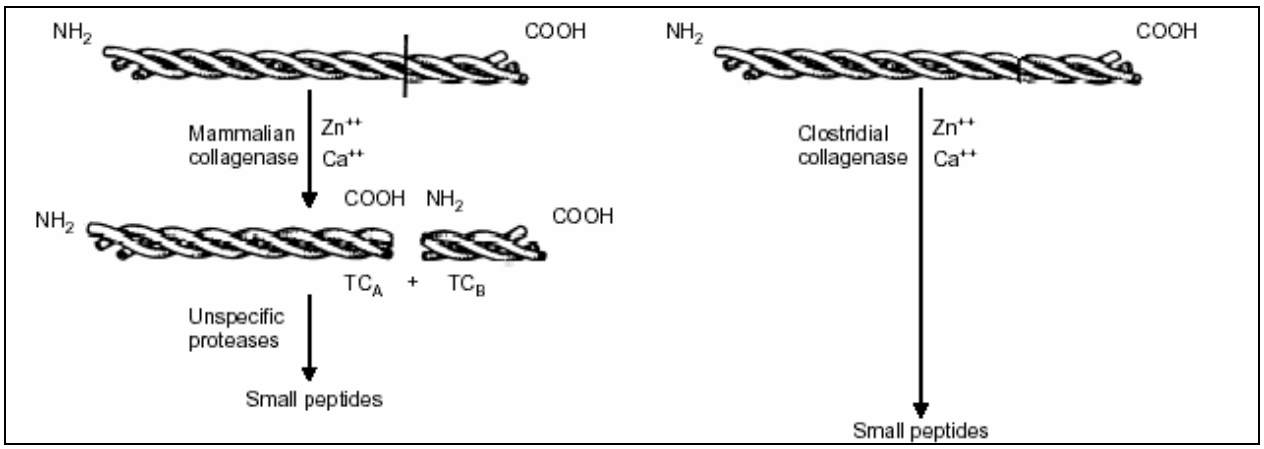

Figura 1. Ação das colagenases de origem animal x colagenases de *Clostridium* sp. Fonte: Jung & Winter, 1998.

Miyoshi & Shinoda (2000) descobriram duas metaloproteases produzidas por *Pseudomonas aeruginosa* capazes de digerir componentes estruturais da célula do hospedeiro o que acelera o processo de invasão da bactéria.

Rodier et al. (1999) purificaram e caracterizaram uma metaloprotease de 95 kDa produzida por *C. albicans* capaz de degradar totalmente o colágeno tipo IV e parcialmente o colágeno tipo I. Esta enzima foi ativa em pH 7,0, podendo facilitar o acesso da levedura ao organismo após atravessar as barreiras endoteliais e pode portanto ser considerada um fator de patogenicidade da *C. albicans.*

Kaminshi et al. (1986) relataram a secreção de uma colagenase produzida por *C. albicans* capaz de degradar o colágeno do dente, e sugeriram que esta enzima deve estar relacionada com a progressão da cárie dentária. Observaram ainda que a mesma não apresentou atividade quando se adicionou ao meio reacional o ácido etilenodiaminotetracético (EDTA) demonstrando tratar-se de uma metaloprotease.

Outros autores também têm demonstrado a produção de enzimas colagenolíticas por espécies do gênero *Candida.* Nishimura et al. (2001) identificaram em seu estudo uma colagenase produzida por *Candida albicans* que não apresentou atividade na presença de EDTA. Costa et al. (2003) relataram a presença de metaloproteases no sobrenadante de cultura de *Candida albicans* capazes de degradar a gelatina. Santos &

Soares (2005) identificaram uma enzima de 50 kDa produzida por *Candida guilliermondii* que foi capaz de degradar colágeno desnaturado.

2.4.1 Colágeno

O colágeno compõe uma família de proteínas fibrosas estruturais e insolúveis presentes em todos os organismos multicelulares. É a principal proteína fibrosa da matriz extracelular dos tecidos conectivos e pode ser encontrado na pele, nos ossos, nos tendões, nos dentes e vasos sangüíneos. Além disto, a degradação do colágeno está envolvida em várias situações fisiológicas e patológicas, como, por exemplo, desenvolvimento ósseo fetal, desenvolvimento embrional, reparação de feridas, invasão de tumores malígnos, ulcerações intestinais, inflamação crônica periodontal e artrite reumatóide (Ravanti & Kähäri, 2000).

A unidade estrutural básica do colágeno é chamada de tropocolágeno, o qual possui uma massa molecular em torno de 285 kDa e é constituído de três cadeias polipeptídicas do mesmo tamanho, as quais variam de acordo com o tipo de colágeno. O colágeno tipo I é o principal componente da matrix extracelular dos animais vertebrados correspondendo a 30% do total de proteínas. A molécula do colágeno tipo I é composta por duas cadeias polipeptídicas $\alpha_{1,}$ com massa molecular de 100 kDa, e uma cadeia polipeptídica $\alpha_{2,}$ com massa molecular de 95 kDa que são superenoveladas umas com as outras (Duarte et al., 2005).

Devido à sua estrutura rígida, a degradação enzimática do colágeno é restrita a um limitado número de proteases e é obtida usando colagenases, uma classe de proteinases altamente específicas para o colágeno (Ravanti & Kähäri, 2000).

O colágeno e os seus peptídeos são interessantes biomateriais, e as aplicações comerciais do colágeno estão crescendo em uma velocidade rápida. Por exemplo, o colágeno é adicionado regularmente como ingrediente na preparação de medicamentos, bebidas, alimentos, cosméticos e em uma variedade de produtos para a saúde (Watanabe, 2004). Vários trabalhos têm demonstrado que os produtos resultantes da degradação proteolítica do colágeno exibem uma variedade de atividades de interesse medicinal e industrial. Podem servir como conservantes não-

alérgicos em medicamentos, ingredientes para alimentos dietéticos e nutrição parenteral e ainda podem ser utilizados na produção de cosméticos (Nakayama et al., 2000).

2.4.2. Aplicações das colagenases

As colagenases têm sido largamente utilizadas na medicina com o propósito de limpar feridas necrosadas, escaras, cicatrizes pós-operatórias, e no tratamento de psoríase e pediculoses (Markovich, 2008), em cicatrizes de queimaduras de crianças (Özcan et al., 2002), em lesões de mamilos de mulheres em aleitamento (Kuşcu et al., 2002) e no tratamento de cicatrizes hipertróficas (Cheng et al., 1999).

Segundo Jung & Winter (1998) existem inúmeras vantagens do uso de colagenases de microrganismo no tratamento de feridas: a) Removem o tecido necrosado com maior eficiência por sua capacidade de hidrolisar vários tipos de colágeno; b) são indolores e não hemorrágicos; c) podem ser usados por longos períodos e também em associação com outros medicamentos; d) atraem macrófagos e fibroblastos para o local da ferida; e) aumentam a formação de tecidos de granulação e estimulam o próprio organismo a promover a cicatrização.

Cheng et al. (1999) investigaram o mecanismo de degradação do colágeno em cicatrizes hipertróficas por colagenases de bactérias onde os experimentos foram conduzidos utilizando-se injeções de colagenases diretamente sobre as cicatrizes hipertróficas em ratos e 13 pacientes. Os resultados demonstraram uma redução de volume da cicatriz de 86% nos ratos e de 46,92% nos pacientes. Em 4 dos 13 pacientes a redução foi maior do que 50% e apenas um entre os 13 pacientes apresentou reaparecimento da cicatriz após o tratamento. Exames histológicos mostraram claramente a dissolução das fibras de colágeno.

Kuşcu et al. (2002), desenvolveram um trabalho utilizando colagenase de *Clostridium histolyticum* na prevenção e tratamento de lesões de mamilo de mulheres em período de aleitamento, as quais se desenvolvem imediatamente ao pós-parto, podendo provocar dores intensas no mamilo, impedindo até a continuidade do aleitamento. Os resultados foram semelhantes aos obtidos com os tratamentos

convencionais ratificando assim, a eficácia da utilização da colagenase neste tipo de lesões.

O Centro de Pesquisa em Cardiologia da Rússia (The Russin Cardiology Research Center) vem desenvolvendo estudos com proteases com atividade colagenolítica de *Bacillus subtilis*, *B. licheniformis*, *Streptomyces* sp. e *Aspergillus niger* para serem utilizadas no tratamento de queimaduras de terceiro grau, os quais demonstraram efeito não tóxico, baixa irritação e um processo de cicatrização duas vezes mais rápido que os observados em feridas não tratadas com estas enzimas (Domogatsky, 2006).

3. OBJETIVOS

3.1. Objetivo geral

Selecionar espécies de *Candida* produtoras de colagenase e caracterizar parcialmente a enzima obtida pela maior produtora.

3.2. Objetivos específicos

- Selecionar uma espécie do gênero *Candida* produtora de colagenase;
- Avaliar qualitativamente e quantitativamente a produção de colagenase da espécie selecionada;
- Determinar dentre os fatores: temperatura, pH inicial do meio, agitação, concentração do substrato, inóculo e tempo de produção, aqueles que podem influenciar a produção de colagenase através de um planejamento fracionário;
- Determinar as melhores condições de produção da colagenase através de planejamento estatístico completo;
- Caracterizar a colagenase quanto aos aspectos físico-químicos, tais como: pH ótimo, temperatura ótima, estabilidade ao pH e à temperatura.

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5. ARTIGO A SER SUBMETIDO PARA PUBLICAÇÃO NO PERIÓDICO BIOCHEMICAL ENGINEERING JOURNAL

PRODUCTION AND PARTIAL CHARACTERIZATION OF A COLLAGENASE FROM *Candida albicans* URM3622

Carolina A. Lima[1,2], Tatiana S. Porto[3], Daniela Araújo Viana[3], José Luiz de Lima Filho[1,2], Ana Lúcia F. Porto [2,4], Maria das Graças Carneiro da Cunha[1,2 (*)].

[1] Department of Biochemistry, UFPE, Av.Professor Moraes Rego s/n., Campus Universitário, 50670-901, Recife, PE, Brazil

[2] Laboratory of Immunopathology Keizo Asami (LIKA), UFPE, Av. Prof. Moraes Lins do Rego, s/n. 50670-901, Recife, PE, Brazil

[3] Departament of Biochemical and Pharmaceutical Technology, University of São Paulo, Av. Prof. Lineu Prestes, 580, B.16. 05508-000, São Paulo, SP, Brazil

[4] Department of Morphology and Animal Physiology, UFRPE, Av. Dom Manoel de Medeiros, s/n. 52171-900, Recife, PE, Brazil

(*) Corresponding author: Address: Department of Biochemistry. University Federal of Pernambuco-UFPE, Av. Professor Moraes Rego s/n., Campus Universitário, 50670-901, Recife, PE, Brazil. Fax: +55-81-2126-8487.

E-mail address: mgcc@ufpe.br

5.1. Abstract

Culture conditions were studied for an extracellular collagenase produced by *Candida albicans* URM3622 using three experimental designs (one 2^{6-2} fractionary factorial and two 2^3 full factorial). The analysis of 2^{6-2} fractionary design data demonstrated for pH, time, temperature, inoculum size, orbital agitation speed and substrate concentration, last two variables were the more significant ones for collagenase production. Based on these results two 2^3 full factorial design were run successively in which the substrate concentration, orbital agitation speed and pH were studied. These two set of experiments showed that all variables studied were significant for the enzyme production, with the maximum collagenolytic activity of 6.7 ± 0.2 (U/ml) was achieved at pH 7.0 with an orbital agitation speed of 160 rpm and 2 % substrate concentration. Maximum collagenolytic activity was observed at pH 8.2 and 45 ºC. The collagenase showed to be stable at pH range of 7.2 – 8.2 and at temperature range of 28–45 °C. These achieved results clearly indicate that *Candida albicans* URM3622 is a viable source of collagenase and could be of interest for pharmaceutical, cosmetic and food industry.

Key-words: Enzyme Activity, Enzyme Production, Submerged Culture, Protease, Collagenase, *Candida albicans.*

5.2. Introduction

Proteases show a wide variety of functions and have various important biotechnological applications. They represent one of the three largest groups of industrial enzymes [1] and occupy 60–65% of the global industrial enzyme market [2,3]. Among the various protease source, microbial proteases play an important role in biotechnological processes accounting for approx 59% of the total enzyme used [4].

Proteases from microorganisms have been extensively utilized in various fields because large amounts of proteases can be produced at a low cost and quickly [5]. With the advent of new frontiers in biotechnology, the spectrum of protease application has expanded into new fields such as clinical, medicinal and analytical chemistry [6]. In this context, the collagenases became an important enzyme. Collagenases are proteolytic enzymes that can hydrolyze both native and denatured collagens. These enzymes are widely used not only in chemical and medical industries but also in food and basic biological science [7]. The enzymatic degradation of collagen produces peptides which have been shown to have several biological activities of industrial and medicinal interest [8], leading to a wide variety of applications, e.g., an immunotherapeutic agent, a moisturizer for cosmetics, a preservative, seasoning and dietary materials [9-12].

It is well known that extracellular protease production in microorganisms was greatly influenced by medium components, especially carbon and nitrogen sources, physical factors, such as pH, temperature, inoculum size, orbital agitation speed and incubation time [13, 14]. To study the influence of these variables on the enzyme production has been used statistical tool.

Statistical design of experiments is a tool widely used for process optimization and control. The multivariate design of experiments is an efficient method for studying

the influence of a certain number of variables on a given response of interest. The significant factors and their effects can be studied with fewer numbers of runs using fractional factorial runs. With the proper design, the significant effects can be identified from a minimum number of experiments at lowest operation cost [15].

The aims of this work were select *Candida* species for collagenase production; determine the best conditions for the enzyme production using experimental designs and characterize the enzyme produced.

5.3. Material and methods

5.3.1. Microorganisms

The microorganisms used in this study (*C. albicas* URM 743, 835, 2252, 3620, 3622, 5324, *C. intermedia* URM 907, 1075, *C. tropicalis* URM 916, 1150, 112, 933, *C. guilliermondii* URM 1020, 1084, 561, 644, 917, 1065, *C. buinensis* URM 4674*, C. butyri* URM 4679*, C. famata* URM 2253) were obtained from Collection of Cultures "Micoteca" (URM) of Department of Mycology of the University Federal of Pernambuco. The microorganisms were maintained at 4 ºC on Sabouraud dextrose agar slants.

5.3.2. Screening of *Candida strains* as collagenase producers

Collagenase of *Candida* strains was obtained from a culture supernatant. Strains were inoculated (calibrated suspensions A_{600} = 0.1) into a 250 ml Erlenmeyer flask containing 50 ml of 1.5% (w/v) malt extract supplemented with 1.0 % (w/v) gelatin, and grown for 72 h at 30 ºC in an orbital incubator shaker with 140 rpm. The cell-free supernatant were clarified by centrifugation at 12,000 x g and 4 ºC for 5 min, and carried out for the screening of collagenase using plates containing 1.0% (w/v) gelatin and 1.5% (w/v) agar. Each plate was incubated during 18 hours at 37 ºC [16]. The ability to break gelatin was shown by the formation of transparent halos on the plates and expressed in mm. The strains showing halos were submitted to the quantitative test.

5.3.3. Azocoll assay for protease activity determination

Azocoll (Sigma Chemical Co. St Louis, MO) assay was carried out by a method developed by CHAVIRA et al. [17] modified. Azocoll was suspended in 0.05 M Tris-HCl

buffer (pH 7.2) containing 1 mM $CaCl_2$ at a final concentration of 5 mg/ml. Briefly, 150 µl of samples (cell-free supernatants) and 150 µl of buffer were mixed with 270 µl of azocoll suspension in a 2.0 ml reaction tube. The reaction tubes were incubated at 37 ºC in a water bath under agitation. After 18 hours of incubation, the reaction was stopped by centrifugation of samples at 10,000 x g (Kubota KR-20000T Japan) and 4 ºC for 10 minutes. The absorbance of the supernatant solution was measured at 520 nm with a spectrophotometer (Micronal B582). One activity unit was defined as the amount of enzyme that produces an increase in the optical density of 0.1 in 18 h at 520 nm.

5.3.4. Protein determination

The protein concentration was determined according to Smith et al. [18], using bovine serum albumin as the standard protein.

5.3.5. Biomass determination

The biomass concentration was determined as cell dry weight after centrifugation at 12,000 x g and 4 ºC for 5 min of 50 ml of culture broth, and dried at 80 ºC overnight until constant weight and expressed in g/l.

5.3.6. Selection of variables that influence the production of collagenase by *C. albicans* URM3622

The collagenase production by *Candida albicans* URM3622 was evaluated using a statistical design. A 2^{6-2} fractional factorial design was carried out to verify the effects and interactions of the substrate concentration (gelatin), medium initial pH, orbital

agitation speed, inoculum size, time and temperature on the production of collagenase. In this design, a set of 20 experiments with four replicates at the central points was performed. The range and levels of the components under study are given in Table 1. The production experiments were carried out using 250 ml Erlenmeyer flasks containing 50 ml of culture medium 1.5 % (w/v) malt extract supplemented with different concentrations of gelatin. At the end of fermentation samples were colleted amd assayed for total protein, collagenolytic activities and biomass. The results were analyzed using “Statistica” software version 7.0 (Statsoft Inc., USA).

5.3.7. Determination of the best conditions for collagenase production by *C. albicans* URM3622

Two successive 2^3 full factorial designs were carried out to study the effects and interactions of medium initial pH, substrate concentration, and orbital agitation speed on the production of collagenase. In these experiments, a set of 8 experiments with four replicates at the central points was performed. The range and levels of the components under study are given in Table 2. The production experiments were carried out using 250 ml Erlenmeyer flasks containing 50 ml of culture medium (1.5 % w/v malt extract supplemented with different concentrations of gelatin) starting with 10^7 cells/ml of inoculum and incubated for 48 h at 37 ºC under different agitation speed. Samples were collected at 48 h of growth and assayed for total protein, collagenolytic activities and biomass. The results were analyzed using “Statistica” software version 7.0 (Statsoft Inc., USA).

5.3.8. Effect of pH on collagenase activity and stability

For determination of optimum pH of the enzyme, the reaction mixture buffer of the 0.5% (w/v) azocoll was varied over the pH range of 3.0 - 10.8. The buffers used were 0.05 M citrate (pH 3.0 - 6.0), 0.05 M Tris-HCl (pH 7.2 - 9.0) and 0.05M carbonate-bicarbonate (10.0 – 10.8). For stability assay, the enzyme was incubated at 5 ºC in buffers of different pH values (pH 3.0 - 10.8, 0.05 M). The incubation time of samples varied from 30 to 240 minutes. Collagenolityc activity of the samples was measured at 37 ºC, using 0.5% (w/v) azocoll in 0.05 M Tris-HCl buffer (pH 8.2).

5.3.9. Effect of temperature on collagenase activity and stability

For the determination of optimum temperature for the enzyme activity, the reaction mixture containing 0.5% (w/v) azocoll was incubated over a temperature range from 28 ºC to 80 ºC using 0.05 M Tris-HCl buffer (pH 8.2). For the determination of the thermal stability, the enzyme was pre-incubated over a temperature range from 28 ºC to 70 ºC. The incubation time of the samples varied from 30 to 240 minutes. After incubation, the samples were submitted to determination of collagenolityc activity, using the 0.5% (w/v) azocoll in 0.05 M Tris-HCl buffer (pH 8.2) at 45 ºC.

5.4. Results and discussion

5.4.1. Screening of *Candida* strains

Among the 21 *Candida* strains studied (Table 3), all *Candida albicans* and three strains of *Candida tropicallis* (URM-112, URM- 916, URM-933) were able to degrade the gelatin and to use the azocoll as substrate. *Candida albicans* strains were those that showed higher qualitative and quantitative collagenolytic activity. The *Candida albicans* (URM-3622) showed the greatest degradation of gelatin with 25 mm degradation hallo in gelatin-agar and quantitative collagenolytic activity of 5.0 U/ml.

Costa et al. [19] identified the presence of enzymes, in the culture of *Candida albicans*, which were capable of degrading gelatin. Kaminihi et al. [20] isolated and characterized a collagenolytic enzyme present in the culture of *Candida albicans* that showed activity against azocoll and native collagen.

The results indicated that strain *Candida albicans* URM-3622 showed the highest collagenolityc activity. Therefore, this strain was selected for the subsequent experiments.

5.4.2. Variables that influence the production process of collagenase by *C. albicans* URM3622

The statistical analysis was performed for all the responses (biomass, collagenolytic activity, total protein and specific activity) however, the collagenolytic activity was the response that really important and was used for the analysis of the efficiency of enzyme production by microorganism.

The results obtained with the 2^{6-2} fractionary design are shown in Table 4. Since this is a resolution IV screening design, its analysis results in contrasts where the main effects are confounded with the three-factor interactions, while the two-factor interactions are confounded with each other. As it is well-known, a factoring model can be interpreted as modeling a given response by a special kind of power series where higher-order terms are assumed to be less important than those of a lesser order, otherwise the series would not converge, and the model would be useless. It is natural, therefore, to assume that third-order terms be much less important than first-order ones, and, in a first approximation at least, may be neglected [21]. Assuming that third-order interactions are negligible, as discussed above, the results for the collagenolytic activity would be interpreted as follows.

The Pareto chart represents the estimated effects of the variables, and the interactions on collagenolytic activity in decreasing order of magnitude. The length of each bar is proportional to the standardized effect. The vertical line can be used to judge which effects are statistically significant. Bars extended beyond this line correspond to the effects statistically significant at a confidence level of 95%. It can observe that the main effects substrate concentration (6) and orbital agitation speed (4) were significant (Figure 1). These two variables showed significant positive effect, suggesting that the increase of the parameters values improve the collagenase production.

It has been fairly well established that extracellular protease secretion in microorganisms is substantially influenced not only by medium components including carbon source, nitrogen source, and trace elements, but also by culture conditions including pH, temperature, orbital agitation speed, and inoculum size. In this study, collagenase production was influenced by the concentration of the substrate and the

orbital agitation speed (Figure 1). Chi et al. [22] performed an optimization study of medium and cultivation conditions for protease production by the yeast *Aureobasidium pullulans*t and observed that the agitation speed influenced the protease production.

It has been reported that effects of a specific nitrogen supplement on protease production differ from organism to organism although complex nitrogen sources are usually used for protease production. In this study, the gelatin concentration was the variable that most influenced on collagenase production (Figure 1). This result shows that the enzyme production can be induced by the increase of gelatin concentration in the culture medium.

In addition, a significant interaction between temperature (1) and inoculum size (2) was also observed (Figure 1). The positive interaction effect means that an increase in temperature with a simultaneous increase in inoculum size led to an increase in collagenolytic activity.

The importance of inoculum size with regard to microbial fermentation processes is generally accepted. However, no appreciable change in enzyme activity was observed with higher inoculum sizes. This demonstrates that inoculum size does not exert an unlimited effect on fermentation processes. There is an optimum value to be achieved, and this appears to be dependent on the microbial species and fermentation system being utilized. As the inoculum size had no significant effect for the production of the enzyme, the concentration of 10^7 cell/mL was set for later studies.

It is known that temperature is one of the most critical parameters that has to be controlled in bioprocess [23]. Several authors have observed the production of collagenase by *Candida albicans* when this yeast is incubated at 37 °C [24-25, 19]. In

this study, the temperature has not effect on the enzyme production, therefore following experiments were performed at 37 ºC.

Despite the pH did not present a significant effect in this study, it has been noted that one important characteristic of most microorganisms is their strong dependence on the extracellular pH for cell growth and enzyme production [26]. According to this comment and due to the fact that fractional factorial designs have many errors of confounding, it was decided to study again the influence of pH in the production of the enzyme.

Based on the analyzed results, three variables, substrate concentration, orbital agitation speed and pH were selected for more detailed subsequent studies, which will determine the best conditions for the collagenase production. Because the other variables were not significant, they were fixed and the conditions selected to continue the experiments were: 10^7 cells/ml of inoculum size, 48 h and 37 ºC for incubation time and temperature, respectively.

5.4.3. Determination of the best conditions for collagenase production by *C. albicans* URM3622

The strategy used to attain the goal of this work was to explore in more detail a dominion around the experimental conditions previously selected for collagenase production by *Candida albicans* URM-3622. For this two successive full factorial designs were performed.

The results of the experiments of the first full factorial design were showed in the Table 5. Analyzing the results, it was verified that the experiment at the number 8

showed the higher value (7.06 U/mL) of collagenolytic activity. This value was higher than that obtained in the first design.

Analyzing the Pareto chart (Figure 2), it appears that all the main variables were significant. All variables showed significant positive effect, suggesting that the increase of the parameters values improve the collagenase production. Although was observed that there was significant interaction between these three variables. This interaction is best examined through the cubic graph (Figure 3).

Figure 3 shows the interaction between the variables agitation, pH and concentration of substrate in function of the collagenolytic activity. It can be observed that the top face of the cube shows the highest values of effect, mainly the corner value 7.284. This condition was corresponding to the 2% substrate concentration, agitation speed (160 rpm) and pH 7.0, i.e., the highest level of the variables.

Similar results were obtained for Patel et al. [27], while studying the concentration of gelatin influence in the production of an extracellular protease produced by *Bacillus* sp.

Chi et al. [22] and Patel et al. [27] observed that the culture medium initial pH influenced the protease production by *Aureobasidium pullulans* and *Bacillus sp*., respectively.

As said previously, the results of the first full factorial design showed an increase in the production of collagenase by *C. albicans* when the agitation speed increased from 120 to 160 rpm. Potumarthi et al. [28] studied the influence of the agitation speed in the production of a protease from *Bacillus licheniformis* and also observed an increase on protease production with an increase in agitation speed from 200 to 300 rpm.

From these results, new levels were selected for the variables of the second full factorial design (Table 2).

The results presented in Table 6 show that the central point of the second full factorial design was the best condition for the collagenase production. It was expected that the values of the second full factorial design was higher than the values of the first, because it had indicated that with the increase in the variables levels would increased simultaneously the collagenolytic activity. Although no increase in activity occurred, the results indicated that the best condition was the run number 8 of the first design, i.e., the central point of the second design, which was the optimum condition for the collagenase production by *Candida albicans*.

After statistical analysis of the results it was noted that only the agitation speed was significant, showing negative effect (Figure 4). This means that when the speed is lower, the activity value will be greater.

5.4.4. Effect of pH on activity and stability of collagenase

Maximum collagenolytic activity was observed at pH 8.2 in a 0.05 M Tris-HCl buffer (Figure 5A). This result is in agreement with those found by Okamoto et al. [29]. These authors identified a collagenase produced by thermophillus *Bacillus* sp. that presented maximum collagenolytic activity at pH 8.0. In our work was also observed a retention of more than 60% of the maximum activity between pH 5.0 and 9.0, and no activity was detected at pH 3.0 and 4.0. These results are not compatible with that obtained by Nakayama et al. [30] that identified a novel collagenolytic enzyme with optimum pH in acid region. However, the results that have been found by Nakayama are

in striking contrast with the characteristics of known collagenases which have an optimum pH of 7-8 [31].

Petrova et al. [32] studied the production of collagenolytic enzymes produced by *Streptomyces* sp. and discovered two collagenases that showed maximum collagenolytic activities at pH 7.5. More than 50% of the maximum activity was detected for these enzymes between pH 6.5 and 9.0. Complete inactivation of collagenase activity was observed at pH value less than 4.5 and greater than 10.5. These results are in agreement with the datas obtained in this work.

Figure 5B shows collagenase stability at pH range of 3.0 - 10.8 during 240 minutes of incubation. The enzyme was stable at pH 7.2 (93.8%) and 8.2 (95.2%) after 120 minutes of incubation, and after 240 minutes of incubation still maintained 63.7 and 73.6 % of the collagenolytic activity, respectively.

5.4.5. Effect of temperature on activity and stability of collagenase

The collagenase was most active at 45 ºC and a reduction in activity was observed at above values being completely inactivated at 80 ºC (Figure 6A). The thermostability profile indicated that the enzyme was stable after 240 minutes at the range of 28-45 ºC and retained c.a. 62.0% of the activity while at 70 ºC 100% of the original activities were lost (Figure 6B). Similar results were found by Kaminishi et al. [20] when isolated and characterized a collagenase produced by *Candida albicans,* which showed a maximum collagenolytic activity at 45 ºC, and approximately 90% of the enzyme activity was lost at 60 ºC, and no activity was detected at 70 ºC.

5.5. Conclusion

There is a growing acceptance for use of experimental designs in biotechnology. Our group obtained satisfactory enzyme production from microbial source where statistical approach was a useful tool. The application of experimental design for selection of culture condition for collagenase production by *Candida albicans* URM3622 allows quick identification of the important factors: pH, orbital agitation speed and substrate concentration and interactions between them, which achieved an increase in the production of collagenase by this microorganism. The present study on collagenase production from *C. albicans* URM3622 clearly indicates the importance of this organism in the field of industrial enzyme production.

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5.6. Acknowledgments

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Table 1 – Factor levels used in the 2^{6-2} design used for studying collagenase production by *C. albicans* URM3622.

Factors	**Level**		
	Lower (-1)	Center (0)	Higher (+1)
Substrate concentration (% w/v)	0.5	1.0	1.5
Medium initial pH	5.0	6.0	7.0
Inoculum size (cells/mL)	10^5	10^7	10^8
Orbital agitation speed of (rpm)	100	120	140
Time of production (hours)	48	72	96
Temperature (º C)	30	34	37

Table 2 – Factor levels used in the first 2^3 and the second 2^3 design used for studying collagenase production by *C. albicans* URM3622.

1st 2^3 Design	**Level**		
Factors	Lower (-1)	Center (0)	Higher (+1)
Substrate concentration % (w/v)	1.0	1.5	2.0
Medium initial pH	5.0	6.0	7.0
Orbital agitation speed (rpm)	120	140	160
2st 2^3 Design	**Level**		
Factors	Lower (-1)	Center (0)	Higher (+1)
Substrate concentration % (w/v)	1.5	2.0	2.5
Medium initial pH	6.0	7.0	8.0
Orbital agitation speed (rpm)	140	160	180

Table 3 – Qualitative and quantitative collagenolytic activity of *Candida* species.

Species	Qualitative test, Zone diameter (mm)	Quantitative test, Collagenolytic activity (U/ml)
C. albicans URM743	18	2.93
C. albicans URM835	16	2.20
C. albicans URM2252	18	2.63
C. albicans URM3620	18	3.95
C. albicans URM3622	25	5.00
C. albicans URM5324	21	1.44
C. buinensis URM4674	-	NT
C. butyri URM4679	-	NT
C. famata URM2253	-	NT
C. guilliermondii URM561	-	NT
C. guilliermondii URM644	-	NT
C. guilliermondii URM917	-	NT
C. guilliermondii URM1020	-	NT
C. guilliermondii URM1065	-	NT
C. guilliermondii URM1084	-	NT
C. intermedia URM907	-	NT
C. intermedia URM1075	-	NT
C. tropicalis URM112	23	2.30
C. tropicalis URM916	13	1.36
C. tropicalis URM933	16	1.70
C. tropicalis URM1150	-	NT

NT– not tested

Table 4 – Conditions and results of the 2^{6-2} fractionary factorial design for colagenase production by *C. albicans* URM3622.

Run	A	B	C	D	E	F	Bm	TP	CA	SA
1	30	10^5	5	100	48	0.5	1.75	1.90	3.83	2.00
2	37	10^5	5	100	96	0.5	2.22	1.92	3.51	1.82
3	30	10^8	5	100	96	1.5	5.90	3.58	5.82	1.62
4	37	10^8	5	100	48	1.5	5.65	3.51	6.03	1.71
5	30	10^5	7	100	96	1.5	5.12	3.84	5.56	1.44
6	37	10^5	7	100	48	1.5	2.24	3.58	4.00	1.11
7	30	10^8	7	100	48	0.5	5.75	1.64	3.30	2.00
8	37	10^8	7	100	96	0.5	5.31	1.74	3.93	2.25
9	30	10^5	5	140	48	1.5	1.31	3.84	6.50	1.69
10	37	10^5	5	140	96	1.5	4.15	3.53	5.30	1.50
11	30	10^8	5	140	96	0.5	7.28	1.60	4.80	2.98
12	37	10^8	5	140	48	0.5	5.70	1.59	4.08	2.56
13	30	10^5	7	140	96	0.5	2.15	1.86	4.30	2.30
14	37	10^5	7	140	48	0.5	2.20	2.00	4.00	1.99
15	30	10^8	7	140	48	1.5	6.92	3.74	5.26	1.40
16c	37	10^8	7	140	96	1.5	5.42	3.62	6.26	1.72
17c	37	10^7	6	120	72	1.0	3.65	2.59	4.30	1.65
18c	34	10^7	6	120	72	1.0	3.63	2.63	4.26	1.61
19c	34	10^7	6	120	72	1.0	4.03	2.64	4.90	1.85
20c	34	10^7	6	120	72	1.0	3.90	2.59	4.26	1.64

A, Temperature (ºC); B, Inoculum size (cell/ml); C, pH; D, Orbital agitation speed (rpm); E, Incubation time (hours); F, Substrate concentration (%); Bm, biomass (g/l); TP, total protein (mg/ml); CA, collagenolytic activity (U/ml); SA, specific activity (U/mg).

Table 5 – Conditions and results of the 2^3 first full factorial design for colagenase production by *C. albicans* URM3622.

Run	A	B	C	Bm	CA	TP	SA
1	1.0	5	120	5.87	1.83	3.22	0.56
2	2.0	5	120	5.86	2.90	5.06	0.57
3	1.0	7	120	6.00	3.46	3.55	0.97
4	2.0	7	120	6,00	3.63	4.91	0.73
5	1.0	5	160	5.68	3.86	3.07	1.25
6	2.0	5	160	6.20	4.20	4.68	0.89
7	1.0	7	160	6.22	5.46	4.33	1.26
8	2.0	7	160	6.42	7.06	3.94	1.79
9c	1.5	6	140	6.21	4.70	4.03	1.16
10c	1.5	6	140	6.03	4.63	3.96	1.15
11c	1.5	6	140	6.15	4.80	4.09	1.17
12c	1.5	6	140	5.89	4.76	4.06	1.17

A, Substrate concentration (%); B, pH; C, Orbital agitation speed (rpm); Bm, Biomass (g/L); TP, Total protein (mg/mL); CA, Collagenolytic activity (U/mL); SA, Specific activity (U/mg).

Table 6 – Conditions and results of the 2^3 second full factorial design for colagenase production by *C. albicans* URM3622.

Run	A	B	C	Bm	CA	TP	SA
1	1.5	6	140	5.41	4.73	4.53	1.04
2	2.5	6	140	5.64	5.16	6.57	0.78
3	1.5	8	140	5.27	4.40	4.86	0.90
4	2.5	8	140	5.35	5.00	5.58	0.89
5	1.5	6	180	5.49	3.93	4.52	0.71
6	2.5	6	180	6.19	4.23	6.01	0.70
7	1.5	8	180	5.52	3.50	4.52	0.77
8	2.5	8	180	6.11	3.70	6.55	0.56
9c	2.0	7	160	5.87	6.04	5.52	1.09
10c	2.0	7	160	5.90	6.83	5.35	1.27
11c	2.0	7	160	6.02	6.56	5.44	1.20
12c	2.0	7	160	5.76	6.66	5.53	1.20

A, Substrate concentration (%); B, pH; C, Orbital agitation speed (rpm); Bm, Biomass (g/L); TP, Total protein (mg/mL); CA, Collagenolytic activity (U/mL); SA, Specific activity (U/mg).

FIGURE CAPTIONS

Figure 1 – Pareto chart for the effects of variables gelatin concentration (6), pH (3), orbital agitation speed (4), inoculum size (2), incubation time (5) and temperature (1) on collagenolytic activity.

Figure 2 – Pareto chart for the effects of variables substrate concentration (1), pH (2) and agitation speed (3) on collagenolytic activity from first full factorial design.

Figure 3 – Cubic plot of the collagenolytic activity obtained in the design of Table 5.

Figure 4 – Pareto chart for the effects of variables substrate concentration (1), pH (2) and agitation speed (3) on collagenolytic activity from second full factorial design.

Figure 5 – (A) Effect of pH on activity of collagenase produced by *Candida albicans* URM-3622. The enzyme activity at pH 8.2 was taken as 100%. (B) – Effect of pH on stability of collagenase produced by *C. albicans* URM-3622. All buffers at 0.05M. Each data point is an average of three experiments and the error bars show the standard deviation.

Figure 6– (A) Effect of temperature on activity of collagenase produced by *Candida albicans* URM-3622. The enzyme activity at 45 ºC was taken as 100%. (B) Thermal stability of collagenase produced by *C. albicans* URM-3622 at different temperatures

during 240 minutes of incubation. Each data point is an average of three experiments and the error bars show the standard deviation.

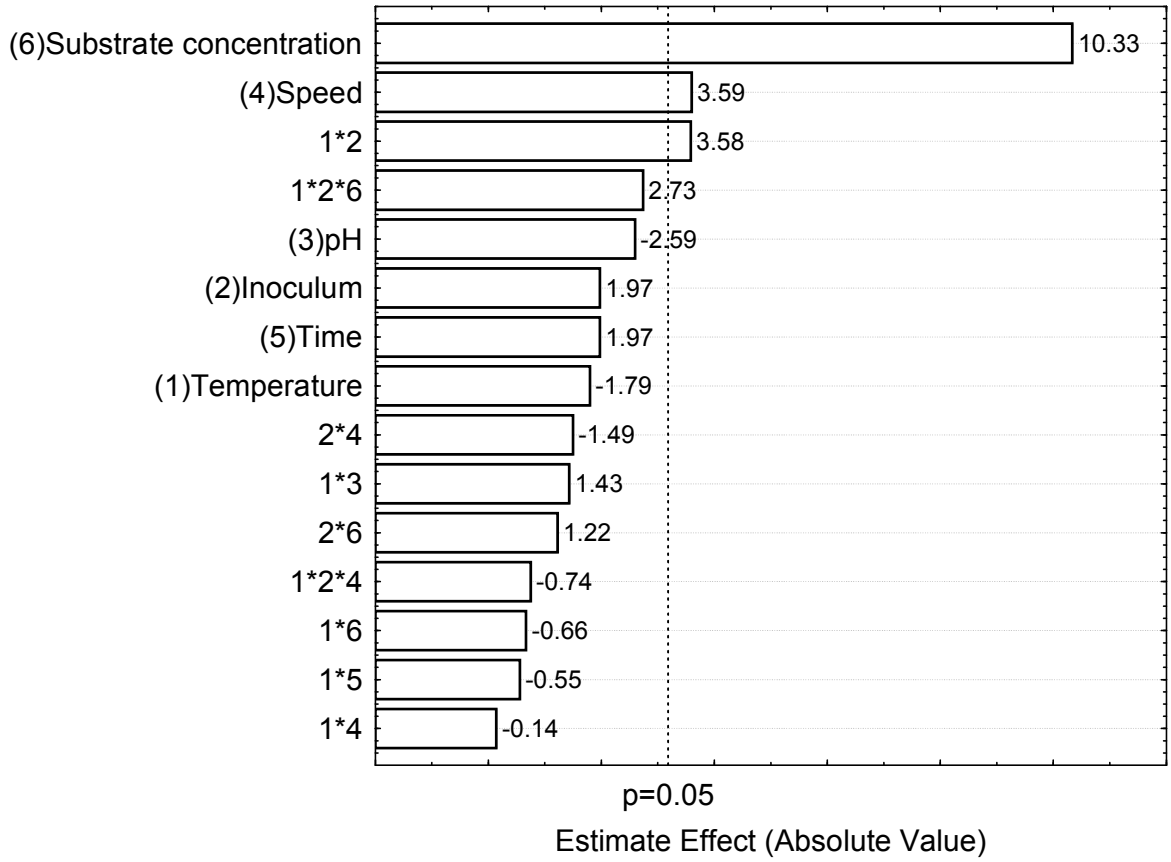
(6)Substrate concentration
10.33
(4)Speed
3.59
1*2
3.58
1*2*6
2.73
(3)pH
-2.59
(2)Inoculum
1.97
(5)Time
1.97
(1)Temperature
-1.79
2*4
-1.49
1*3
1.43
2*6
1.22
1*2*4
-0.74
1*6
-0.66
1*5
-0.55
1*4
-0.14
p=0.05
Estimate Effect (Absolute Value)

Figure 1

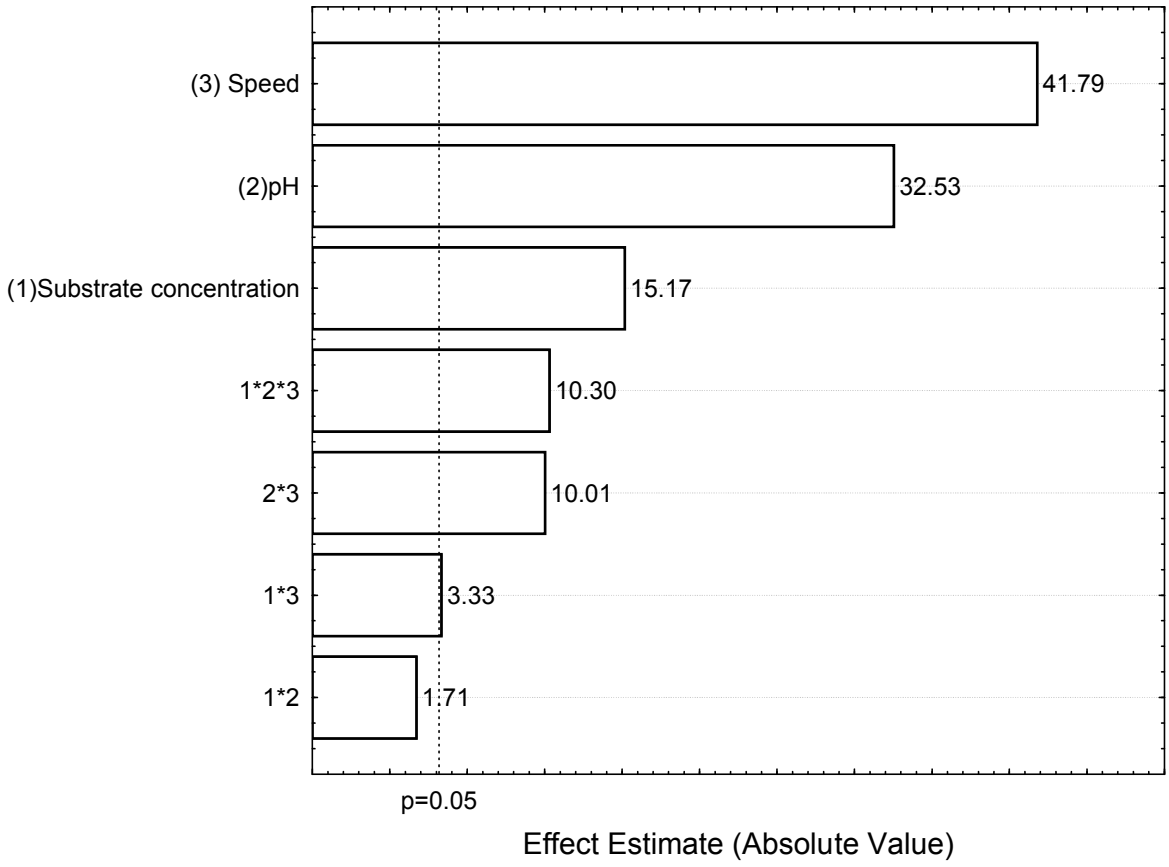
(3) Speed
41.79
(2)pH
32.53
(1)Substrate concentration
15.17
1*2*3
10.30
2*3
10.01
1*3
3.33
1*2
1.71
p=0.05
Effect Estimate (Absolute Value)

Figure 2

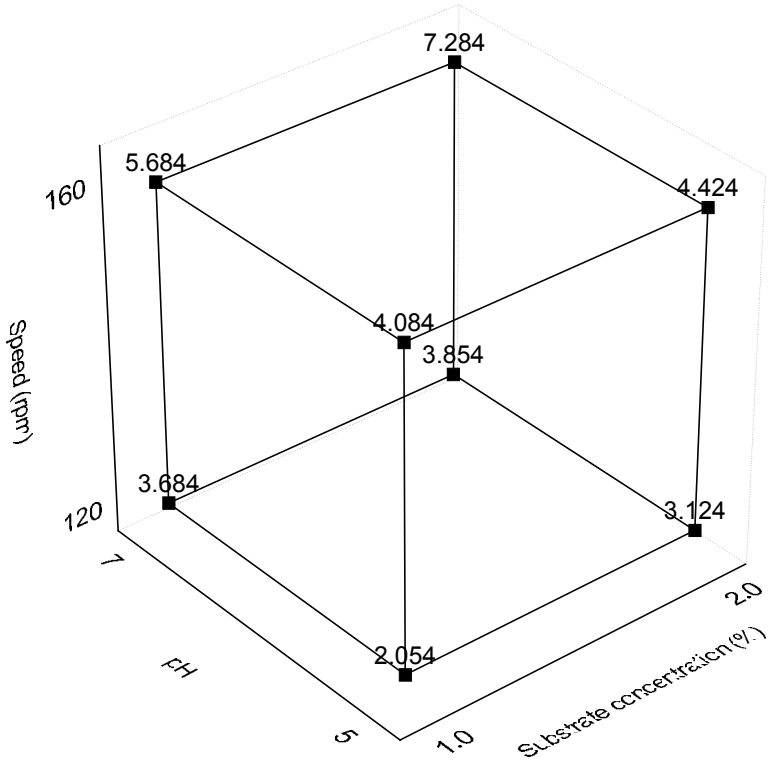
7.284
5.684
4.424
4.084
3.854
3.684
3.124
2.054
160
120
Speed (rpm)
7
5
pH
1.0
2.0
Substrate concentration (%)

Figure 3

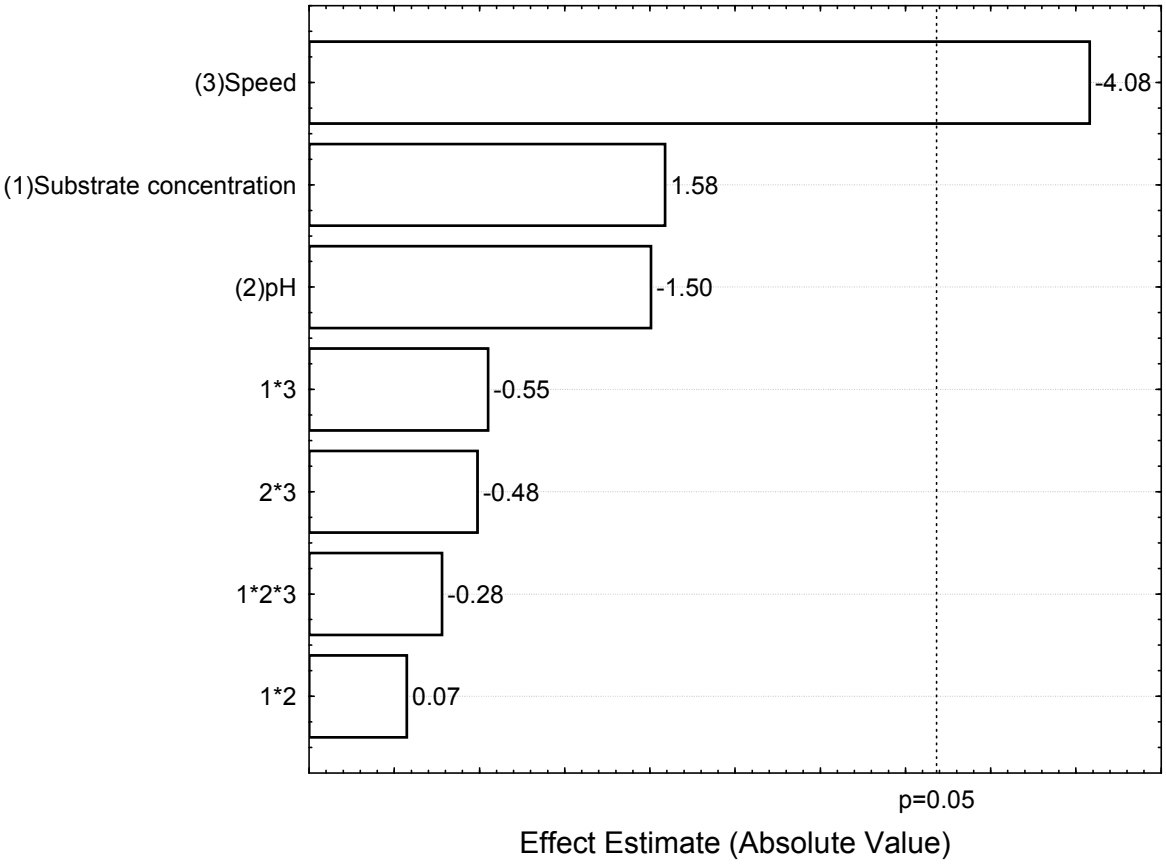
(3)Speed
-4.08
(1)Substrate concentration
1.58
(2)pH
-1.50
1*3
-0.55
2*3
-0.48
1*2*3
-0.28
1*2
0.07
p=0.05
Effect Estimate (Absolute Value)

Figure 4

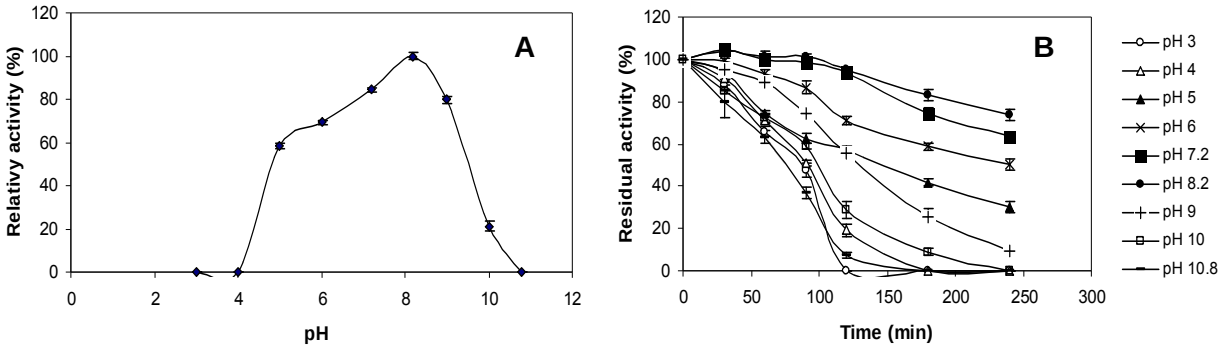
A
Relativy activity (%)
pH
B
Residual activity (%)
Time (min)
pH 3
pH 4
pH 5
pH 6
pH 7.2
pH 8.2
pH 9
pH 10
pH 10.8

Figure 5

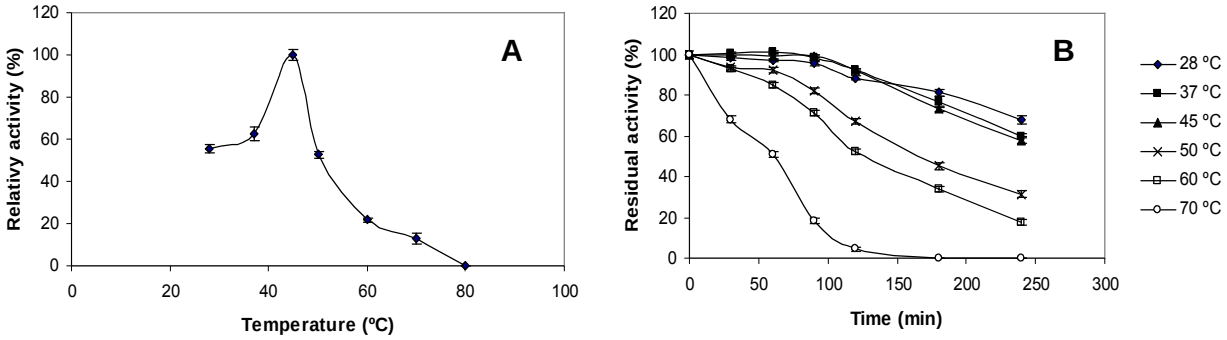
A
Relativy activity (%)
Temperature (ºC)
B
Residual activity (%)
Time (min)
28 ºC
37 ºC
45 ºC
50 ºC
60 ºC
70 ºC

Figure 6

6. CONCLUSÕES

Os estudos desenvolvidos no presente trabalho mostraram que:

- Entre as espécies de *Candida* estudadas, a *Candida albicans* URM3622 foi à espécie que apresentou a maior atividade colagenolítica;

- A análise do planejamento estatístico 2^{6-2} demonstrou que as variáveis: concentração de substrato e velocidade de agitação foram aquelas que influenciaram a produção da colagenase por *Candida albicans* URM3622;

- Os melhores resultados para a produção da colagenase em cultura submersa foram obtidos quando se utilizou uma velocidade de agitação de 160 rpm, pH 7 e concentração de substrato de 2%;

- A colagenase produzida por *Candida albicans* URM3622 apresentou atividade máxima ao pH 8,2 e temperatura de 45 ºC;

- A colagenase mostrou-se estável na faixa de pH entre 7,2-8,2 e na faixa de temperatura entre 28-45 ºC;

- A *Candida albicans* URM3622 apresenta potencialidade como produtora de colagenase, a qual pode ser utilizada para fins biotecnológicos.

7. ANEXOS

7.1. Normas para a redação de artigos para a revista "*Biochemical Engineering Jornal*"

Guide for Authors

Submission of Papers

Submission to this journal proceeds totally on-line via the Elsevier Editorial System at http://ees.elsevier.com/bej. There you will be guided stepwise through the creation and uploading of the various manuscript files. The system automatically converts source files to a single Adobe Acrobat PDF version of the article, which is used in the peer-review process. Please note that even though manuscript source files are converted to PDF at submission for the review process, these source files are needed for further processing after acceptance. All correspondence, including notification of the Editor's decision and requests for revision, takes place by e-mail and via the Author's homepage, removing the need for a hard-copy paper trail.

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7.2. Indicadores de Produção 2006-2008.

7.2.1. Resumos em Congressos

LIMA, C. A; RODRIGUES, P. M. B; BEZERRA, R. S.; CARNEIRO-DA-CUNHA, M. G. Pré-purification of an alkaline protease from intestine of Nile Tilapia (*Oreochromis niloticus)* using reversed micelles. VIII Reunião Regional Nordeste da Sociedade de Brasileira de Bioquímica e Biologia Molecular-SBBq, realizada em Natal, Rio Grande do Norte, no período de 6 a 8 de Dezembro de 2006.

RODRIGUES, P. M. B.; **LIMA, C. A.**; MOREIRA, K. A.; MOTTA, C.M.S. ; LIMA-FILHO, J. L.; PORTO, A.L.F.; CARNEIRO-DA-CUNHA, M. G. Production and partial characterization of protease from *Peniccillium aurantiogriseum*. VII Reunião Regional Nordeste da Sociedade Brasileira de Bioquímica e Biologia Molecular-SBBq, realizada em Natal, Rio Grande do Norte, no período de 6 a 8 de Dezembro de 2006.

LIMA, C. A.; LIMA-FILHO, J. L., PORTO, A. L. F.; CARNEIRO-DA-CUNHA, M. G. Evaluation of strains C*andida* as collagenases producers. XXXVI Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular – SBBq, realizada em Salvador, Bahia, no período de 21 a 25 de maio de 2007.

LIMA, C. A.; CUNHA, M.; PORTO, T. S.; PORTO, A. L. F.; CARNEIRO-DA-CUNHA, M. G. Produção de colagenase por *Candida albicans* URM-3622. 5º Congresso Brasileiro de Micologia, realizado em Recife, Pernambuco 12 a 16 de novembro de 2007.

CUNHA, M.; ALBERTINE, A.; **LIMA, C. A.**; CAVALCANTI, M. T. H.; MOREIRA, K. A.; BARROS-NETO, B.; Viana, D. A.; LIMA-FILHO, J. L.; MOTTA, C. S.; PORTO, A. L. F. Produção de xilanase por planejamento estatístico utilizando bagaço de cana-de-açúcar como substrato. 5º Congresso Brasileiro de Micologia, realizado em Recife, Pernambuco 12 a 16 de novembro de 2007.