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MARIA CAROLINA DE ALBUQUERQUE WANDERLEY

PRODUÇÃO, CARACTERIZAÇÃO PARCIAL E PURIFICAÇÃO DE COLAGENASE PRODUZIDA POR *Penicillium* sp. UCP 1286 ISOLADO DA CAATINGA

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Orientadora: Prof. Dra. Ana Lúcia Figueiredo Porto
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Recife, 28 de março de 2016

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“Freedom of thought is best promoted by the gradual illumination of men´s minds which follows from the advance of science.”
Charles Darwin

RESUMO

Colagenases são enzimas específicas capazes de degradar a tripla hélice do colágeno nativo ou desnaturado. A busca por novas colagenases microbianas têm aumentado bastante ao longo dos anos. Uma nova cepa de *Penicillium* sp. (UCP 1286) isolada do solo da Caatinga, um bioma exclusivamente brasileiro, foi selecionada para produção de colagenase. O meio de cultura utilizado foi constituído apenas de gelatina como fonte de carbono e nitrogênio. A atividade colagenolítica obtida foi cerca de 2,7 vezes maior que os valores descritos na literatura, tanto para a atividade volumétrica (379,79 U/mL) quanto específica (1.460,77 U/mg), no intervalo de tempo de 126 h de produção. A partir da aplicação de planejamento fatorial, produção da enzima aumentou 65% em comparação aos resultados preliminares obtidos, sendo equivalente a 632,70 U/mL de atividade colagenolítica. A caracterização da enzima mostrou que o pH ótimo foi 9,0 e a temperatura ótima, 37 °C. Considerando a inibição total pelo fenilmetilsulfonil fluoreto, a enzima parece estar classificada na família das serinocolagenases. Com relação à especificidade, a colagenase produzida por *Penicillium* sp. UCP 1286 apresentou maior atividade quando utilizado o azocoll como substrato, não apresentando atividade relevante quando testada frente à azocaseína. Comparando a capacidade de degradação da enzima produzida por *Penicillium* sp. com a enzima comercial de *Clostridium histolyticum*, observou-se que apresentou maior especificidade para o colágeno tipo V e gelatina, e a principal banda observada através de eletroforese correspondeu ao peso molecular de 37 kDa e o zimograma confirmou atividade colagenolítica. A purificação por Sistema Duas Fases Aquosas (SDFA) foi eficiente para a colagenase produzida por *Penicillium* sp. UCP 1286. Sendo os maiores valores de rendimento e coeficiente de partição obtidos no planejamento fatorial [PEG 3350 g/mol a 15% (m/m) de concentração, e fosfato com pH 7 e concentração 12,5% (m/m)]. Os resultados sugerem que a enzima produzida apresenta-se como um produto biotecnológico promissor com aplicabilidade na área da saúde.

Palavras-chave: Enzima Colagenolítica, *Penicillium*, Colágeno, SDFA

ABSTRACT

Collagen specific enzymes are capable of degrading triple helix of the native or denatured collagen. The search for new microbial collagenases has greatly increased over the years. A new strain of *Penicillium* sp. (UCP 1286) isolated from soil of Caatinga, an exclusively Brazilian biome, was selected for the production of collagenase. The culture medium used had only gelatin as a source of carbon and nitrogen. The collagenolytic activity achieved was about 2.7 times higher than the values reported in the literature, both for volumetric activity (379.79 U/mL) and specific activity (1460.77 U/mg) in a time interval of 126 hours of production. With factorial design application, enzyme production increased 65% compared to the preliminary results, equivalent to 632.70 U/mL of collagenolytic activity. The characterization of the enzyme showed that the optimum pH and temperature were, respectively, 9.0 and 37 °C. Due to the total inhibition by phenylmethylsulfonyl fluoride, the enzyme seems to be classified in the family of serinocollagenases. With regard to specificity, collagenase produced by *Penicillium* sp. UCP 1286 showed higher activity when used as a substrate azocoll, showing no activity when tested against the azocasein. Comparing the enzyme degradation capacity produced by *Penicillium* SP. With commercial enzyme produced by *Clostridium histolyticum*, the first presented more specific to type V collagen and gelatin. The major band observed in electrophoresis corresponded to the molecular weight of 37 kDa, and zimogram confirmed collagenolytic activity. The purification technique via aqueous two-phase system (ATPS) was effective for collagenase produced by *Penicillium* sp. UCP 1286. The run with better values of yield and partition coefficient were at runs on center point, using PEG 3350 g/mol at 15.0% (w/w) concentration, and phosphate at pH 7.0 and concentration 12.5% (w/w). Results indicate that the enzyme is a promising biotechnological product.

Keywords: Collagenolytic Enzyme, *Penicillium*, Collagen, Factorial Design, ATPS

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LISTA DE ABREVIAÇÕES E SIGLAS

UCP	Universidade Católica de Pernambuco
Azocoll	Azo dye-impregnated collagen
kDa	Quilo Dalton
PMSF	Fenilmetilsulfonil fluoreto
SDS	Sódio dodecil sulfato
SDS-PAGE	Eletroforese em gel de poliacrilamida utilizando SDS
EDTA	Ácido etileno diamino tetracético
Gly	Glicina
4-Hyp	4-Hidroxiprolina
Ileu	Isoleucina
Leu	Leucina
MMPs	Metaloproteinases da matriz
Pro	Prolina
EC	Enzyme commission
SDFA	Sistema Duas Fases Aquosas
ATPS	Aqueous Two-Phase System
PF	Purification Factor
PEG	Polyethilene Glycol
K	Partition coefficient
Y	Activity yield

SUMÁRIO

1 INTRODUÇÃO

Proteases colagenolíticas apresentam a capacidade de hidrolisar tanto colágeno nativo quanto desnaturado, e estão tornando-se cada vez mais importantes comercialmente (Lima et al., 2009). As colagenases produzidas por micro-organismos são preferíveis, quando comparadas com as dos mamíferos ou plantas, por causa da diversidade bioquímica e susceptibilidade à manipulação genética que suas fontes possuem (Pandey et al., 2006; Rao et al., 1998).

Os fungos possuem grande vantagem dentre os micro-organismos produtores de enzimas colagenolíticas. A produção enzimática ocorre extracelularmente, o que torna particularmente mais fácil a sua recuperação após o final da fermentação (Sandhya et al., 2005).

Colagenases têm sido descritas como utilizadas em diversos segmentos industriais, como: medicinal, farmacêutico, alimentício, cosmético e têxtil (Goshev et al., 2005; Kanth et al., 2008). Quanto à aplicação médica, há relatos na literatura de sua aplicação no tratamento de úlceras e queimaduras (Agren et al., 1992; Püllen et al., 2002), para eliminar escaras (Shmoilov et al., 2006), no tratamento da doença de Dupuytren's, em vários tipos de fibroses (como cirrose), no preparo de amostras para diagnóstico (Lima et al., 2013), na produção de peptídeos bioativos com atividades antioxidante e antimicrobiana (Lima et al., 2014), além de possuírem um importante papel no sucesso de cirurgias para transplantes de alguns órgãos (Shmoilov et al., 2006).

A busca por novas colagenases microbianas têm aumentado bastante ao longo dos anos, e alguns autores relatam que sua produção representa, atualmente, uma das maiores dentro da indústria de produção enzimática (Abidi et al., 2013; Graminho et al., 2013). Se faz necessário o desenvolvimento e investimento em estudos que comprovem a aplicação de novas colagenases produzidas por micro-organismos, uma vez que possuem vasto espectro de aplicação com alto potencial biotecnológico e valor comercial agregados.

Diversos processos têm sido desenvolvidos para otimizar o isolamento e purificação de colagenases, devido ao seu vasto potencial de aplicação biotecnológico. À medida que são desenvolvidos métodos mais rentáveis, sua utilização também se expande, o que implica na necessidade de um entendimento mais aprofundado a respeito dessas enzimas (Daboor et al., 2010).

Existem vários métodos descritos na literatura para a separação e purificação de proteases. A ultrafiltração, precipitação e cromatografia são métodos comuns(Li et al. 2006). No entanto, para a escala industrial, tais procedimentos são considerados viáveis devido ao elevado custo e longo tempo de processamento (McMaster 2007). Assim, a busca de métodos alternativos de purificação de proteases tornou-se cada vez mais comum (Yavari et al. 2013). A extração líquido-líquido utilizando sistemas duas fases aquosas (SDFA) é um dos processos bioseparação mais promissora, que pode ser usado nas fases iniciais do processo de purificação, substituindo as separações sólido-líquido difíceis, ou mesmo em etapas de purificação subsequente (Rosso et al. 2012).

O presente projeto teve como proposta produzir colagenase a partir de *Penicillium* sp. UCP 1286 isolado da Caatinga, estudar os parâmetros fermentativos mais relevantes para o melhoramento da produção através da utilização de planejamento estatístico e caracterizar parcialmente a enzima. Além de purificar a enzima utilizando SDFA.

2 OBJETIVOS

2.1 Geral

Produzir, caracterizar parcialmente e purificar a colagenase do *Penicillium* sp. UCP 1286, avaliar os parâmetros fermentativos mais relevantes para o melhoramento da produção através da utilização de planejamento estatístico.

2.2 Específicos

- Selecionar, entre as linhagens provenientes da Caatinga disponíveis na Coleção UCP da Universidade Católica de Pernambuco (UNICAP), a melhor produtora de colagenase;
- Avaliar a influência do pH inicial do meio de cultura, da temperatura, da velocidade de agitação orbital e da concentração inicial do substrato na produção da colagenase através de planejamento fatorial completo 2^4;
- Caracterizar a colagenase quanto aos aspectos físico-químicos, tais como: pH ótimo, temperatura ótima, estabilidade ao pH e à temperatura, ação de inibidores e especificade a substratos;
- Analisar as variáveis de resposta: coeficiente de partição (K), recuperação em atividade (Y) e fator de purificação (FP) através do planejamento fatorial do SDFA;
- Avaliar e analisar a influência das variáveis: massa molar do Polietilenoglicol (PEG), concentração do PEG, concentração de fosfato e pH do fosfato, sobre a extração da colagenase através do SDFA, utilizando planejamento fatorial 2^4.

3 CAPÍTULO I

3.1 Revisão de Literatura

3.1.1 *Mercado global das enzimas*

As enzimas possuem vasta aplicação nas indústrias de alimentos, detergentes e couro, além de serem importantes no estudo da estrutura de proteínas e peptídeos. A crescente preocupação com a consciência ambiental causada pela poluição das indústrias químicas exigiu o desenvolvimento de processos à base de enzimas como alternativa de substituição parcial ou total aos processos químicos que apresentam toxicidade (Laxman et al., 2005). Estudos recentes apontam um aumento no uso das enzimas em processos industriais e reações químicas (Jisha et al., 2013; Kumar et al., 2014).

A utilização industrial de enzimas é extremamente ampla e possui inúmeras aplicações (Adrio e Demain, 2014). Estima-se que existem cerca de 500 produtos industriais sendo produzidos a partir de enzimas (Johannes e Zhao, 2006; Kumar e Singh, 2012). A grande parte das enzimas (cerca de 65%) são utilizadas nas indústrias de detergentes, amido, couro, têxtil, produtos para cuidados pessoais e de papel e celulose. Enzimas alimentícias representam o segundo maior segmento com 25% do mercado, incluindo aplicações nas indústrias da cerveja, vinho, suco, pães, gorduras e óleos. Por último, ocupando 10% do mercado, estão as enzimas utilizadas na produção de suplementos alimentares para animais (Cherry e Fidantsef, 2003).

Os processos industriais envolvidos na produção de produtos químicos e farmacêuticos têm muitas desvantagens em relação aos processos enzimáticos, tais como: uma baixa eficiência catalítica, a necessidade de altas temperaturas, baixo pH e pressões elevadas, além disso, geram uma grande quantidade de resíduos orgânicos e a poluição devida ao uso de solventes orgânicos (Adrio e Demain, 2014; Cherry e Fidantsef, 2003). Nos últimos anos, o aumento na utilização de enzimas com a finalidade de catalisadores industriais pôde ser observado, devido a elas funcionam sob condições de reação brandas, são derivadas de fontes renováveis, são biodegradáveis e apresentam especificidade e seletividade (Cherry e Fidantsef, 2003).

A busca de novas enzimas continua a ser um desafio constante, principalmente devido à necessidade de desenvolvimento de condições de produção mais sustentáveis (Adrio e Demain, 2014; Jisha et al., 2013; Sumantha et al., 2005). Com o avanço da biotecnologia, enzimas microbianas têm recebido cada vez mais atenção e processos que possam envolver a utilização da maquinaria metabólica microbiana para a produção de enzimas têm sido foco de interesse (Laxman et al., 2005).

O mercado global das enzimas industriais é competitivo, com a Novozymes representando a principal empresa dominante, seguida da DSM e Dupont, entre outras. A competição ocorre pela busca de um produto com qualidade e performance, pelo uso dos direitos de propriedade intelectual e habilidade de inovação. Os continentes pioneiros no consumo das enzimas são a Europa e América do Norte, seguidos dos países asiáticos como China, Japão e Índia (Adrio e Demain, 2014).

3.1.2 *Proteases*

As proteases (Enzyme Commission - EC 3:4, 11-19, 20-24, 99) provavelmente surgiram nos primeiros estágios de evolução das proteínas, atuando como enzimas destrutivas simples necessárias ao catabolismo protéico e geração de aminoácidos em organismos primitivos (López-Otín e Bond, 2008). Enzimas com capacidade de clivar proteínas compõem a maior família das enzimas, constituindo cerca de 2% do genoma humano (Marnett e Crai, 2005; Schilling e Overall, 2008). Podem ser consideradas executoras eficientes de uma reação química comum: a hidrólise de ligações peptídicas (López-Otín e Bond, 2008).

As proteases pertencem a um grupo de enzimas altamente complexo que diferem entre si na especificidade ao substrato, mecanismo catalítico e sítio ativo (Sumantha et al., 2006). Possuem uma série de funções e várias aplicações biotecnológicas importantes que variam desde aditivos em detergentes a produtos terapêuticos efetivos (Figura 1) (Li et al., 2013; Lima et al., 2009).

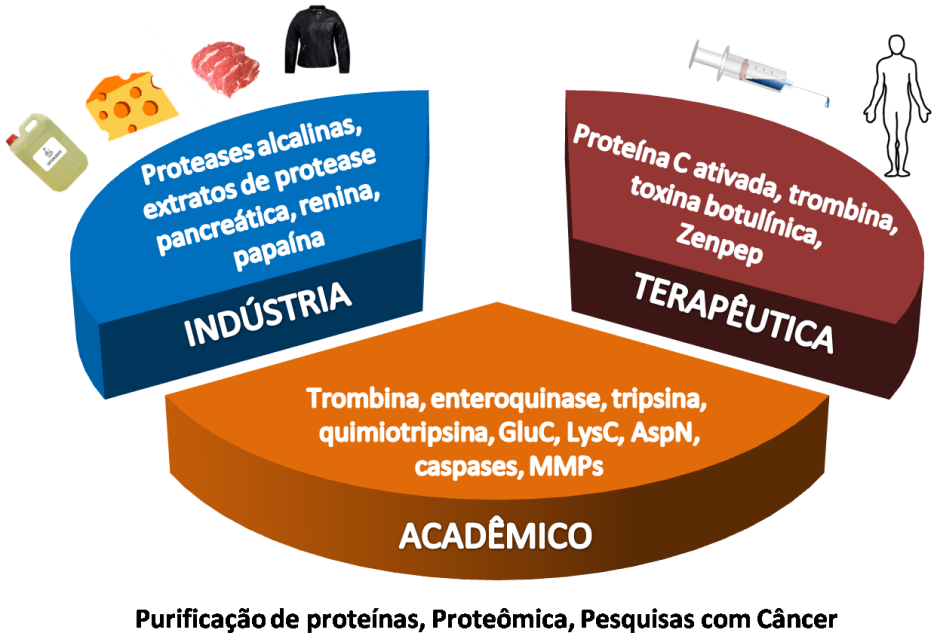


Figura 1. Um panorama das aplicações de proteases (Adaptado de Li et al. 2013)

3.1.3 *Fontes de proteases*

Uma vez que as proteases são fisiologicamente necessárias para a vida de todos os organismos, elas são ubíquas e podem ser encontradas em vasta diversidade de fontes, como animais, plantas e micro-organismos (Rao et al., 1998).

Papaína, bromelina e queratinases são exemplos de proteases originadas de plantas. Entretanto, a produção a partir de plantas é um processo que demanda bastante tempo, além de vários fatores estarem envolvidos, como espaço de terra para cultivo e condições climáticas favoráveis ao crescimento. Já as proteases mais comuns obtidas de animais são: tripsina, quimiotripsina, pepsina e reninas. Contudo, esta produção depende da disponibilidade de animais, que por sua vez é governada por políticas públicas e agrícolas (Jisha et al., 2013; Rao et al., 1998).

Dentre as várias fontes de proteases, as que são produzidas por micro-organismos têm um papel importante em processos biotecnológicos e estão sendo utilizadas com mais frequência, pois grandes quantidades dessas enzimas podem ser produzidas rapidamente e com baixo custo (Barret et al., 2004). Em geral, proteases microbianas são preferíveis às enzimas obtidas a partir de plantas e animais devido à possibilidade de manipulação genética e a diversidade bioquímica dos micro-organismos (Pandey et al., 2006; Rao et al., 1998).

Com o crescimento da biotecnologia, as aplicações das proteases têm expandido para novas áreas, como clínica, medicinal e química analítica (Shanmughapriya et al., 2008). A busca de novas proteases microbianas tem aumentado ao longo dos anos e, atualmente, representam uma das maiores indústrias de enzimas (Abidi et al., 2013; Graminho et al., 2013).

3.1.4 *Classificação de proteases*

Devido as suas diversidades estruturais e funcionais, as proteases são capazes de realizar funções que podem variar entre a reciclagem de uma proteína intracelular para a digestão de um nutriente e a amplificação do sistema de cascata da resposta imunológica. A diversificação dos papéis biológicos é decorrente da evolução das inúmeras estruturas protéicas, que convergiram para locais ativos semelhantes e conseguem reconhecer substratos variados (Li et al., 2013).

As proteases podem ser divididas em dois grupos principais: exopeptidases e endopeptidades, dependendo do seu local de ação (Figura 2). Exopeptidases hidrolisam ligações peptídicas próximas ao terminal amino (aminopeptidases) ou carboxi (carboxipeptidases), enquanto as endopeptidases clivam ligações peptídicas distantes das extremidades aminoterminal e carboxiterminal (López-Otín e Bond, 2008; Rao et al., 1998).

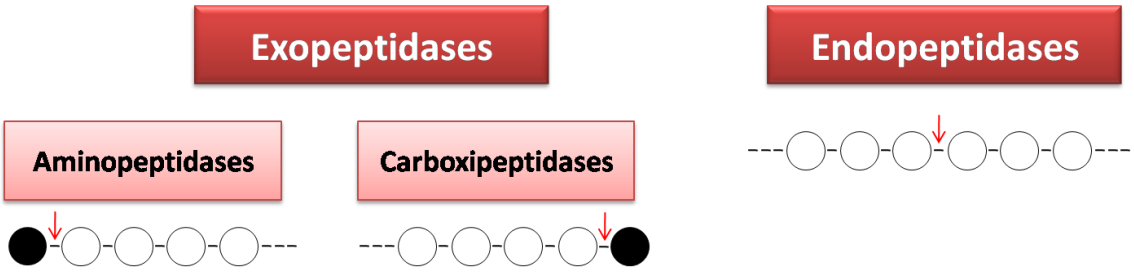


Figura 2. Mecanismo de ação das proteases (Adaptado de Rao et al., 1998)

Com relação à estrutura e mecanismo de ação dessas enzimas, novos esquemas de classificação foram propostos. Baseado nos mecanismos de catálises, por exemplo, proteases são classificadas em seis classes distintas: aspártico, glutâmico, metalo, cisteíno, serino e treonina proteases, embora as glutâmicas não tenham sido encontradas em mamíferos até agora (Figura 3) (López-Otín e Bond, 2008).

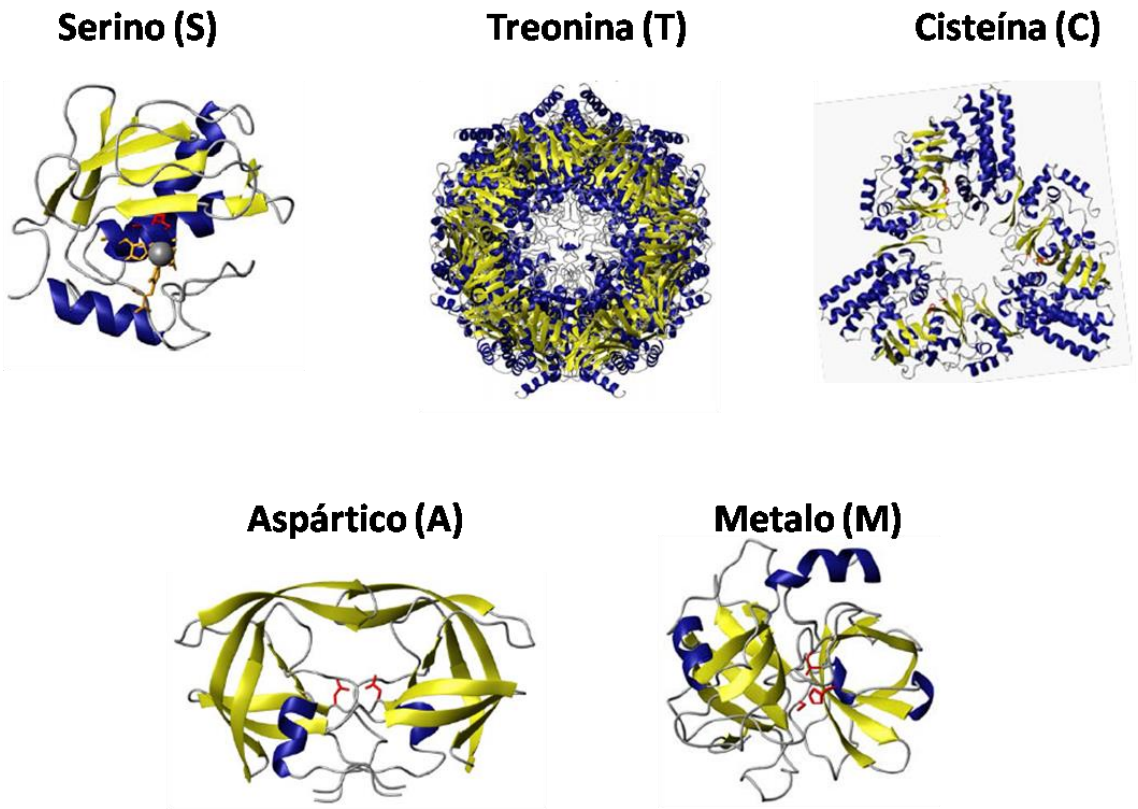


Figura 3. Conformação das principais classes de proteases (Adaptado de López-Otín e Bond, 2008)

As aspárticoproteases (EC 3. 4. 23) possuem um resíduo de ácido aspártico para propiciar suas atividades catalíticas. As metaloproteases (EC 3. 4. 17) são carboxipeptidases (proteases que liberam um único resíduo C-terminal de um polipeptídeo) que usam íons metálicos em seu mecanismo de catálise. Cisteinoproteases (EC 3. 4. 22) possuem uma cisteína em seu centro ativo. Serinoproteases (EC 3. 4. 21) são classificadas como endopeptidases (proteases que clivam ligações internas em cadeias polipeptídicas) que possuem uma serina em seu centro ativo, que está envolvida no processo catalítico (Jisha et al., 2013). As proteases também podem ser classificadas em diferentes famílias, dependendo das suas sequências de aminoácidos e relações evolutivas. Considerando-se o pH ótimo, as proteases podem ser classificadas em ácidas, neutras ou alcalinas (Rao et al., 1998).

As metalo e serinoproteases são as classes de proteases com mais componentes, sendo 194 e 176 membros, respectivamente, seguidos de 150

cisteínoproteases, enquanto que a classe das treonina proteases contém 28 membros e aspártico proteases apenas 21 (López-Otín e Bond, 2008). Ambas podem ser tanto endo quanto exopeptidases (Rao et al., 1998). Dentre as metaloproteases de importância farmacêuticas, estão encontradas as colagenases.

3.1.5 *Colagenases*

Poucas enzimas possuem capacidade de degradação da estrutura do colágeno, e as que apresentam essa propriedade são conhecidas como enzimas ou proteases colagenolíticas (Daboor et al., 2010). Proteases colagenolíticas estão tornando-se cada vez mais importantes comercialmente (Lima et al., 2009).

As colagenases têm sido isoladas e caracterizadas a partir de diferentes fontes, como o trato digestivo de peixes e invertebrados, incluindo da barbatana caudal do girino (Gross e Nagai, 1965; Nagai et al., 1966), bacalhau do Atlântico (Kristjánsson et al., 1995), caramujo (*Achatina fulica*) (Indra et al., 2005), camarão tropical (*Penaeus vannamei*) (Sellos e Van Wormhoudt, 1999; Van Wormhoudt et al., 1992), peixe-gato (*Parasilurus asotus*) (Klimova et al., 1990; Sellos e Van Wormhoudt, 1992), cavala (*Scomber japonicas*) (Park et al., 2002); além de plantas, como o gengibre (*Zingiber officinale*) (Kim et al., 2007).

Colagenases produzidas por micro-organismos são preferíveis por causa da diversidade bioquímica e susceptibilidade à manipulação genética que suas fontes possuem (Lima et al., 2011; Rao et al., 1998). Além disso, as colagenases microbianas têm capacidade de clivar a tripla hélice do colágeno em múltiplos sítios, enquanto que as colagenases de mamíferos clivam em um único sítio (Hamdy, 2008; Jain e Jain, 2010).

Colagenases microbianas têm sido recuperadas de micro-organismos patogênicos, principalmente o *Clostridium histolyticum*, que é a fonte mais utilizada para produção dessas enzimas (Daboor et al., 2010). Outras bactérias como *Bacillus cereus* e *Klebsiella pneumoniae* (Suphatharaprateep et al., 2011), *Bacillus pumilus* (Wu et al., 2010), *Bacillus licheniformis* (Asdornnithee et al., 1994; Baehaki et al., 2014, 2012) também têm sido relatadas.

Estudos relatam a biossíntese de colagenase através dos fungos pertencentes a diferentes gêneros como *Aspergillus, Cladosporium, Alternaria, Penicillium* (de Siqueira et al., 2014; Lima et al., 2011; Rosso et al., 2012; Yakovleva

et al., 2006), *Candida* (Lima et al., 2009), *Microsporum* (Viani et al., 2007) e *Rhizoctonia* (Hamdy, 2008). Espécies do gênero *Penicillium* apresentam potencial biotecnológico maior comparado aos outros gêneros citados, tanto pela produção de proteases e outras enzimas, como pela capacidade de crescimento deste micro-organismo em diversas condições de cultivo, utilizando ampla variedade de substratos como nutrientes (Ikram-Ul-Haq e Mukhtar, 2007).

Dentre os micro-organismos produtores de enzimas colagenolíticas, os fungos possuem grandes vantagens, tais como a elevada produtividade, baixo custo de produção, rápido desenvolvimento e a possibilidade de a enzima resultante poder ser modificada e recuperada mais facilmente. A produção enzimática ocorre extracelularmente, o que torna particularmente mais fácil a sua recuperação após o final da fermentação (Lima et al., 2011; Sandhya et al., 2005). Além disso, colagenases microbianas são mais versáteis, possuem ampla especificidade ao substrato e são capazes de hidrolisar tanto o colágeno nativo insolúvel, quando o colágeno desnaturado solúvel (Daboor et al., 2010; Mookthiar et al., 1985; Peterkofsky, 1982). Como proteases fúngicas são capazes de hidrolisar muitas outras proteínas além do colágeno, a procura de colagenases a partir desses micro-organismos com as características adequadas, como, elevada especificidade, é um ponto muito importante a ser mais estudado (Sharkova et al., 2015).

Diversos processos têm sido desenvolvidos para otimizar o isolamento e purificação de colagenases, devido ao seu vasto potencial de aplicação biotecnológico. À medida que são desenvolvidos métodos mais rentáveis, sua utilização também se expande, o que implica na necessidade de um entendimento mais aprofundado a respeito dessas enzimas (Daboor et al., 2010).

A Tabela 1 descreve alguns micro-organismos relatados na literatura, com potencial para produção de colagenase, bem como os meios de cultura utilizados e as respectivas atividades enzimáticas.

Tabela 1. Descrição dos artigos publicados na literatura relacionados à produção de colagenase

Micro-organismo	Meio de cultura	A_c (U/mL)[a]	Referências
Arthrobotrys oligospora	LMZ com gelatina	Sim[b]	Minglian et al. (2004)
Aspergillus terreus	Farelo de trigo	Sim[b]	de Siqueira et al. (2014)
Aspergillus flavus	Meio dependente de colágeno	82,95	Mahmoud et al. (2007)
Bacillus cereus	Glicerol e gelatina	23,07	Suphatharaprateep et al. (2011)
Bacillus licheniformis	Extrato de levedura e colágeno	3,10	Baehaki et al. (2012)
Bacillus licheniformis	Luria Broth e colágeno	0,55	Baehaki et al. (2014)
Bacillus pumilus	Gelatina e peptona	35,97	Wu et al. (2010)
Bacillus subtilis	Extrato de levedura e gelatina	3,07	Tran and Nagano (2002)
Candida albicans	Extrato de malte e gelatina	5,00	Lima et al. (2009)
Coccidioides immitis	Czapek	Sim[b]	Lopes et al. (2008)
Entomophtora coronate	Casaminoácidos, extrato de levedura e solução de Berthelot	Sim[b]	Hurion et al. (1977)
Klebsiella pneumoniae	Glicerol e gelatina	10,53	Suphatharaprateep et al. (2011)
Lagenidium giganteum	Extrato de levedura e glicose	8,00	Dean e Domnas (1983)
Microsporum canis	Meio com colágeno tipo I	1,0	Viani et al. (2007)
Paracoccidioides brasiliensis	Base de carbono para levedura, colágeno, solução de	1,2	Voltan et al. (2008)

	vitamina, neopeptona		
Penicillium aurantiogriseum	Farinha de soja	164,00	Lima et al. (2011a)
Penicillium aurantiogriseum	Farinha de soja	231,00	Lima et al. (2011b)
Penicillium chrysogenum	Meio liquid LMZ	Sim[b]	Benito et al. (2002)
Rhizoctonia solani	Sabouraud, glicose e colágeno	212,33	Hamdy (2008)
Streptomyces exfoliatus	Farinha de soja	43,50	Jain and Jain (2010)
Zygosaccharomyces rouxii	Extrato de levedura, peptona e glicose	70,40	Ok and Hashinaga (1996)

a = atividade volumétrica; b = atividade qualitativa

Os fungos listados na Tabela 1 são como fungos filamentosos (dos gêneros *Penicillium, Aspergillus, Arthrobotrys, Microsporum, Entomophthora* e *Lagenidium*), dimórficos (*Coccidioides* e *Paracoccidioides)* ou leveduras (*Candida* e *Zygosaccharomyces*). Fungos filamentosos são mais estudados quando comparados aos outros tipos de fungos em relação à produção de colagenase.

Do ponto de vista industrial, a patogenicidade pode influenciar negativamente na escolha do micro-organismo para o desenvolvimento do bioprocesso. Muitos artigos listados na Tabela 1 contêm fungos patogênicos, os quais foram estudados para melhor compreensão dos mecanismos causadores de suas respectivas doenças, e não pela produção de enzima colagenolítica propriamente dita. A produção de colagenase por micro-organismos representam um fator de virulência, uma vez que possuem papel importante na destruição de tecidos (Awad et al., 2000). Considerando esse aspecto, além da atividade enzimática e especificidade, a espécie *Penicillium aurantiogriseum* foi a que apresentou melhor produção de colagenase (Lima et al., 2011).

Cerca de 30 a 40% do custo de produção estão relacionados ao custo do meio de cultura (Joo et al., 2002). A possibilidade de variar a composição do meio de cultura, utilizando materiais de baixo custo, pode interferir positivamente no custo final da produção. Dos artigos listados na Tabela 1, doze utilizaram alguma fonte de

colágeno para indução da produção de colagenase. Entretanto, Lima et al. (2011b) relataram o uso de um meio de cultura de baixo custo para a produção de colagenase por *P. aurantiogriseum*, utilizando farinha de soja como substrato principal, alcançando 231,00 U/mL da enzima.

Hamdy (2008) utilizou meio de cultura contendo Sabouraud, glicose e colágeno insolúvel tipo I para a produção de colagenase por *R. solani* e obteve a segunda maior atividade enzimática (212,33 U/mL). Mahmoud et al. (2007) observaram uma atividade enzimática igual a 82,95 U/mL da colagenase produzida por *A. flavus* utilizando meio de cultura contendo gelatina, glicose, extrato de levedura e colágeno nativo bovino. Ok e Hashinaga (1996) também obtiveram resultados satisfatórios de produção de colagenase por *Z. rouxii* (70,40 U/mL), utilizando meio contendo peptona, extrato de levedura e glicose.

3.1.6 *Classificação das colagenases*

As colagenases podem ser classificadas como serinocolagenases ou metalocolagenases, baseado em suas funções fisiológicas diferentes (Daboor et al., 2010).

As serinocolagenases, assim como as serinoproteases, contém um resíduo de serina em seus sítios catalíticos (Daboor et al., 2010; Rao et al., 1998). Possuem pesos moleculares entre 24 e 36 kDa e estão normalmente associadas a órgãos digestivos, sendo capazes de clivar a tripla hélice dos colágenos tipos I, II e III, e geralmente estão envolvidas na produção de hormônios, degradação de proteínas e fibrinólise (Neurath e Walsh, 1976; Roy et al., 1994).

Relatos na literatura já descreveram a produção de serinocolagenase a partir do bacalhau do Atlântico (*Gadus morhua*) (Kristjánsson et al., 1995), caranguejo da costa verde (*Carcinus maenas*) (Roy et al., 1994), *Thermoactinomyces* sp. (Petrova et al., 2006), *Alicyclobacillus sendaiensis* (Tsuruoka et al., 2003), *Penicillium aurantiogriseum* (C. a. Lima et al., 2013; C. A. Lima et al., 2011a; Lima et al., 2011b; Rosso et al., 2012), *Coccidioides immitis* (Lopes et al., 2008), entre outros.

Metalocolagenases são membros da família das Metaloproteinases de Matriz (MMP), com pesos moleculares variando entre 30 e 150 kDa. Bem como todas as MMPs, metalocolagenases são enzimas zinco-dependentes e são inibidas por qualquer quelante que se ligue a esses íons (Daboor et al., 2010). De acordo com

Freije et al. (1994), apenas as MMPs 1, 8, 13, 14 e 18 possuem atividade contra os colágenos nativos tipos I, II, III, VII e X. As metalocolagenases têm sido extraídas de tecidos animais e de peixes, como ossos, barbatanas, peles e também do hepatopâncreas de caranguejos marinhos (Sivakumar et al., 1999).

As MMPs de mamíferos constituem uma família de enzimas que incluem: colagenases intersticiais, estromelisinas, gelatinases e metaloproteinases tipos de membrana, e diferem das proteases microbianas quanto à estrutura (Ravanti e Kahari, 2000). As MMPs catalisam uma única clivagem proteolítica nas cadeias helicoidais do colágeno, resultando em dois fragmentos que são, posteriormente, acessíveis às proteases menos específicas (Figura 4) (Jung e Winter, 1998).

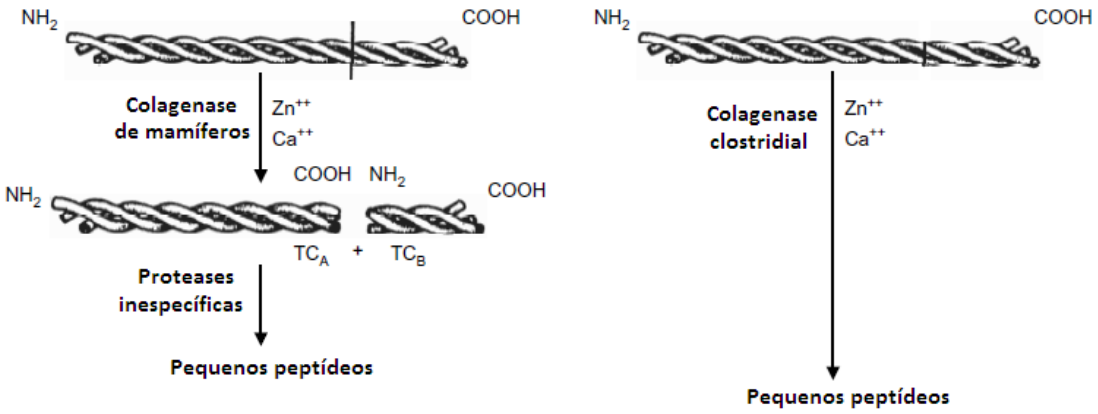


Figura 4. Ação das colagenases de mamíferos e clostridial. TC = tropocolágeno (Adaptado de Jung e Winter, 1998).

3.1.7 *Condições de produção das colagenases*

Um dos pontos principais para a produção de enzimas é a otimização das condições de cultivo, com o objetivo de reduzir o custo e aumentar a eficiência (Laxman et al., 2005). Do ponto de vista industrial, a otimização de processos produtivos pode promover também um aumento nos rendimentos das proteases (Haddar et al., 2009; Lima et al., 2011). .

A escolha do pH inicial do meio de cultura influencia diversos processos enzimáticos, tais como a produção de enzimas, transporte de células através de membranas e expressão de proteases extracelulares (Anandan et al., 2007; Reddy et al., 2008). Lima et al. (2011a) realizaram planejamento fatorial para definir as melhores condições de cultivos e afirmaram que o pH inicial (8,0) foi um fator

importante para a produção de colagenase por *Penicillium aurantiogriseum*. Hamdy (2008) obteve atividade colagenolítica máxima utilizando pH igual a 5,5, embora a maior atividade colagenolítica e menor atividade caseinolítica foi atingida com pH igual a 6,5, ou seja, a especificidade da enzima foi maior.

A influência da temperatura na produção de proteases por micro-organismos é um fator importante (Thys et al., 2006). A temperatura pode regular alguns componentes como síntese enzimática, secreção da enzima e a duração da fase de produção da enzima, além de propriedades de parede celular (Anandan et al., 2007; Chellapan et al., 2006).

A temperatura de produção de colagenase adequada depende da espécie do fungo. Vários artigos relacionados à colagenase afirmaram que a temperatura ótima de produção de fungos é de 30 °C (de Siqueira et al., 2014; Hamdy, 2008; Hurion et al., 1979, 1977; Olutiola e Nwaogwugwu, 1982). Entretanto, alguns autores utilizaram a temperatura entre 24 °C e 26 °C (Benito et al., 2002; Dean e Domnas, 1983; Ibrahim-Granet et al., 1996; Minglian et al., 2004; Ok e Hashinaga, 1996; Wang et al., 2006; Yang et al., 2005). De acordo com de Siqueira et al. (2014), a temperatura de incubação interfere no crescimento e metabolismo dos fungos, e consequentemente na produção de peptidases.

3.1.8 Sistema Duas Fases Aquosas (SDFA)

A recuperação de biomoléculas faz parte da fase crítica do processo de produção. A purificação de um produto voltado para o mercado industrial deve conter algumas características tais como: robustez, confiabilidade, facilidade no escalonamento do processo e na remoção de impurezas, velocidade de processo, alto rendimento de recuperação e baixo custo (Rosa et al, 2011). Para a fabricação de uma biomolécula, devem ser levadas em consideração a taxa de recuperação de produto e sua purificação, onde se encontra o desafio do processo, atualmente dominado por várias etapas cromatográficas (PrzybycienPujar& Steele, 2004).

A extração líquido-líquido utilizando Sistemas de Duas Fases Aquosas (SDFA) é um dos processos mais promissores de biosseparação, que pode ser utilizada nas etapas iniciais do processo de purificação, em substituição às difíceis separações sólido-líquido, ou até mesmo em etapas de purificação subsequentes

(Rosso *et al,* 2012). Os SDFA foram uma das alternativas em potencial para o emprego na indústria (Pandey, 2011).

Os SDFA são resultantes da incompatibilidade entre duas soluções aquosas de componentes estruturalmente diferentes tais como dois polímeros (por exemplo, polietileno glicol - PEG e dextrana), ou um polímero e um sal (por exemplo, PEG e fosfato), que são separados em duas fases em meio aquoso (Albertsson, 1986). Este fenômeno foi descrito inicialmente por Beijerinck no século 19, que descobriu a formação de duas fases entre ágar e gelatina, quando misturadas em determinadas concentrações (Rosa et al., 2010)

As vantagens desses sistemas incluem o tempo de processamento, baixo custo de material e baixa energia de consumo (Yavari *et al*, 2013). O sistema bifásico tem sido utilizado no desenvolvimento de bioprocessos para a recuperação e purificação de vários produtos biológicos como proteínas, material genético, bionanopartículas, células e organelas (Asenjo and Andrews 2011).

A literatura descreve o uso do SDFA em moléculas como anticorpos humanos (Azevedo et al. 2009), catalase (Kavakçioğlu e Tarhan 2013), poligalacturonases (Maciel et al. 2014), citrinina (Pimentel et al. 2013), xilanase (Rahimpour et al. 2007), lipase (Ramakrishnan et al. 2016), elastase (Xu et al. 2005), protease alcalina (Yavari et al. 2013), proteases fibrinolíticas (Silva et al. 2013), e outros. O sistema formado por PEG e fosfato foi relatado para extração de colagenase, uma vez que o PEG possui propriedades físicas favoráveis, como viscosidade e densidade entre as diferentes fases (Rosso et al. 2012; Lima et al. 2013).

3.1.9 *Aplicações Médicas da Colagenase*

Colagenases têm sido bastante usadas na produção de medicamentos. Em aplicações médicas, pode ser usado em queimaduras e tratamento de úlceras (Agren et al., 1992; Püllen et al., 2002), para eliminar cicatrizes, no tratamento da doença de Dupuytren, em vários tipos de fibrose (como cirrose hepática) (Shmoilov et al., 2006), no preparo de amostras para o diagnóstico (Lima et al., 2013), na produção de peptídeos com atividades antioxidante e antimicrobiana (Lima et al., 2014). As colagenases também podem desempenhar papel importante no sucesso da cirurgia de transplante de alguns órgãos específicos (Graminho et al., 2013; Shmoilov et al., 2006).

De acordo com Jung e Winter (1998), as colagenases possuem papel fundamental na cicatrização de feridas. Em pessoas saudáveis, a atividade de colagenases endógenas é suficiente para a remoção dos tecidos mortos. Entretanto, em pacientes com feridas crônicas não-cicatrizadas, como úlceras venosas e úlceras diabéticas, o estado nutricional, o tratamento medicamentoso ou o envelhecimento da pele podem tornar insuficiente a produção de colagenase endógena, necessitando de suplementação a partir de colagenase bacteriana para auxiliar na limpeza da ferida, iniciando o processo de cura (Figura 5). Ensaios clínicos comprovam o efeito no desbridamento de feridas, contribuindo para o processo de cura em si, bem como sua importância na migração de células com atividades importantes, tais como macrófagos, fibroblastos e queratinócitos.

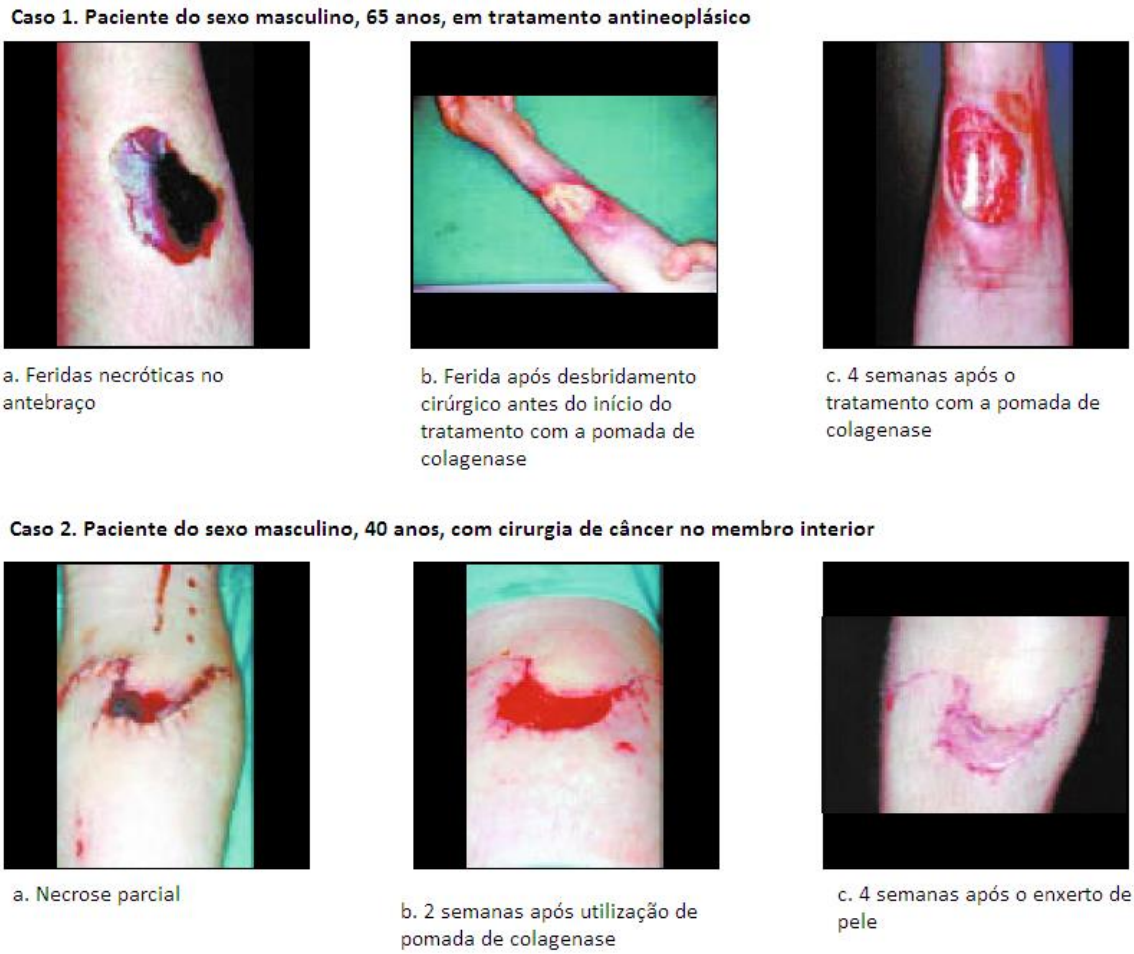
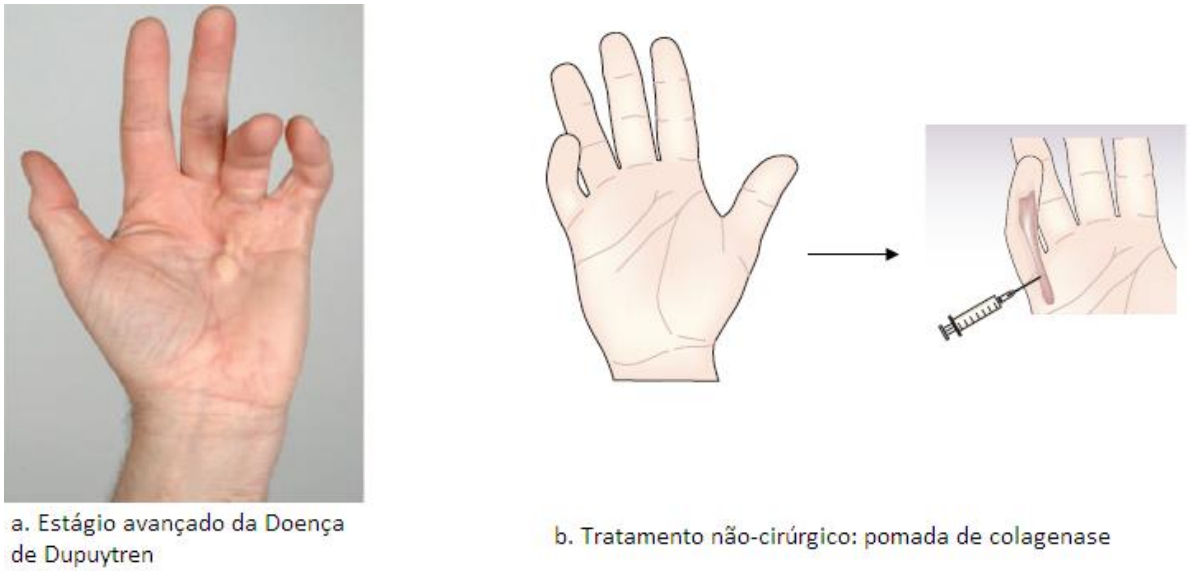


Figura 5. Casos clínicos que comprovam a eficácia do uso da pomada com colagenase (Adaptado de Jung e Winter, 1998)

A Doença de Dupuytren é uma desordem fibroproliferativa de etiologia desconhecida, que muitas vezes resulta em encurtamento e engrossamento da fáscia palmar, levando à contratura irreversível da flexão dos dígitos. O tratamento mais comum é a liberação cirúrgica dos tecidos palmodigitais afetados, entretanto os sintomas retornam com frequência. O tratamento efetivo, não invasivo e bastante promissor, é a injeção de colagenase de *Clostridium histolyticum* (Figura 6) (Shih e Bayat 2010).



a. Estágio avançado da Doença de Dupuytren

b. Tratamento não-cirúrgico: pomada de colagenase

Figura 6. Representação clínica da doença de Dupuytren (a) e tratamento à base de colagenase (b)

A busca por novas colagenases microbianas têm aumentado bastante ao longo dos anos, e alguns autores relatam que sua produção representa, atualmente, uma das maiores dentro da indústria de produção enzimática (Abidi et al., 2013; Graminho et al., 2013). Fazem-se necessários o desenvolvimento e investimento em estudos que comprovem a aplicação de novas colagenases produzidas por micro-organismos, uma vez que elas possuem vasto espectro de aplicação com alto potencial biotecnológico e valor comercial agregados.

A utilização do colágeno e seus peptídeos como biomateriais, bem como suas aplicações comerciais, têm aumentado cada vez mais. O colágeno é comercializado como ingrediente em medicamentos, cosméticos, além de uma variedade de produtos de cuidados de saúde. Produtos naturais com múltiplas funções, como o

colágeno e seus peptídeos, atraem interesse e necessidade de estudos voltados a seus desenvolvimentos (Watanabe, 2004).

3.1.10 *Colágeno*

O colágeno é o principal elemento fibroso constituinte da pele, tendões, ossos, dentes, vasos sanguíneos, intestinos e cartilagens, correspondendo a 30% da proteína total e a 6% em peso do corpo humano (Di Lullo et al., 2002; Jain e Jain, 2010; Müller, 2003; Suphatharaprateep et al., 2011). Está presente na matriz extracelular e nos tecidos conectivos. É uma família de proteínas relacionadas, produzidas por genes diferentes, cuja principal função é estrutural (Vargas et al., 1997).

A unidade estrutural básica da maioria dos colágenos é formada pelo entrelaçamento em tripla hélice de três cadeias polipeptídicas chamadas cadeias alfa. A cada três cadeias alfa polipeptídicas, é formado o monômero do colágeno, o tropocolágeno (Figura 7). De acordo com o tipo de colágeno, as três cadeias interlaçadas do tropocolágeno podem ser idênticas (como é o caso dos colágenos tipos II e III) ou diferentes (Asghar e Henrickson, 1982; Chung e Uitto, 2010). Essa estrutura protéica justifica as propriedades físicas e biológicas dos colágenos: rigidez, solidez e estabilidade, além de oferecer suporte tanto para células quanto tecidos (Chung e Uitto, 2010; Kadler et al., 1996; Vargas et al., 1997).

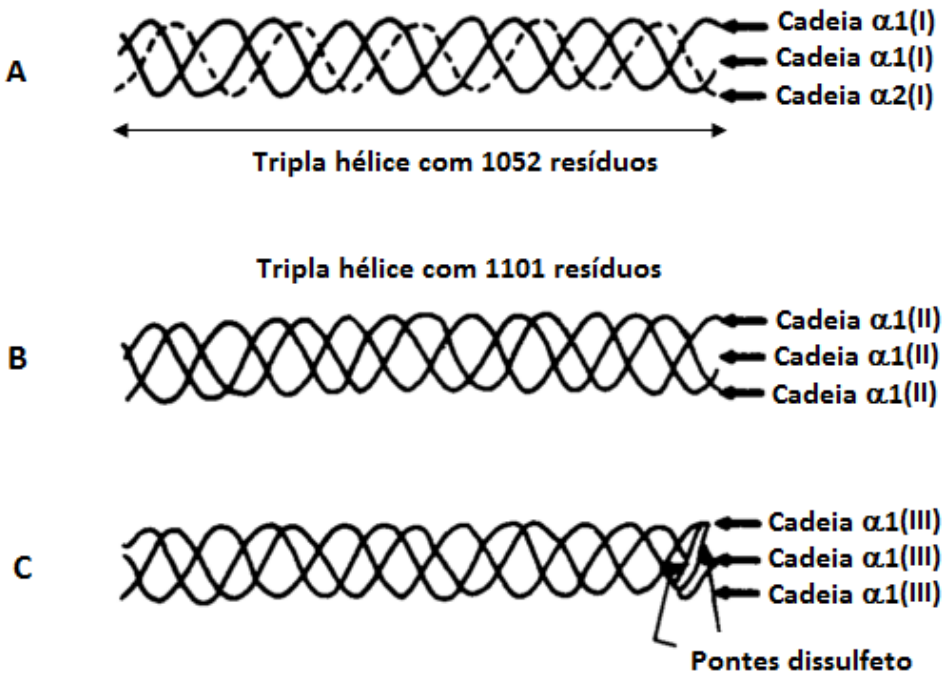


Figura 7. Parte helicoidal das cadeias α em diferentes tipos de colágeno. (A) Colágeno tipo I, com duas cadeias α idênticas e uma diferente. (B) Colágeno tipo II, com três cadeias α idênticas. (C) Colágeno tipo III, com três cadeias α idênticas. Neste último, ligações de pontes de sulfeto intermolecular estão presentes na região helicoidal.

A estrutura do colágeno é estabilizada a partir de pontes de hidrogênio intermoleculares, e é um produto de uma contínua repetição da sequência Glicina-X-Y-, onde X é em sua maioria, Prolina, e Y, Hidroxiprolina, formando as α-cadeias (Figura 8). Apenas uma pequena porção das regiões N- e C- terminais não formam a estrutura em tripla hélice nos colágenos fibrilares. Entre quatro e oito moléculas de colágeno em seção transversal são estabilizados por ligações covalentes cruzadas, constituindo a unidade básica das fibras de colágeno. Assim, essa proteína assume a natureza forte dos tecidos que são compostos por ela, como pele, tendões e ossos (Asghar e Henrickson, 1982; Gelse et al., 2003; Gomez-Guillen et al., 2011).

A montagem da tripla hélice precisa de um pré-requisito estrutural: o resíduo de Glicina (Gly), o menor aminoácido, em cada cadeia polipeptídica, ocupando a terceira posição. As hélices α montam-se em torno de um eixo central, de maneira que todos os resíduos de Glicina são posicionadas no centro da tripla hélice, enquanto que as cadeias laterais mais volumosas de outros aminoácidos ocupam as

posições exteriores (Gelse et al., 2003). A sequência Gly-X-Y pode conter imperfeições como interrupções na conformação da tripla hélice (Uitto et al., 2008). Essas interrupções, além de promover flexibilidade às moléculas de colágeno tipo haste, também pode gerar sítios de clivagem proteolítica não específicos para a sequência primária (Chung e Uitto, 2010).

O comprimento da molécula de colágeno é variável de acordo com cada diferente tipo dessa proteína. Colágenos dos tipos I, II e III possuem cerca de 1000 resíduos de aminoácidos compondo a tripla hélice. Alguns domínios podem ser muito menores, ou conter interrupções por moléculas não-tripla hélice. Colagenos tipo VI e X apresentam cerca de 200 ou 460 resíduos de aminoácidos, respectivamente (Bateman et al., 1996; von der Mark, 1999).

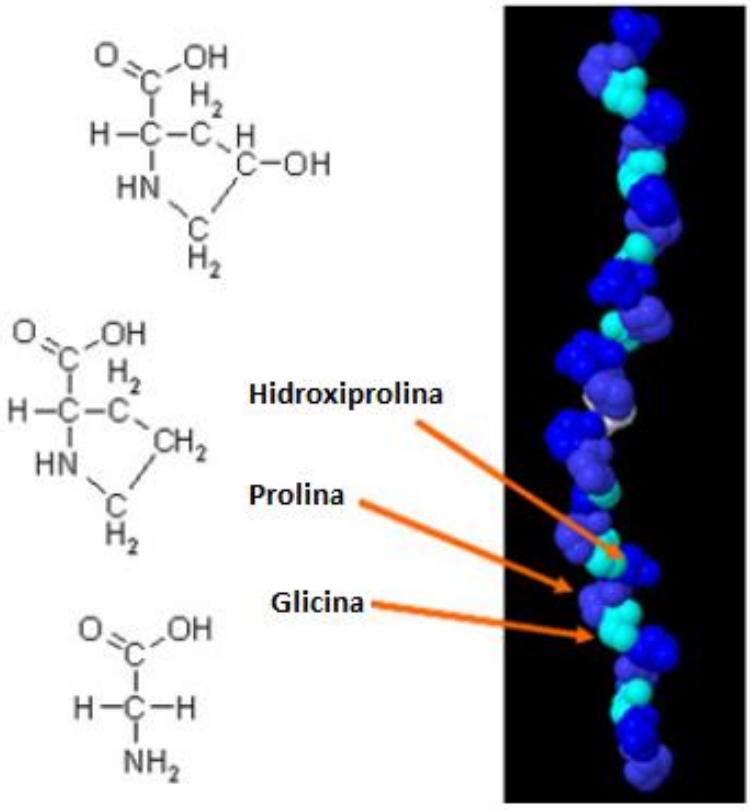


Figura 8. Estrutura do colágeno (Adaptado de Morris and Gonsalves, 2010)

Existem cerca de 29 tipos de colágenos geneticamente distintos, caracterizados por complexidade considerável e diversidade em sua estrutura, subunidades (cadeias α), variantes em suas conexões, presença de domínios adicionais, domínios não-helicoidais, montagem e função (Elango et al., 2015; Gelse

et al., 2003; Gomez-Guillen et al., 2011). Os colágenos podem ser designados por números romanos de I a XXIX, seguindo a ordem de suas respectivas descobertas (Chung e Uitto, 2010). As cadeias dos colágenos podem ser formadas por três cadeias idênticas (homotriméricas) como nos tipos: II, III, VII, VIII, X e outros, ou por duas ou mais diferentes cadeias (heterotrimétricas), como nos tipos I, IV, V, VI, IX e XI (Gelse et al., 2003). Cada uma das três α-cadeias no interior da molécula forma uma hélice com um total de 18 aminoácidos por vez (Hofmann et al., 1978).

Com base em sua estrutura e organização supramolecular, os diferentes tipos de colágeno podem ser agrupados nas seguintes famílias: colágenos formadores de fibrilas, colágenos associados às fibrilas, colágenos formadores de rede, fibrilas de ancoragem, colágenos transmembrana, colágenos da membrana basal e outros com funções únicas. A família mais abundante, com cerca de 90% do total de colágeno, é representada pelos colágenos formadores de fibrilas. A Tabela 2 descreve os diferentes tipos de colágeno, bem como sua composição molecular e distribuição nos tecidos (Gelse et al., 2003).

Tabela 2. Classificação dos diferentes tipos de colágeno de acordo com as famílias, composição molecular e distribuição nos tecidos (Adaptado de (Gelse et al., 2003)

Tipo de colágeno	Composição molecular	Distribuição nos tecidos
Colágenos formadores de fibrila		
I	$[\alpha1(I)_2\alpha2(I)$	Osso, derme, tendões, ligamentos, córnea
II	$[\alpha1(II)]_3$	Cartilagem, corpo vítreo, núcleo pulposo
III	$[\alpha1(III)]_3$	Pele, parede dos vasos, fibras reticulares da maioria dos tecidos (pulmão, fígado, baço)
V	$\alpha1(V)$, $\alpha2(V)$, $\alpha3(V)$	Pulmão, córnea, osso, membranas fetais; junto com colágeno tipo I

XI	α1(XI) α2(XI) α3(X)	Cartilagem, corpo vítreo
Colágeno da membrana basal		
IV	[α1](IV)$_2$$\alpha$2(IV; α1-α6	Membranas basais
Colágenos microfibrilares		
VI	α1(VI), α2(VI), α3(VI)	Amplamente distribuído: pele, cartilagem, placenta, pulmões, parede dos vasos, disco intervertebral
Fibrilas de ancoragem		
VII	[α1)(VII)$_3$	Pele, junções derme-epiderme, mucosa oral, cérvix
Colágenos formadores de rede		
VII	[α1(VIII)$_2$$\alpha$2(VIII)	Células endoceliais, membrana de Descemet
X	[α3(X)]$_3$	Cartilagem hipertrófica
Colágenos associados às fibrilas		
IX	α1(IX) α2(IX) α3(IX)	Cartilagem, humor vítreo, córnea
XII	[α1(II)]$_3$	Pericôndrio, ligamentos, tendões
XIV	[α1(XIV)]$_3$	Derme, tendões, paredes dos vasos, placenta, pulmões e fígado
XX	[α1(XX)]$_3$	Epitélio da córna, pele embrionária, cartilagem externa, tendões
XXI	α1(XXI)]$_3$	Parede dos vasos sanguíneos
Colágenos transmembrana		

XIII	$[\alpha 1(XIII)]_3$	Epiderme, folículo piloso, intestino, condrócitos, pulmões, fígado
XVII	$[\alpha 1(XVII)]_3$	Junções derme-epiderme

Os principais colágenos na pele humana são os tipos I e III, que representam cerca de 80% e 10% da massa total de colágeno, respectivamente. A associação entre esses dois colágenos forma as amplas fibras extracelulares, características da derme humana. O colágeno tipo V está presente na maioria dos tecidos conjuntivos, incluindo derme (menos de 5% do total de colágeno). Na derme, o colágeno tipo V está localizado na superfície das fibras de colágeno formadas pelos tipos I e III, e a regulação do crescimento lateral dessas fibras é feita pelo tipo V (Chung e Uitto, 2010). O colágeno tipo IV possui um arranjo da tripla hélice mais flexível, restrito apenas às membranas basais (von der Mark, 1999).

3.1.11 *Aplicação do colágeno*

As matérias-primas mais comuns utilizadas na extração de colágeno e gelatina são pele, ossos, tendões e cartilagens. A pele do porco foi a primeira matéria-prima usada na manufatura de gelatina na década de 1930 e continua sendo o material mais usado em larga escala de produção industrial, chegando a 46% do total de substratos utilizados. Mais recentemente, subprodutos do peixe e indústria aviária têm recebido atenção considerável (Gómez-Guillén et al., 2011).

Relatos na literatura a respeito de fontes alternativas e novas funcionalidades do colágeno têm aumentando bastante nos últimos 15 anos. Tal interesse pode ser graças à valorização econômica de subprodutos industriais (principalmente da indústria da carne e pesqueira), gerenciamento de resíduos a favor do meio ambiente e a busca por condições inovadoras de processamento desses produtos, além de potenciais novas aplicações. Colágeno e gelatina têm ampla aplicação nas indústrias alimentícia, fotográfica, cosmética e farmacêutica, devido à capacidade que esses substratos possuem na formação de géis e suas propriedades viscoelásticas. Ou seja, podem ter funções como emulsificantes, agentes anti-espumantes, estabilizadores de colóides, agentes de finalização além de agentes de microencapsulamento (Gómez-Guillén et al., 2011).

Outras características que tornam o colágeno um substrato de interesse para o uso nas indústrias médica, coméstica e alimentícia, são: biodegradabilidade, baixa imunogenicidade e a facilidade de produção em larga escala (Gelse et al., 2003). Estudos recentes também relatam que a utilização dos peptídeos de colágeno com propriedades biológicas é umas das aplicações promissoras deste substrato (Gómez-Guillén et al., 2011). Lima et al. (2014) obtiveram peptídeos bioativos do colágeno, com propriedades anti-oxidante e antimicrobianas, e sugeriram a utilização dos hidrolisados de colágeno na indústria alimentícia, com o objetivo de aumentar a vida dos produtos, além do desenvolvimento de alimentos funcionais.

3.1.12 *Caatinga*

A Caatinga é um bioma exclusivamente brasileiro, localizado no Nordeste do Brasil, com cerca de um milhão de quilômetros quadrados, estando inserido na região semi-árida, habitada por aproximadamente 23 milhões de pessoas (Ferreira et al., 2014; Menezes et al., 2012). Os solos são um ambiente rico para microorganismos e as suas características afetam diretamente microbiota (Pacchioni et al., 2014).

A diversidade microbiana dos solos da Caatinga ainda é pouco estudada, mas esse bioma tem condições climáticas severas, como alta temperatura, elevada exposição UV e longos períodos de seca que podem promover a presença de microrganismos com composição taxonômica e funcional distintas em relação a outros biomas (Menezes et al., 2012; Pacchioni et al., 2014).

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5 CAPÍTULO II

Collagenolytic enzymes produced by fungi: A systematic review

Artigo submetido no periódico Brazilian Journal of Microbiology

Brazil, January 11th, 2015

Dear Editor,

Please find attached the paper entitled: "**Collagenolytic enzymes produced by fungi: A systematic review**", by Wanderley, M. C. A. *et al.*, which the authors would like to submit to **Brazilian Journal of Microbiology**.

On the behalf of the authors, I would like to state that all authors agree to submit the manuscript to **Brazilian Journal of Microbiology** and this work has not been submitted or under consideration in any other journal. The present manuscript has 7,369 words, 2 figures, 3 tables and 69 references. There is no conflict of interest. The manuscript is prepared strictly according to the Journal format as provided in the instruction to authors.

Looking forward to hearing from you, I remain

Yours sincerely

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Conflict of Interest Policy

Article Title: **Collagenolytic enzymes produced by fungi: A systematic review**	Corresponding Author name: **Ana Lúcia Figueiredo Porto**

Declarations

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

The authors are aware of the ethical responsibilities and the manuscript has no conflict of interest.

Article Title: **Collagenolytic enzymes produced by fungi: A systematic review**	Corresponding Author name: **Ana Lúcia Figueiredo Porto**

Authors Agreement

All authors agree to submit the manuscript to **Brazilian Journal of Microbiology**

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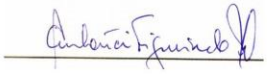

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Collagenolytic enzymes produced by fungi: A systematic review

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Collagenolytic enzymes produced by fungi: A systematic review

Abstract

Specific proteases capable of degrading native triple helical or denatured collagen have been required for many years and have a large spectrum of applications. Different sources have been used for collagenase recovery, but the most widely used commercial source has been pathogenic bacteria *Clostridium histolyticum*. There are few complete reports that fully uncover production, characterization and purification of fungi collagenases. In this review, authors searched through four scientific on line data bases using the following keywords: (collagenolytic OR collagenase) AND (fungi OR fungus OR fungal) AND (production OR synthesis OR synthesize) AND (characterization). Scientific criteria were adopted in this review to classify found articles by score (from 0-10). A total of 2074 articles were found in the following databases: Science Direct, Scopus, PubMed and Web of Science. After exclusion criteria, 21 articles were selected. None obtained the maximum of 10 points defined by the methodology, which indicates a deficiency in studies dealing simultaneously with production, characterization and purification of collagenase by fungi. A clear gap in literature about collagenase production by fungi was verified, which prevents further development in the area and increases the need for further studies, particularly full characterization of fungal collagenases with high specificity to collagen.

Keywords: Collagenase; Fungus; Characterization; Purification; Production.

1 Introduction

Collagen is a fibrous protein found in skin, tendons, bones, teeth, blood vessels, intestines and cartilage, corresponding to 30% of the total protein[1]. Collagen is present in the extracellular matrix and connective tissues and is a family of related proteins, genetically distinct, whose main function is structural[2].

There are more than 26 genetically distinct types of collagens, characterized by considerable complexity and diversity in their structure, their splice variants, presence of additional, non-helical domains, their assembly and their function[3,4]. Collagen types I, II and III are the most abundant in human. Each collagen molecule is a small, hard stick formed by interlacing in a triple helix of three polypeptide chains called alpha chains (Figure 1). This protein structure justifies the physical and biological properties of collagen: stiffness, strength and stability[2].

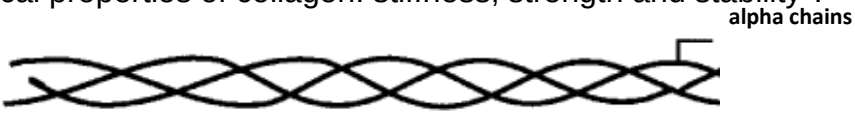

Figure 1. Collagen Molecule: intertwining three alpha chains triple helix[2]

Proteases, in general, from microbial sources are preferred to the enzymes from plant and animal sources for its biochemical diversity and genetic manipulation possibility[5,6]. There are several reports of proteinase producing fungi capable of hydrolyzing insoluble substrates, such as fibrin and collagen - a considerable portion are soil saprophages, as well as parasites on insects and plants[7].

Specific proteases capable of degrading native triple helical or denatured collagen (collagenases) have been required for many years[8]. Collagenases have been isolated and characterized from different sources, as digestive tracts of fish and invertebrates including: tadpole tailfin[9,10], Atlantic cod[11], land snail (*Achatina fulica*)[12], tropical shrimp (*Penaeus vannamei*)[13,14], catfish (*Parasilurus asotus*)[13,15], mackerel (*Scomber japonicas*)[16]; besides plants (*Zingiber officinale*)[17]; bacteria as: *Bacillus cereus* and *Klebsiella pneumoniae*[18], *Bacillus pumilus*[19], *Bacillus licheniformis*[20–22] and fungi, shown in this review.

Microbial collagenase have been recovered from pathogenic micro-organisms, especially *Clostridium histolyticum*, which is the most widely used commercial

source[23]. Other studies reported collagenase producing fungi of genera *Aspergillus*, *Cladosporium*, *Penicillium* and *Alternaria*[24].

Among microorganisms that produce collagenolytic enzymes, filamentous fungi have great advantages such as high productivity and low production cost, rapid development, and the resulting enzyme may be modified and recovered more easily[25]. Enzyme production occurs extracellularly, which makes it particularly easier to recover afterwards[26]. As fungal proteases are capable of hydrolyzing many other proteins besides collagen, the demand for collagenases from fungi with suitable characteristics, namely high specificity, is a very significant research direction to be taken[7]. Collagenases are capable of hydrolyzing both native and denatured collagen, and are becoming increasingly important commercially[27].

Collagenases have been used in medical, pharmaceuticals, food, cosmetics and textiles segments and have applications in fur and hide tanning to help ensure the uniform dying of leathers[28,29]. In medical applications, it can be used in burns and ulcers treatment[30,31], to eliminate scars[32], for Dupuytren's disease treatment in addition to various types of fibrosis such as liver cirrhosis, to preparing samples for diagnosis [33], for production of peptides with antioxidant and antimicrobial activities [34], and play an extremely important role in the transplant surgery success of some specific organs[32].

The search for new microbial collagenases has increased over the years and its production currently represents one of the biggest enzyme industries[35,36]. The development of new production methods, including the search for producing micro-organisms, alternative sources of substrates, and better extraction conditions and purification of collagenase, has been of great importance, since it has a wide application spectrum with high biotechnological potential. In this context, the authors felt the need to better understand the state of the art regarding production, characterization and purification of collagenolytic enzymes by fungi.

2 Material and Methods

The first step on this process, was to make electronic searches in the Scopus (http://www.scopus.com/), ScienceDirect (http://www.sciencedirect.com/), ISI Web of Science (http://apps.isiknowledge.com) and PubMed (http://www.ncbi.nlm.nih.gov/pubmed) databases, using the following keywords:

(collagenolytic OR collagenase) AND (fungi OR fungus OR fungal) AND (production OR synthesis OR synthesize) AND (characterization).

This procedure allowed selecting published papers on the production and characterization of collagenolytic enzyme produced by fungi. Papers that did not report on the enzyme production process were excluded. There were no limitations regarding the year and date of publication, due to lack of publications about this issue. No restrictions were made for methodology used, types of analysis and quantification of results. In addition, there were no restriction on type of micro-organism, collagenolytic activity methodology, culture conditions and characterization assays.

Two independent searches were made and the conformity of the selected papers validated, considering the inclusion criteria described. In case of divergence among the papers, all of the criteria were reviewed and discussed. When in the article title only protease production was mentioned, lacking collagen related terms, researchers proceeded to summary evaluation, looking for methodologies for activity determination involving collagen or gelatin as substrate.

Papers selection criteria were defined to evaluate both better conditions for collagenolytic enzyme production by fungus with biotechnological potential applicability and methodological quality in the characterization of the enzyme. Scientific criteria adopted in this review were according to the ones proposed by Greenhalgh[37]. The parameters were classified on the scale: adequate (score: 2), partially adequate (score: 1) and inadequate (score: 0) or adequate (score: 1) and inadequate (0).

Production process: papers that studied the best growing conditions for producing collagenolytic enzyme received a score of 2, papers that did not conduct studies to improve growing conditions, using collagen or gelatin as substrate, received score of 1, and those which used nonspecific means for collagen production received a score of 0. **Characterization of the enzyme:** papers that reported biochemical characterization of enzyme and included other tests as well as optimum pH and temperature and enzyme inhibition tests, received a score of 2. Those which evaluated only optimum pH and temperature and the effect of inhibitors received a score of 1. Papers that did not have at least these three factors in enzyme characterization were considered inadequate and received a score of 0. **Quantification method of collagenolytic activity:** methods that used chromogenic

substrates (OrangeCollagen or Azocoll) for quantification of collagenolytic activity, received a score of 2. Papers with other quantitative methodologies for collagenolytic activity, received a score of 1, and those that held only qualitative analysis activity, received a score of 0. **Purification:** purification by chromatography methods received a score of 2, those which used other purification methods, received a score of 1, and those that did not do any kind of purification, received 0. **Micro-organism:** articles that used non-pathogenic fungi for collagenolytic enzyme production received a score of 1, while those using pathogenic fungi were considered inadequate and received a score of 0. **Substrate specificity:** enzymes with specific activity over collagen, received a score of 1; those who presented a wide hydrolysis spectrum or have not been tested, received a score of 0.

Maximum overall score was 10 points. Other parameters such as production time, year of publication, satisfactory collagenolytic activity, among others, did not scored but were taken into consideration, as they were relevant to subsequent discussion. The parameters scored are summarized in Table 1.

Table 1. Score of selected parameters for critical evaluation of the systematic review

Criteria for determining the scores	**Pointing**		
	2	**1**	**0**
(A) Production	Specific for collagenase, with controlled variables	Specific for collagenase, with uncontrolled variables	No specific for collagenase
(B) Characterization	Complete	Partial	Absent
(C) Microorganism		Non-pathogenic	Pathogenic
(D) Collagenolytic Activity Method	Azocoll or OrangeCollagen	Others	Absent
(E) Purification	Complete	Partial	Absent
(F) Substrate		Collagenase (specific)	Non-Specific

3 Results and Discussion

By applying the established search procedure, a total of 1346 articles were found in Science Direct database, 678 articles in Scopus database, 45 articles in PubMed, and 5 articles in Web of Science, totaling 2074 articles. Based on defined inclusion and exclusion criteria, 21 articles were selected for this review, distributed as shown in Figure 2.

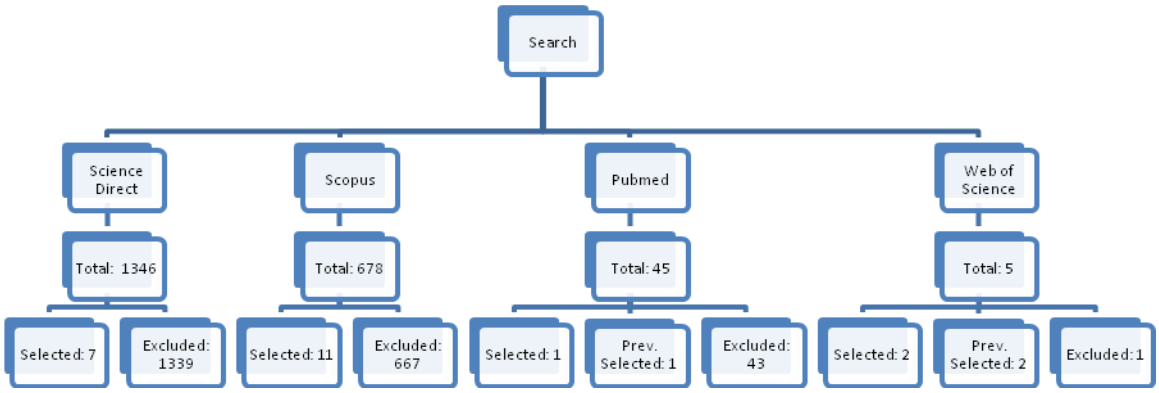

Figure 2. Total articles selected in 4 different databases using the described methodology.

Regarding the scores obtained for each selected article, none obtained the maximum of 10 points defined by the methodology. According to the distribution in Table 2, only one article hit a score of 9 (4.77% of selected articles), two articles obtained the score of 8 (9.52%), and three articles reached the score of 7 (14.29%). 71.43% of the articles achieved scores below 7, which indicates a deficiency in studies dealing simultaneously with production, characterization and purification of collagenase by fungi. Where the enzyme obtained should present specificity to substrate and have its activity quantified by the method adopted as the most appropriate (Azocoll).

As described in the methodology, no time interval has been defined. However, only 11 articles have been published in the last ten years. Of these 11 articles, only 4 were published in the last five years, clearly indicating a need for further research related to the production of collagenase by fungi.

Table 2. Scores distribution of selected articles

Authors	(A)	(B)	(C)	(D)	(E)	(F)	Total
Hurion et al. 1977	1	0	0	2	2	0	**4**
Hurion et al. 1979	1	0	0	2	2	0	**4**
Olutiola and Nwaogwugwu 1982	0	2	1	0	0	0	**4**
Dean and Domnas 1983	0	2	1	1	1	0	**6**
Zhu et al. 1990	0	0	0	0	0	0	**0**
Tomee et al. 1994	1	0	0	0	0	0	**3**
Ibrahim-Granet et al. 1996	0	1	0	2	2	0	**4**
Ok and Hashinaga 1996	2	1	1	1	1	1	**7**
Benito et al. 2002	0	2	1	2	2	0	**7**
Minglian et al. 2004	1	2	1	2	2	0	**8**
Yang et al. 2005	1	2	1	1	1	0	**6**
Wang et al. 2006	1	2	1	2	2	0	**7**
Mahmoud et al. 2007	2	1	0	2	2	0	**6**
Viani et al. 2007	1	0	1	0	0	0	**3**
Hamdy 2008	2	2	1	2	2	0	**8**
Lopes et al. 2008	0	1	0	0	0	0	**1**
Voltan et al. 2008	1	0	0	1	1	0	**4**
Lima et al. 2011a	2	2	1	1	1	1	**9**
Lima et al. 2011b	2	0	1	0	0	0	**5**
de Siqueira et al. 2014	0	2	1	1	1	0	**6**
Sharkova et al. 2015	0	0	1	0	0	0	**3**

(A) Production: Specific for collagenase production with controlled variables (score 2), specific for collagenase production with uncontrolled variables (score 1), non-specific for collagenase (score 0)

(B) Characterization: Complete characterization (score 2), partial characterization (score 1), absent (score 0)

(C) Microorganism: Non-pathogenic microorganism (score 1), pathogenic microorganism (score 0)

(D) Collagenolytic Activity: Chromogenic substrate for collagenolytic activity method (score 2), others quantitative methods (score 1), qualitative (score 0)

(E) Purification: Purification by chromatography (score 2), partial purification (score 1), absent (score 0)

(F) Substrate Specificity: Collagenase with specificity for collagen (score 1), non-specific (score 0)

Table 3 summarizes the relevant information on the criteria applied for the selected papers.

Table 3. Summary of selected articles relevant data according to the criteria adopted on the review

Authors	Hurion et al. 1977	Hurion et al. 1979	Olutiola and	Dean and Domnas	Zhu et al. 1990	Tomee et al. 1994	Ibrahim-Granet et	Ok and Hashinaga	Benito et al. 2002	Minglian et al. 2004
Microorg.	*E. coronata*	*E. coronata*	*A. aculeatus*	*L. gigganteum*	*A. flavus*	*Aspergillus*	*T. schoenleini*	*Z. rouxii*	*P. chrysogen*	*A. oligospora*
Culture Medium	Casamino acids	Casamino acids	Glicose, salts, l-	Peptone, yeast	M9 (without	Yeast carbon	Sabouraud	YPG	LMZ	LMZ - with gelatin
Culture Conditio	pH 5.6, 30° C, without	pH 5.6, 30° C, without,	7 days, 30 °C	Gyrotory shaker	2 weeks, T.A.	5 days, 37 °C, 150	25°C, pH 6, 7 days	pH 7, 25°C, 50h,	26 °C, 200 rpm, 7	25-18°C,6 days,150-
Col. Activ.	Synthetic peptide	Synthetic peptide	Achilles tendon	Azocoll	SDS-PAGE	Orange collagen e	C-18 e SDS-	Ninhydrin	Azocoll	Azocoll
Col. Activ.	X	X	X	8 U/mL	X	X	X	70.4 U/mL	1% e 2%	0.0134 U/mL/m
Molecular	23 - 40	X	X	X	X	32	82	X	35	38
Isoeletric Point	X	X	X	X	X	X	X	X	X	4.9
pH and temper.	X	X	pH 7, 35°	pH 8.4, 60°	X	X	X	pH 8.2	pH 10, 55°	pH 6-8, 45°
Inhibitors	X	EDTA, DFP,	Ca^{2+}, Na^{+}, EDTA, 2,4-	PMSF, TPCK,	EDTA, fenantrolin,	X	Fenantrolina, EDTA,	X	PMSF, EDTA,	PMSF e SSI
Specific Activity	0.088 nkat/mg	X	3.6 U/mg	X	X	0.39 U/mg	X	70.4 U/mg	0.37 U/mg	1.12 U/mg
Substrate	BAE (trypsin-	X	Casein, Elastin,	BAPA, TAME	X	X	Synthetic Peptides,	Synthetic Collagen,	Casein, BSA,	Casein, Gelatin,
Purif.	Croma-tography	Ultrafiltration,	Ammonium	X	$(NH_4)_2SO_4$ Ammoniu	Cation exchange	GF, Orange 3,	X	Ammonium	Ammonium

Yang et al. 2005	Wang et al. 2006	Mahmoud et al. 2007	Viani et al. 2007	Hamdy 2008	Lopes et al. 2008	Voltan et	Lima et al. 2011a	Lima et al. 2011b	de Siqueira et	Sharkova et al.
L. psalliota	*M. microscaphoid*	*A. flavus*	*M. canis*	*R. solani*	*C. immitis*	*P. brasilien*	*P. aurantiogri*	*P. aurantiogri*	*A. terreus*	*Micromycetes*
Glucose, gelatin	Protease Inducing -	Gelatin, glucose,	Medium with type I	Sabouraud -glucose-	Czapek	Yeast carbon	Soybean flour,	Soybean flour,Gluco	Solid medium of	Several
26°C, 100 rpm,	6 days, 26 °C, 200 rpm	6 days,37 °C	14 days	108h, 175rpm,	pH 5.5, 9 days, T.A.	150 rpm, 35 °C,	0,75% gelatin,	pH 7.0, 24 °C, 24 h	$2.0x10^5$esp ores, 72h,	200 rpm, 28°C, 4
Non describe	Folin	Ninhydrin	Synthetic peptide	Ninhydrin	Zymogram	Azocoll	Azocoll	Azocoll	Azocoll	Azocoll
14%	Collagen 15.9%,	82.95 U/mL	1 Unit of collagenas	212.33 U/mL	X	1.2 U/mL	164 U/mL	231 U/mL	0.165 OD/mL	113.2 e 332 x 10^-
32	39 KDa	72-92 KDa	X	66 KDa	25 KDa	20 - 200 kDa	X	X	X	X
X	6.8	X	X	X	X	X	X	X	X	X
pH 10, 70°	pH 9, 60°	X	X	pH 5, 40°	X	X	pH 9, 37°	X	pH 6.5, 55°	X
Leupeptin,	PMSF	Cetrimide	X	EDTA, Iodoacetat	PMSF	PMSF, EDTA,	PMSF, iodoacetic	X	PMSF, EDTA, IAA	X
48 U/mg	X	92.17 U/mg	X	$18064.7x10^3$ U/mg	X	X	319 U/mg	X	X	X
Casein, BSA,	Casein, BSA, Skimmed milk,	X	X	Collagen, Casein,	Casein, Gelatin,	Casein, Elastase	Azocoll, Type I	X	Casein, Keratin	Plasmin, Plasmino
Ultrafiltration,	Source 15Q, Phenyl	Ammonium	X	Ammonium	X	X	X	X	X	X

3.1 Microorganism

The significance and frequency of main types of microorganisms as bioactive metabolites producers had varied significantly during the last decades[57]. The microbial collagenases are preferable than plant or animal enzymes, considering the biochemical diversity and susceptibility to genetic manipulation that their sources have[25,58].

Based on this systematic review, 21 articles were selected, of which 17 were carried out with ten different genera of filamentous fungi (*Penicillium, Aspergillus, Arthrobotrys, Monacrosporium, Trichophyton, Microsporum, Lecanicillium, Entomophthora, Micromycetes* e *Lagenidium*). Two genera found were classified as dimorphic (*Coccidioides* and *Paracoccidioides*), and only one had a yeast morphology (*Zygosaccharomyces*).

From the industrial point of view, pathogenicity can negatively influence microorganism choice for bioprocess development. Interestingly, approximately 40% of fungi cited in selected articles are described as classic pathogens. The pathogenic species that were associated with good collagenolytic enzyme production were *Rhizoctonia solani* with a production of 212.3 U/ml[52] and *Penicillium aurantiogriseum* with 231 U/ml[25] and 164 U/mL[55].

A great diversity of collagenolytic enzymes producing fungi could be observed (more than twenty different taxa). Most belonging to phylum *Ascomycota*, other to phyla *Basidiomycota* (*Rhizoctonia solani*), *Entomophthoromycota* (*Conidiobolus coronatus*) and *Oomycetes* (*Lagenidium giganteum*). Filamentous fungi are clearly more studied in comparison to yeasts for collagenolytic enzyme production. Many articles contain pathogenic fungi in order to better understand its pathogenesis mechanisms and not in order to study enzymatic production itself. The genus *Aspergillus* was the most frequent, followed by *Penicillium* and *Entomophthora* genres. Considering pathogenesis, enzyme activity and specificity, the fungi better qualified for enzyme production were the filamentous fungus *Penicillium aurantiogriseum* and *Zygosaccharomyces rouxii* yeast.

3.2 Culture Medium

Culture medium selection is of great importance for collagenase production, since this factor will directly affect final process cost. As said earlier, one of the advantages of working with microorganisms is the possibility to vary the composition of the culture medium, using lower cost materials, such as byproducts of the fishing industry, for example, as substrate. Nine of the selected papers presented a culture medium containing collagen or gelatin in its composition, other studies used other sources of carbon and nitrogen, mainly yeast extract. Some studies involving bacteria indicate that adding gelatin or casein in the medium increases the collagenase yield. However, the work of Ok and Hashinaga[45] with *Z. rouxii* yeast, observed that adding gelatin in YPG medium was not essential for the production of collagenase. Lima et al.[25] reported the use of a inexpensive culture medium for *P. aurantiogriseum* collagenase production, using soy flour as main substrate, and the same medium was used by authors Lima et al.[55], reaching one of the best collagenolytic activity values found during this review (Table 3).

Regarding collagenolytic enzyme activity, Hamdy[52] used a medium containing sabouraud, glucose and insoluble collagen type I, with filamentous fungus *Rhizoctonia solani* and obtained a good activity result (212.33 U/ml). Mahmoud et al.[50] found an enzyme activity of 82.95 U/ml using the *A. flavus* and culture medium containing gelatin, glucose, yeast extract and native bovine collagen. Ok and Hashinaga[45] showed satisfactory results for the selected yeast *Z. rouxii* (70.4 U/ml), using culture media containing peptone, yeast extract and glucose.

Voltan et al.[54] observed different collagenase production with dimorphic fungus *P. brasiliensis* in yeast and filamentous forms, using different mediums, were with elastin and collagen the production was stimulated. Minglian et al.[47] had protease activity in culture filtrates increased with the concentration of gelatin as an inducing substrate when added to LMZ medium.

Sharkova et al.[7] stated that the highest levels of collagenolytic activity for *T. inflatum* k1 in a medium containing glucose, starch, NaCl, KH_2PO_4, $MgSO_4$ and inorganic nitrogen sources (NH_4NO_3, $NHNO_4$) was higher than in the protein rich medium (332.0×10^{-3} and 23.0×10^{-3} U/mL, respectively). A similar relationship was observed for the collagenase activity calculated per mg of *T. inflatum* k1 mycelium grown on these media. Fungi inhabiting soils and consuming plant and animal debris

seem to have no need for proteases with such narrow substrate specificity as those utilized by animals for hemostatic regulation. The data obtained for the 83 micromycete strains studied suggest entomopathogenic fungi as the most promising potential collagenolytic activities producers.

According to Hamdy[52], the use of different batch or collagen types may interfere in enzymes production (enzyme activity) and collagenases from different microorganisms have affinity for specific types of collagen[59]. The production of different fungi in different media must be the subject of extended studies.

3.3 Culture Conditions

Process development is also a factor to be considered since optimization of culture conditions can promote an increase in the yields of protease and reduction in production costs, a major issue from an industrial point of view[55,60].

The culture medium initial pH influences many enzymatic processes, such as enzyme production, cell transport across membranes and extracellular proteases expression[61,62]. The pH of the culture medium used in the selected articles ranged from 5.5 to 8.0, while temperature ranged from 18 to 37 °C. Regarding agitation, only Hurion et al.[39] showed non mixed enzyme production, with microorganism *E. coronata*. In most of the works, ranged an agitation was in the range 100-200 rpm.

Fermentation time to collagenase production varied widely, from 24 hours to 14 days, a time of 6 to 7 days being reported by 8 papers. Several studies showed activity decay after the 7th day of fermentation. Zhu et al.[42] demonstrated that, in medium containing insoluble collagen, after 2 weeks, fungus grows only to half the mass obtained in milk medium for 1 week. The articles that studied time influence on enzyme production reported higher production during stationary phase.

The work of Lima et al.[25] presented a factorial design to define the best growing conditions for the production of collagenase. Authors stated that initial pH, temperature and concentration of substrate are significant factors for collagenase production by *P. aurantiogriseum* using soybean flour medium. Results indicate the best production conditions as: 0.75% substrate concentration (soybean flour), 200 rpm agitation, culture medium initial pH of 8.0 and incubation temperature of 28 °C.

Hamdy[52] reported that maximum yield (212.33 U/mL) was reached after 108 hours of incubation, in pH 5.5, 30 °C and 175 rpm, although higher collagenolytic

activity with lower caseinolytic activity was achieved at pH 6.5, enhanced the specificity of the production process.

Temperature influence on protease production by microorganisms is an important factor[63]. Temperature can regulate some components as enzymatic synthesis, enzyme secretion and length of the enzyme´s synthesis phase, besides the properties of cell wall[62,64]. In general, studies used temperatures between 18 and 37 °C during production. The papers that studied different temperatures showed 30 °C as the optimum temperature for collagenolytic protease production. According to de Siqueira et al.[56], incubation temperature interferes with fungus growth and metabolism, and consequently, peptidase production, the best temperature being 30°C, according to Hamdy[52]. Lima et al.[25] reported that the best conditions for volumetric collagenolytic activity and biomass production were 24 °C and pH 7.0.

Among works that discriminated the shaking speed, 150-200 rpm were most used, except for Yang et al.[48], that used 100 rpm. Hamdy[52] showed in his results that although there is little difference, the agitation of 175 rpm was the best for enzyme production.

3.4 Collagenolytic Activity

Collagenolytic activity can be described as collagen hydrolysis by collagenase with peptides or amino acids release. Different methods are described in literature to measure this activity: colorimetric, fluorescence, turbidity and viscometry or radioactivity, among others. All these methods are quite time-consuming, the time needed ranging from 3 to 18 h. On the other hand, their major advantage is that most of them use native collagens[23,65].

The radioactive or fluorescent methods require more time to produce substrate and more specific measuring equipment, as well as immunological methods. Moreover, synthetic oligopeptide is not an entirely specific substrate for collagenase [65]. Another used technique was developed by Mandl et al.[59], using collagen *in natura* as substrate and ninhydrin as coloring reagent. The ninhydrin method measures free amino acids release, which difficult continuous activity monitoring or may underestimate enzymes activity if it releases peptides and not free amino acids. Besides, in this method the ninhydrin can react with free amino acids existing in solution, which limits the technique sensitivity[66].

Among colorimetric methods, there is the Azocoll based[67]. The Azocoll is an azo dye-impregnated collagen, which is a specific substrate for collagenase, since it allows observing hydrolysis by release of dye-impregnated soluble peptides that are measured by spectrophotometry, increasing the method sensitivity.

All 21 articles selected in this review have different methodologies to quantify collagenase activity. Eight of the articles used Azocoll as a substrate for measurement of collagenolytic activity. Other papers used other quantitative methods, such as: ninhydrin (4 items), Folin (1 item), synthetic peptide (4 items) and OrangeCollagen (1 item).

Table 3 shows the results obtained for collagenolytic activity between the different methods. Lima et al.[55] reported the highest volumetric activity (231 U/mL) of the articles in this systematic review, using colagenase from *P. aurantiogriseum.* Hamdy[52] used the *R. solani*, and obtained 212.33 U/mL. Lima et al.[25] also conducted a study with *P. aurantiogriseum* and obtained a collagenolytic activity of 164 U/mL. Other authors reported enzymatic activities less than 83 U/mL, or did not perform quantitative assays, or described as relative activity in percentage.

Regarding the specific activity, less than half the articles quantify this parameter. Interestingly Hamdy[52] reported a specific activity value well above the others (18064.7 x 10^3 U/mg). Another article that presented a good specific activity was Lima et al.[25], with 319 U/mg. In general, the specific activity varied significantly (from 0.37 to 18064.7x103 U/mg). The highest activities were observed in studies involving production optimization. However, effectiveness of production tends to be evaluated by volumetric collagenolytic activity due to the industrial relevance of this parameter Lima et al.[55].

3.5 Enzyme Characterization

3.5.1 Isoeletric Point

From selected articles, only two values for isoelectric point of collagenolytic enzyme were reported. The values found by Minglian et al.[47] and Wang et al.[49] were respectively 4.9 to an enzyme produced by *A. oligospora* and 6.8 to another produced by *M. microscaphoides*. However, in these studies no significant

collagenolytic activity was reported when compared to other activities found, as can be seen in Table 3.

3.5.2 pH and Temperature Optimal

The optimum pH for enzyme activity varied considerably (pH 5-10). For the best results regarding collagenolytic activity, Lima et al.[55] and Mahmoud et al.[50], the optimum pH of the enzyme was not evaluated. Ok and Hashinaga[45] evaluated the optimal pH (8.2) of the enzyme produced by *Z. rouxii* yeast. Lima et al.[25] found that pH of 9.0 was the best for collagenolytic enzyme produced by *P. aurantiogriseu*m. Only the enzyme produced by *R. solani* presented an acid optimum pH, 5.0[52]. As pH, optimum enzyme activity temperature also varied greatly (from 35 to 70 °C). Only one of the works have produced a *in natura* collagen specific collagenase and evaluated optimum temperature, 37 °C[25].

3.5.3 Inhibitors

Enzyme inhibitors are molecules that interact with enzyme or compounds that chelate metal ions required by the enzyme to maintain its conformation[23]. Some compounds can inactivate irreversibly to collagenase, such as dithiothreitol (DTT) and mercaptoethanol[68,69]. Other inhibitors tested are phenylmethylsulphonyl fluoride (PMSF) for serine proteases, ethylenediaminetetraacetic acid (EDTA) for metalloproteases, and iodoacetic acid (IAA) for cysteine proteases[55].

Of the 21 selected articles, most conducted inhibitors tests (14 articles). Six concluded that the enzyme belongs to serine proteases group, four concluded belongs to metalloproteinases, two articles to both of the groups and in the remainder articles no conclusion were obtained. The collagenolytic enzyme produced by *R. solani* was inhibited by Hg^{2+}, iodoacetate, arsenate, arsenite, cystein and EDTA[52]. Lima et al.[25] reported the inhibition of the collagenase enzyme produced by *P. aurantiogriseum* by PMSF, indicating that the enzyme is a serine protease.

3.5.4 Substrate Specificity

For certain industrial applications, such as medical and cosmetic areas, the enzyme specificity is one of the most important parameters to consider. From the 21 selected articles, 15 conducted substrate specificity tests using other protein sources. None performed specificity tests using different types of collagen. Hamdy[52] tested the enzyme produced by *R. solani* on collagen, casein and gelatin, and the best results were obtained with collagen. Lima et al.[25] reported enzyme specificity tests produced by *P. aurantiogriseum* on Azocoll, type I collagen, gelatin and azocasein, where the best results were found for the first substrate, Azocoll.

3.5.5 Molecular Weight

The identified size of collagenolytic enzymes found in the different papers ranged from 25 to 82 kDa. However, the majority of the values (5 of 11 papers) is between 32 and 39 kDa. None of the two studies that have specific activity for collagen succeeded in obtaining the precise enzyme molecular weight. Among the articles that presented largest enzymatic activity, only Hamdy[52] determined the enzyme size by electrophoresis, reporting a value of 66 kDa, with 212.33 U/ml of enzyme activity.

3.6 Purification

Once a crude collagenase extract is recovered, it must be purified using one of several chromatographic methods that can be classified as: gel filtration, ion exchange, hydrophobic interaction or affinity[23]. Furthermore, there are traditional enzymatic extraction methods, such as ammonium sulfate precipitation, ultrafiltration, Tris-HCl buffer extraction, with sodium bicarbonate buffer, among others[23,58].

From the 21 articles selected, 12 had some kind of purification, 11 of them using chromatographic techniques and only one exclusive by ammonium sulfate[40]. Mahmoud et al.[50] purified the enzyme produced by *A. flavus* using the DEAE-Cellulose column and obtained a yield of 39.43%. Hamdy[52] could yield 60.49% with the purification using gel filtration chromatography, but the enzyme activity had reduced the amount to 128.4 U/ml.

The others papers reporting good enzymatic activities did not undergo any purification activities [25,45,55]. Other selected articles showed no significant amount of enzyme nor quantify the collagenase produced.

4 Conclusions

From the 21 select papers, 11 were published in the last ten years and only four in the last five years. According to the scoring methodology criteria, only 5 studies showed score ≥ 7. This paper summarized the main findings on production of fungal collagenase. Only two studies reported enzymes with high specificity to collagen over other protein substrates. Among microorganisms studied the *Penicillium aurantiogriseum* and *Rhizoctonia solani* stood out in volumetric and specific collagenase activity, and are non-pathogenic filamentous fungi and extracellular enzyme producers. In the culture medium composition the use of collagen-based compounds favored collagenolytic enzymes production. For enzymes characterization, articles found differed a lot regarding parameters analyzed. The articles with better scores did not undergo an appropriate purification process. It was possible to observe a gap in literature about collagenase production by fungi and its characterization, which prevents further development in the area and increases the need for further studies, particularly for full characterization of fungal collagenases with high specificity. It was also observed that studied fungal collagenases presents promising and competitive biotechnology characteristics when compared with bacterial enzymes, most used commercially.

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6 Ethical Statement/Conflict of Interest

The authors are aware of the ethical responsibilities and the manuscript has no conflict of interest.

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6 CAPÍTULO III

Production and Characterization of a Collagenase by Penicillium sp. UCP 1286 Isolated From the Soil of Caatinga

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Abstract:	A new Penicillium sp. strain isolated from the soil of Caatinga, a Brazilian Biome (UCP 1286) was selected for collagenase production. Using gelatin as a sole carbon and nitrogen source, a fermentation system allowing the obtention of a collagenolytic activity about 2.7 times higher than existing data was developed, with the highest values of collagenolytic activity (379.80 U/mL) and specific activity (1460.77 U/mg) obtained after 126 hours of production. By applying a factorial design, the enzyme production was further increased by about 65% compared to the preliminary results obtained, with 632.70 U/mL of collagenolytic activity. The application of the factorial design demonstrated the existence of two factors with statistical significance on the production of the enzyme: pH and temperature, both with negative effects. The enzyme was found to be more active at pH 9.0 and 37 °C and also to be very stable in comparison with the collagenase produced by other microorganisms. The enzyme seems to belong to collagenolytic serine proteases family because of its inhibition by phenylmethylsulfonyl fluoride. Concerning the substrate specificity, it was observed

	that the highest enzyme activity corresponds to azocoll, there was no relevant activity on azocasein and the enzyme showed to be more specific to type V collagen and gelatin than the commercial colagenase produced by Clostridium histolyticum. The major band observed at electrophoresis was approximately 37 kDa. The zymogram analysis confirmed the collagenolytic activity. All data indicates this enzyme as promising biotechnology product.
Response to Reviewers:	Dear Editor in Chief, Dr. Nasib Qureshi The article has been revised and amended as the required requests. The title page was remade and article layout settings were also modified. Yours sincerely , Dr. Ana Lucia Figueiredo Porto.

Production and Characterization of a Collagenase by *Penicillium* sp. UCP 1286 Isolated From the Soil of Caatinga

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Production and Characterization of a Collagenase by *Penicillium* sp. UCP 1286 Isolated From the Soil of Caatinga

Abstract

A new *Penicillium* sp. strain isolated from the soil of Caatinga, a Brazilian Biome (UCP 1286) was selected for collagenase production. Using gelatin as a sole carbon and nitrogen source, a fermentation system allowing the obtention of a collagenolytic activity about 2.7 times higher than existing data was developed, with the highest values of collagenolytic activity (379.80 U/mL) and specific activity (1460.77 U/mg) obtained after 126 hours of production. By applying a factorial design, the enzyme production was further increased by about 65% compared to the preliminary results obtained, with 632.70 U/mL of collagenolytic activity. The application of the factorial design demonstrated the existence of two factors with statistical significance on the production of the enzyme: pH and temperature, both with negative effects. The enzyme was found to be more active at pH 9.0 and 37 °C and also to be very stable in comparison with the collagenase produced by other microorganisms. The enzyme seems to belong to collagenolytic serine proteases family because of its inhibition by phenylmethylsulfonyl fluoride. Concerning the substrate specificity, it was observed that the highest enzyme activity corresponds to azocoll, there was no relevant activity on azocasein and the enzyme showed to be more specific to type V collagen and gelatin than the commercial colagenase produced by *Clostridium histolyticum*. The major band observed at electrophoresis was approximately 37 kDa. The zymogram analysis confirmed the collagenolytic activity. All data indicates this enzyme as promising biotechnology product.

Keywords: Collagenolytic; Enzymes; Factorial Design; Fermentation; Filamentous Fungi; Specificity.

Introduction

Collagen is the major fibrous element of skin, bones, tendons, cartilage, blood vessels and teeth found in all animals (Jain e Jain 2010; Suphatharaprateep et al. 2011). Collagen is found in connective tissues, making up approximately 30% of the protein in human body (Di Lullo et al. 2002; Müller 2003). Because of the rigid structure of collagen (three helically wound polypeptide fibrils) its degradation is restricted to a few proteases (Suphatharaprateep et al. 2011).

Collagenases are specific enzymes that can hydrolyze both native and denatured collagens (Tran e Nagano 2002; Wu et al. 2010). These enzymes can degrade native triple helix of collagen to small fragments and play an important role in connective tissue metabolism (Dresden 1971; Lima et al. 2011b). Collagenolytic enzymes are a kind of proteases that are related to various physiological and pathological processes and have several applications in industry, medicine and biotechnology (Ravanti e Kahari 2000; Wu et al. 2010; Lima et al. 2011a; Lima et al. 2013). With biotechnology accelerated growth, applications of proteases have expanded to new areas such as clinical, medicinal and analytical chemistry (Shanmughapriya et al. 2008).

Among various sources of proteases, those produced by microorganisms play an important role in biotechnological processes and are used with increasing frequency, as large amounts of these enzymes can be produced quickly and at low cost (Barret et al. 2004). Search for microbial collagenases has been increasing due to their wide application, as they are able to cleave collagen helix at multiple sites, while mammalian collagenases cleave at a single site (Hamdy 2008; Jain e Jain 2010).

Microorganisms are preferred as source of collagenolytic enzymes due to their biochemical diversity and susceptibility to genetic manipulation (Rao et al. 1998). Furthermore, microbial collagenases are quite versatile, possess broad substrate specificities and are able to hydrolyze both water-insoluble native collagens and water-soluble denatured collagens (Mookthiar et al. 985; Peterkofsky 1982; Daboor et al. 2010).

The extracellular production of fungi collagenases makes particularly easy its recovery after the end of fermentation (Sandhya et al. 2005). Studies have reported

the biosynthesis of collagenases by fungi belonging to the different genera, such as *Aspergillus, Cladosporium, Alternaria, Penicillium* (Yakovleva et al. 2006; Lima et al. 2011b; Rosso et al. 2012; de Siqueira et al. 2014), *Candida* (Lima et al. 2009), *Microsporum* (Viani et al. 2007) and *Rhizoctonia* (Hamdy 2008). Species of *Penicillium* genus have a higher biotechnological potential compared to other genera cited, both for production of proteases and other enzymes, as they have the capacity of growth in different culture conditions, using a wide variety of substrates as nutrients (Ikram-Ul-Haq e Mukhtar 2007).

Caatinga is an exclusively Brazilian Biome, located in Northeast Brazil, with about one million square kilometers, mostly overlapping with semiarid region, inhabited by approximately 23 million people (Menezes et al. 2012; Ferreira et al. 2014). Soils are a rich environment for microorganisms and their characteristics affect directly microbiota (Pacchioni et al. 2014). The microbial diversity of Caatinga soils is still poorly studied, but this biome has severe climate conditions, such as high temperature, high UV exposure and long periods of drought which promote the presence of microorganisms with distinct taxonomic and functional composition in relation to other biomes (Menezes et al. 2012; Pacchioni et al. 2014).

The aim of this work was to report a new producer strain of collagenase, isolated from the soil Caatinga (Pernambuco – Brazil), and determine the best conditions for the production of the enzyme using an inexpensive culture medium. Furthermore, collagenase was characterized under the most favorable conditions and a variety of substrates was tested, to study its specificity to azocasein and different forms of collagen.

Material and methods

Microorganism

The *Penicillium* sp. strain (UCP 1286) isolated from Serra Talhada city soil, in Caatinga biome (Pernambuco – Brazil), were obtained from UCP - the Catholic University of Pernambuco Collection, UNICAP.

Culture medium

The maintenance medium used was malt extract agar containing: malt extract (0.5%), peptone (0.1%), glucose (2%) agar 1.5 (%). The culture medium used for enzyme production is composed of: gelatin (0.5% w/v), $MgSO_4 \cdot 7H_2O$ (0.025 w/v), K_2HPO_4 (1.5 w/v), $FeSO_4 \cdot 7H_2O$ (0.015 w/v), $CaCl_2$ (0.025 w/v) and mineral solution (1% v/v), according Lima et al. (2011b) modified. The mineral solution was prepared by adding, per 100 mL of distilled water, 100 mg $FeSO_4 \cdot 7H_2O$, 100 mg $MnCl_2 \cdot 4H_2O$, 100 mg $ZnSO_4 \cdot H_2O$, and 100 mg $CaCl_2 \cdot H_2O$, and it was used 1% from total volume of fermentation. Both medium were sterilized in autoclave at 121 °C for 15 min.

Kinetic of growth and collagenolytic enzyme production

Inoculum spores were produced in maintenance medium plates containing a cell culture grown for 5 days at 28 °C, and then suspended in 3 mL of a 0.9% (w/v) NaCl and 0.01% (v/v) Tween 80 solution, previously sterilized at 121 °C for 20 min. After inoculation with a 150 µL spores suspension (10^6 spores/mL), fermentation was carried out for 8 days at 28 ºC and 150 rpm in 1 L Erlenmeyer flasks containing 250mL of the culture medium. At 6-hour intervals, 2 mL samples were taken for protein content and collagenolytic activity determination. Cotton caps were used to minimize water evaporation. The broth obtained at the end of fermentation was vacuum filtered through 0.45 µm-pore diameter nitrocellulose membranes to remove the mycelium.

Screening of significant variables for collagenolytic enzyme production

To evaluate the influence of the initial pH, initial concentration of gelatin, temperature and agitation on extracellular collagenolytic enzyme production by *Penicillium* sp. UCP 1286 isolated from Caatinga soil, a 2^4 factorial design was carried out at all combinations of levels given in Table 1.

Table 1

The center point was performed in quadruplicate, to provide an variance estimate of pure experimental error responses. From this, it was predicated the effects of experimental errors and used to assess the significance of the effects and interactions of the independent variables - pH, concentration of carbon and nitrogen source (gelatin), temperature and orbital agitation speed - on the production of collagenolytic enzyme. All statistical and graphical analyzes were performed with 95 % confidence using the Statistica 8.0 software (StatSoft Inc., Tulsa, OK, USA).

Azocoll assay for collagenolytic enzyme activity determination

The Azo dye-impregnated collagen (Azocoll; Sigma Chemical Co., St Louis, MO) assay was carried out according to a modified version of the method developed by Chavira et al. (Chavira et al. 1984). Azocoll was washed and suspended in 0.05 M Tris-HCl buffer (pH 7.2) containing 1 mM $CaCl_2$ up to a final concentration of 0.5% (w/v). Subsequently, 150 µL of cell-free filtrate 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 °C in a water bath under agitation. After 18 h of incubation, the samples were chilled in ice for 5 min to stop the reaction and centrifuged at 10,000 × *g* and 4 °C for 20 min (model KR-20000T; Kubota Seisakusho, Tokyo, Japan). The absorbance of the supernatant was measured at 520 nm by a UV-Vis spectrophotometer (model B582; Micronal, São Paulo, Brazil). One unit of enzyme activity (U) was defined as the amount of enzyme, per millilitre, that led, after 18 h of incubation, to an absorbance increase of 0.1 at 520 nm, because of the formation of azo dye-linked soluble peptides.

Saline precipitation with ammonium sulfate

Crude extract was placed in an ice bath and $(NH_4)_2SO_4$ was added, at different concentrations of saturation (0 – 20%, 20 – 40%, 40 – 60%, 60 – 80% and 80 – 90%), with constant stirring for 2 hours. Precipitated protein was removed by centrifugation at 10.000 rpm, for 15 min at 4 °C and the supernatant was discarded. The precipitated protein was dissolved in 4 mL of 0.05M Tris-HCl buffer of pH 9.0. The 60 – 80% was selected based on its specific collagenolytic activity. Ammonium sulfate was removed using dialysis.

Protein and Biomass determination

Protein concentration was determined by the method of Bradford (1976) modified using the dye as "Coomassie Blue Bright G-250" to detect protein in enzymatic samples. The calibration curve was obtained from stock solutions of bovine serum albumin (BSA).

Total volume of fermentation flasks was filtered for biomass determination. Biomass was determined by the dry weight method using pre-weighted nitrocellulose membranes with 0.45 µm-pore diameter, after drying at 80 ºC for 24 h.

Effects of pH and temperature on collagenolytic enzyme activity and stability

To evaluate pH effect on collagenolytic enzyme activity, the pH of the reaction mixture containing 0.5% (w/v) of azocoll was varied over the range 3.0 ~ 11.0. The buffers used were 0.05 M citrate (pH 3.0 ~ 6.0), 0.05 M Tris-HCl (pH 7.0 ~ 9.0), and 0.05 M carbonate-bicarbonate (pH 10.0 ~ 11.0). For stability tests, the culture filtrated was incubated at 4 ºC (selected as a cold storage reference temperature at which the enzyme thermo inactivation is expected to be very low) in the above buffers at different pH values. The incubation time of samples varied from 1 to 24 h.

An analogous study was done for the effect of temperature in the same azocoll-buffer solution. To determine optimum temperature for enzyme activity, the reaction mixture was incubated at the selected temperature (from 25 to 70 ºC), while for thermo stability tests the enzyme was pre-incubated for 15 ~ 180 min at the same temperatures.

The residual activity was calculated as the ratio between the enzymatic activity, observed at the end of each incubation run, and that at the beginning, and expressed as percentage (%).

Substrate specificity

To test substrate specificity, the proteolytic activity of extracellular collagenolytic enzyme produced by *Penicillium* sp. UCP 1286 and commercial collagenase was also assayed on insoluble collagen (type I and type V), gelatin and azocasein. Azocasein was used as comparison substrate to check on enzyme specificity.

The activity was assayed on insoluble collagen according to Endo et al. (1987). The standard reaction mixture, containing 25 mg collagen (type I and V, from bovine Achilles tendon) in 5 mL of 0.05 M Tris-HCl buffer (pH 7.0), was incubated with 1 mL enzyme samples at 37ºC. The amount of free amino groups released was measured by the ninhydrin method of Rosen (1975). One activity unit (U) was defined as the number of µmol of L-leucine released as a result of the action of 1 mL culture filtrate containing collagenolytic enzyme, after 18 h at 37ºC.

Collagenolytic enzyme activity on gelatin was assayed by the method of Moore and Stein (1948), slightly modified. Reaction was carried out at 37ºC for 18 h after the addition of 0.1 mL of the enzyme solution to 1.0 mL of a solution containing 2 mg gelatin in 0.05 M Tris-HCl buffer (pH 7.5). The reaction was stopped by the addition of 0.1 mL of 10% (w/v) trichloroacetic acid. The medium was centrifuged at 10,000 x g for 10 min. The supernatant (0.2 mL) was mixed with 0.5 mL of ninhydrin solution, heated at 100ºC for 10 min, cooled in ice water for 5 min, and the mixture was then diluted with 2.5 mL of 50% (v/v) 1-propanol. After centrifugation at 12,000 x g for 10 min, the absorbance of the mixture was measured at 570 nm. One unit (U) of enzyme activity was expressed as µmol of L-leucine equivalents released per min.

The enzyme activity on azocasein was determined according to Leighton et al. (1973), with 1% (w/v) azocasein in a 0.1 M Tris-HCl buffer (pH 7.2). One unit (U) of protease activity was defined as the amount of enzyme required to raise the optical density at 440 nm by one unit after 1 h.

Effect of inhibitors

Protease inhibitors effect was investigated following the procedures of the manufacturer's guide of inhibitors. The inhibitors tested were: phenylmethylsulphonyl fluoride (PMSF) for serine proteases, ethylenediaminetetraacetic acid (EDTA) for metalloproteases, and iodoacetic acid (IAA) for cysteine proteases, at the concentration of 10 mM. For sensitivity determination, the enzyme was pre-incubated for 30 min at 37ºC with the inhibitors. The residual activity was determined as the percentage of the proteolytic activity in an inhibitor-free control sample. After all, proceeded collagenolytic activity using the azocoll method.

Polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were analyzed by SDS-PAGE in a 15% polyacrylamide gel, according to Laemmli (1970). The protein molecular markers were phosphorylase b (97.0 kDa), bovine serum albumin (66.0 kDa), ovalbumin (54.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). The gel was loaded with 20 μL of concentrated enzyme by lyophilization, and subject to electrophoresis at a constant current of 100 V. Gel was stained with 0.25% (w/v) Comassie Brilliant Blue R-250 in methanol-acetic acid- water (45:10:45) and distained in the same solution without dye.

Zymogram

Proteolytic activity of enzyme bands was confirmed by zymogram analysis. To prepare a zymogram, concentrated enzyme was mixed under non-reducing conditions with SDS-PAGE sample buffer and electrophoresed in a 15% polyacrylamide with 0.1% (w/v) gelatin as substrate incorporated in the gel. Gel was loaded with 20 μL of concentrated supernatant, subject to electrophoresis at a constant current of 100 V at 4°C and incubated for 1 h at room temperature with 2.5% (v/v) Triton X-100 and for 18 h at 37 °C in 50 mM Tris-HCl buffer, pH 9.0. Gel was stained and distained as described in the previous section.

Results

Enzyme production kinetics

Figure 1 shows the results of enzyme kinetics production. At 96 hours it can be observed a sharp increase in the enzyme activity. Collagenolytic enzyme reached its maximum activity after 126 hours of fermentation (379.80 U/mL). Based on results, the incubation time used for further study was 126 hours. It can be observed a comparison between total protein in the culture broth and collagenolytic activity over fermentation time at Figure 1. The specific activity of collagenolytic enzyme was calculated as 1460.77 U/mg after 126 hours.

Figure 1

2^4 Factorial Design

Table 2 shows fermentations conditions and results after 126 hours of production. The highest values of collagenolytic activity (632.70 U/mL) and specific activity (3954.38 U/mg) were obtained in run 9, conducted using 0.25% (w/v) gelatin, 200 rpm, pH 6.0 and 24°C.

Table 2

A full factorial model was designed to compare the activity data. This model included four main effects, six two-factor, four three-factor and one four-factor interactions. The statistically significant estimates of the effects (at the 95% confidence level) are listed of Table 3. The values of the significant effects indicate that, on average, higher activities were obtained when factors 1 (pH) and 3 (temperature) were selected at their lowest levels. With respect to the effect of the factors on the biomass concentration, the gelatin concentration was the only one that presented positive significant effect.

Table 3

Effect of pH on collagenolytic activity and stability

Figure 2(A) shows the pH dependence of collagenolytic activity in azocoll. The enzyme was found to be very active between pH 7.0 and 9.0. At pH 6.0, only 20% of the activity remained, and the results were even worse for the most acidic conditions (pH 3.0 to 5.0) and more basic (pH 10 and 11). Figure 2(B) shows the stability at pH between 3.0 and 11.0, during 24 hours of incubation at 4 °C, the selected storage temperature. The enzyme was quite stable between pH 7.0 and 9.0, keeping about 85 to 90% of stability, after 24 hours. With the pH 5.0 and 6.0, the residual activity decreased to 70%. In others values of pH, the activity was below 30%.

Figure 2

Effect of temperature on collagenolytic activity and stability

To find optimum temperature for enzyme action, collagenolytic activity was measured at different temperatures between 25 and 75°C. Figure 3(A) shows that the highest value is observed at 37 °C. At 45°C, approximately 85% of the enzymatic activity still remained, and then a gradual decrease occurs until almost all activity is lost at 75 °C.

Figure 3(B) shows the results of enzyme stability over 6 hours at the different temperatures evaluated. A gradual decrease on enzyme stability was observed for 25 and 37 °C, while for the other temperatures considered, after the first 30 minutes, no collagenolytic activity was observed.

Figure 3

Substrate specificity

Five protein substrates were tested (azocoll, type I collagen, type V collagen, gelatin and azocasein). Besides azocasein, the culture filtrate showed activity on all of the substrates. Higher enzyme activity was observed with azocoll as substrate (692.65 U/mL and 3463.25 U/mg). The enzyme´s activity towards azocoll was

assumed as 100% (Figure 4). The relative activity on other substrates was lower, but it can be observed that the enzyme produced by *Penicillium* sp. UCP 1286 has much higher affinity for collagen-derived substrates when compared to noncollagen-derived substrate azocasein.

Figure 4

Commercial enzyme produced by *Clostridium hystolyticum* exhibits highest affinity for collagen type I (32.22% relative activity) when compared to the collagenolytic enzyme produced in this work, that presented higher relative values for type V collagen (46.90%) and gelatin (22.41%) (Figure 4).

Effects of inhibitors

The culture filtrates obtained from *Penicillium* sp. UCP 1286 was subjected to inhibition by phenylmethylsulfonyl fluoride (PMSF), which is a method to establish whether or not one enzyme is a serine protease. After 30 minutes of incubation at 37 °C in the presence of 10 mM of PMSF, the enzyme activity was reduced to 0, whereas in the presence of 10 mM iodoacetic acid (that inhibits cysteine proteases) there was maintenance of a residual activity of 88.31%. The collagenolytic enzyme was slightly inhibited by EDTA, known to inhibit metalloproteinases (a loss of only about 6% of activity was observed).

Electrophoresis and Zymogram

Electrophoresis and zymogram of the culture filtrate and 60-80% fraction from saline precipitation are presented in Figure 5A and 5B, respectively. The electrophoresis presented a major band corresponding to a molecular mass (MM) of approximately 37 kDa, while others proteins can be observed at 28 and 29 kDa (Figure 5A).

Figure 5

The zymogram analysis (Figure 5B) confirmed the bands collagenolytic activity. It can be observed that the 60-80% fraction showed 4 bands at the lowest protein volume concentration applied (10 μg), with approximately 140 kDa, 120 kDa, 100 kDa and 37 kDa with activity towards gelatin.

Discussion

Microorganism

Table 4 reports data from earlier work related to the production of collagenolytic enzyme by different microorganisms. Among some fungi and bacteria, it can be seen that the *Penicillium* genus provides the highest values. Thus, this genus was selected for enzyme production. Moreover, there are few reports in the literature describing the production of collagenase by *Penicillium*.

Table 4

It must also be pointed out that the values for collagenolytic activity reported on this work are about 2.7 times higher than existing data. The composition of the culture medium is another aspect that must noticed. It is known that the costs of culture media and substrates are key factors on the production of industrial enzymes being thus necessary to develop processes that make use of inexpensive and easy to prepare substrates (Lima et al. 2011b). The use, as in the present work, of a low cost and readily available substrate, as is the case of gelatin and trace elements, may be an important step in the advancement of these processes.

Enzyme production kinetics

At Figure 1, after 126 hours it can be observed a gradual decrease in the biosynthesis of the enzyme, probably due to a period of microorganism adaptation to the medium containing only salts and gelatin or due to depletion of nutrients. The production effectiveness was evaluated by volumetric collagenolytic activity due to the industrial relevance of this parameter (Lima et al. 2011b).

In addition, the enzyme concentration remained constant (a slight increase was observed) for the first 84 hours, most likely caused by the fungus need to get adapted to the poor culture medium, as compared to the other media listed in Table 4.

The maximum specific collagenolytic activity was obtained also at 126 hours of fermentation (1460.77 U/mg) (Figure 1). This value is much higher than other published results. Baehaki et al. (2014) achieved a specific activity of 0.546 U/mg, with *Bacillus licheniformis*; Jain and Jain (2010) found 15.66 U/mg of specific activity by *Streptomyces exfoliates*, Wu et al. (2010) obtained 2.77 U/mg using *Bacillus pumilus*, Mahmoud et al. (2007), 97.12 U/mg with *Aspergillus flavus* and Ok and Hashinaga (1996), 70.4 U/mg with *Zygosaccharomyces rouxii*, all of them with more than 15 times lower activity than the produced by *Penicillium* sp. UCP 1286 .

The literature describes the importance of defining parameters that have a significant influence on the extracellular enzyme production by microorganisms - not only the composition of the culture medium as a carbon and nitrogen sources and trace elements should be considered but also the culture conditions such as pH, temperature and stirring speed (Lima et al. 2009).

2^4 Factorial Design

Table 2 shows the results of the performed experimental design, where the final fermentation time was 126 hours, which is in accordance with the previously determined production kinetics. The factorial design increased the enzyme production by about 65% compared to the preliminary results achieved on the kinetics experiments, obtaining a collagenolytic activity of 632.70 U/mL.

According to Jain and Jain (Jain e Jain 2010), the maximum production obtained for *Streptomyces exfoliates* (43.5 U/mL) was observed after 5 days of culture at 30 °C and 150 rpm. Lima et al. (2009) conducted a 2^3 full factorial for the production of collagenase with *Candida albicans* and found the highest value (7.6 U/mL) with a 2% substrate concentration, agitation of 160 rpm and pH 7.0. For the case of *Penicillium aurantiogriseum*, Lima et al. (2011a) reported that the highest values of collagenolytic activity (164 U/mL) and biomass concentration (1.8 g/L) were obtained with 0.75% substrate concentration, 200 rpm, pH 8.0 and 28°C. The results described in this paper show that the concentration of substrate (0.25%) used for the

maximum production of collagenolytic enzyme (632.70 U/mL) was lower than those reported in the literature, which can represent an economy in production. With respect to the agitation, the results were similar to those related by Lima et al. (2011a) (200 rpm). Temperature found in present work was lower than others papers (24 °C), and pH was slightly higher (9.0) showing that maximum enzyme production conditions are milder than those reported.

Statistical analysis of the experimental design is listed in Table 3. The results show that only two factors were statistically significant: (1) pH and (3) temperature, both with negative effects. Therefore, as lower their levels, the higher the values of collagenolytic activities.

Temperature is an important factor in regulating enzymatic synthesis (Chellapan et al. 2006). In a study reporting the production of collagenase by *Penicillium aurantiogriseum*, the temperature also had a negative effect (Lima et al. 2011a). Since the initial pH of the culture medium influences many enzymatic processes, enzyme production, cell transport across membranes and expression of extracellular proteases, we observed, a gradual decrease in collagenase production when the external pH was raised from 6 to 8. Suphatharaprateep et al. (2011) reported that the optimal initial pH for collagenase production from *Klebsiella pneumonia* and *Bacillus cereus* were 7.5 and 6.0, respectively.

Regarding the effect of the different factors on biomass concentration, only the concentration of gelatin (2) proved to be statistically significant. Jain and Jain (2010) showed that the maximum collagenase production (43.50 U/mL) was observed with higher biomass concentration (5.60 cm^3 of cells). The initial pH of the medium was shown by Lima et al. (2011a) to be the factor that exhibited a significantly higher effect on the biomass, with an increase in pH causing a decrease in cell growth.

With a 2^4 experimental design, it was possible to establish fermenting conditions that allowed for a 66% increase in the enzyme activity as compared to the initially obtained values. It is observed that maximum collagenolytic production occurred when lowest concentration of substrate and temperature were utilized, which facilitates industrial process, reducing costs.

Enzyme characterization

Concerning pH effect on collagenolytic activity, Jain and Jain (2010) determined the collagenase activity from *Streptomyces exfoliatus* at different pH (4-10) and found that the enzyme retained 65% residual activity at pH 10 as compared to its optimum activity at pH 7. The collagenase from *Klebsiella pneumoniae* had an optimum pH range from 6.0 to 8.0, with maximal activity at 7.0 in Tris-HCl buffer while *Bacillus cereus* collagenase activity was recorded as the highest in the pH range of 5.4-8.2 (Suphatharaprateep et al. 2011). The collagenase from *Bacillus pumilus* (Wu et al. 2010) was evaluated in a range from 3 to 10 being the highest activity at pH 7.5. Baehaki et al. (2014) used a pH range between 2 and 12 and observed the maximum activity at pH 9.0. Lima et al. (2011a) showed that the enzyme produced by *P. aurantiogriseum* was very active at the pH range 8 to 10, and the highest activity occurred at pH 9.0, as in the present work. Ok and Hashinaga (1996) related that optimum pH to collagenase produced by *Zygosaccharomyces rouxii* was 8.2. Only the enzyme produced by *Rhizoctonia solani* was produced under acid pH (5.0) (Hamdy 2008). The results described at present work are in agreement with those reported in literature that show that collagenases exhibit optimum activity values under neutral or alkaline conditions (Sakurai et al. 2009; Lima et al. 2009). These results indicate that this collagenolytic enzyme belongs to the group of alkaline proteases.

Lima et al. (2011a) evaluated collagenase stability at pH range 3.0 to 11.0. The enzyme was stable between pH 6.0 and 10.0 during the first 8 h, but after 28 h its stability was restricted to a pH range from 7.0 (50.7%) to 9.0 (75.1%). In accordance with Wu et al. (2010), collagenase from *B. pumilus* is stable between pH 6.5 and 8, the enzyme retained above 84% of full activity after 30 min. By retaining more than 80% of its stability at pH 8 and 9, the enzyme described in the present work proved to be very stable in comparison with the collagenase produced by other microorganisms.

Comparing results of temperature assays, Wu et al. (2010) found for *B. pumilus* collagenase an optimum temperature of 45 °C, and with heating, the collagenase retained above 50% activity at 70 °C. Baehaki et al. (2014) assayed the collagenase activity between 30 and 90 °C, being observed an increase in enzymatic activity between 30 and 50 °C, with a strong reduction occurring above 60 °C. *S.*

exfoliatus showed the maximum collagenase activity at 70 °C (Jain e Jain 2010). In the study of Suphatharaprateep et al. (2011), both *K. pneumoniae* and *B. cereus* were shown to produce collagenases with the optimal temperature of 37 °C. Hamdy (2008) related optimal temperature for *R. solani* collagenase at 40 °C. For *P. aurantiogriseum* collagenase, the optimal temperature was also 37 °C; at temperature lower or higher than 37 °C, the collagenase production decreased, as well as in the present work (Lima et al. 2011a).

Figure 3B shows collagenase stability to temperature. At 25 and 37 °C, the enzyme retained about 90% and 60% of its enzymatic activity after 1.5 hours of incubation. After this period, the values were gradually decreased until reaching 15% at 37 °C after 6 hours and about 10%, at 25 °C after 5.5 hours. Regarding the others temperatures, may have occurred protein denaturation.

The enzyme from *P. aurantiogriseum* was stable after 1.5 h incubation in the temperature range 25 to 45 ºC, retaining 96.2, 96.3, and 81.6% of its initial activity at 25, 37, and 45 ºC, respectively while at 70 ºC its activity was completely lost after only 15 min (Lima et al. 2011a). The results for a collagenase from *B. licheniformis* showed that the enzyme remained relatively stable and retained above 50% activity under 20 min incubation at 50 and 70 °C. However, the enzyme activity decreased gradually after 20 min incubation (Baehaki et al. 2014).

The action of collagenase is very specific, acting only on collagen and gelatin and not on any of the other usual protein substrates (Rao et al. 1998). Lima et al. (2011a) showed that *P. aurantiogriseum* collagenase highest activity was also obtained with azocoll (164.00 U/mL and 393.00 U/mg). Hamdy (2008) related specificity of collagenase produced by *R. solani* to gelatin and collagen (33.23 U/mg and 120 U/mg, respectively). Ok and Hashinaga (1996) tested collagenase activity using soluble (27.1 U/mL) and insoluble collagen (5.6 U/mL), besides synthetic peptides as Cbz-GPLGP (21.1 U/mL) and FALGPA (0.41 U/mL).

The collagenase produced by *B. licheniformis* exhibited the highest activity on casein, being also able to hydrolyze collagen, gelatin and fibrin (Baehaki et al. 2014). For *B. pumilus* collagenase, in addition to hydrolyzing the native collagen from bovine Achilles tendon, it was also able to act on gelatin, with a cleavage rate of 50.72% and 62.56%, respectively (Wu et al. 2010).

Among the collagen types, type V is classified as a member of the family of fibrillar collagens, based on their primary structure and their potential to form fibrils in

the interstice (Kiriviko e Mlyllyla 1984; Adachi e Hayashi 1985). This type of collagen has been described as involved in maintenance processes of vascular injury in patients with certain diseases, such as systemic lupus erythematosus and vasculitis (Moreland et al. 1991). More specific studies are required to better understand the action of the enzyme produced by *Penicillium* sp. UCP 1286 against type V collagen, but already indicates a potential biotechnological application for this protease (Figure 4).

For further characterization of the enzyme, the culture filtrate was tested against enzyme inhibitors. Enzyme inhibitors are molecules that interact with the enzyme or compounds that chelate metal ions required by the enzyme to maintain its conformation (Daboor et al. 2010). In particular, the conditions selected for tests with PMSF were consistent with the observations of James (1978), and those for tests with EDTA according Hamdy (2008). Although more tests for a complete characterization of the enzyme are required, the preliminary results suggest that the enzyme belongs to the family of collagenolytic serine proteases, since it was completely inhibited by their reversible inhibitor of serine proteases.

The collagenolytic enzyme from *P. aurantiogriseum* kept only 24% on its activity in presence of PMSF, and retained 100 and 93.6% of its activity in the presence of iodoacetic acid and EDTA (Lima et al. 2011a). The enzyme produced by *Bacillus pumilus* was strongly inhibited by EDTA (Wu et al. 2010). As in the present work, Jain and Jain (2010) showed that the collagenase produced by *S. exfoliates* completely lost its activity in the presence of PMSF and retained 65.91% in the presence of EDTA. Mahmoud et al. (2007) tested inhibition of collagenase produced by *A. flavus* using cetrimide, a reversible competitive inhibitor against collagenase, and collagenolytic activity was 0 with 1 mg/mL of cetrimide. The results of Hamdy (2008) showed that collagenase by *R. solani* was inhibited by EDTA (31% of relative activity), iodoacetate (18%) and sodium arsenite (13%).

Electrophoresis was used for collagenolytic enzyme characterization, estimating its molecular weight. Reported molecular weights vary significantly based on the enzyme type (serine or metallocollagenase) and the source (microbial or animal tissue) (Daboor et al. 2010).

At zymogram was observed 4 bands with approximately 140 kDa, 120 kDa, 100 kDa and 37 kDa (Figure 5B). Multiple collagenases have been reported at literature by zymography (Baehaki et al. 2012; Baehaki et al. 2014).

Roy et al. (1994) reported that serine collagenases have, typically, molecular weights in the range of 24 and 36 kDa. Baehaki et al. (2014) found several protein bands and zymography analysis indicated that the molecular mass of collagenase fractions were approximately 124, 35, 31 and 26 kDa from *B. licheniformis*. Baehaki et al. (2012) reported multiple collagenases in range 14.5 – 210 kDa produced by *B. licheniformis*. Sakurai et al. (2009) produced a purified collagenase from *S. parvulus* with a relative molecular mass of 52 kDa. Matsushita et al. (1994) reported that collagenases isolated from related species of *C. perfringens* had molecular weights ranging from 80 to 120 kDa. Mahmoud et al. (2007) related a collagenase produced from *A. flavus* with molecular weights between 72 and 92 kDa. Hamdy (2008) produced a purified collagenase from *R. solani* that showed a molecular weight at 66 kDa.

Some researchers (Kristjánsson et al. 1995; Sivakumar et al. 1999) isolated serine collagenases from digestive glands of marine organism with molecular weights < 60 kDa. The wide range of molecular weight is to be expected for an enzyme such as collagenase that does not have a single structure (Daboor et al. 2010).

Conclusions

The *Penicillium* sp. UCP 1286 fungus isolated from Caatinga was shown to produce large amounts of extracellular collagenase, using only gelatin as carbon and nitrogen source. Moreover, the activity of produced collagenolytic enzyme was much higher that other microbial production systems reported in the literature. This makes this production system as a very promising alternative for collagenase production as it associates a high producer microbial with the use of an inexpensive and readily available substrate.

Complementary, optimal enzyme production conditions were established, with the temperature showing the greatest effect. The enzymes was seems to be a serine alkaline protease, having the optimal collagenolytic activity at 37°C and pH 9.0.

Concerning specificity, the produced enzyme hydrolyses different types of collagen, including azocoll, type I, type V and gelatin. The data indicates that the produced enzyme presents a higher affinity to type V collagen and gelatin, when compared to commercial colagenase. Also, low azocasein activity indicates a collagen specificity of this produced enzyme, desirable property for many applications.

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Ethical Statement/Conflict of Interest

The authors are aware of the ethical responsibilities and the manuscript has no conflict of interest.

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Figure legends

Figure 1. Collagenolytic activity and total protein content produced by *Penicillium* UCP 1286 isolated from Caatinga in gelatin culture medium.

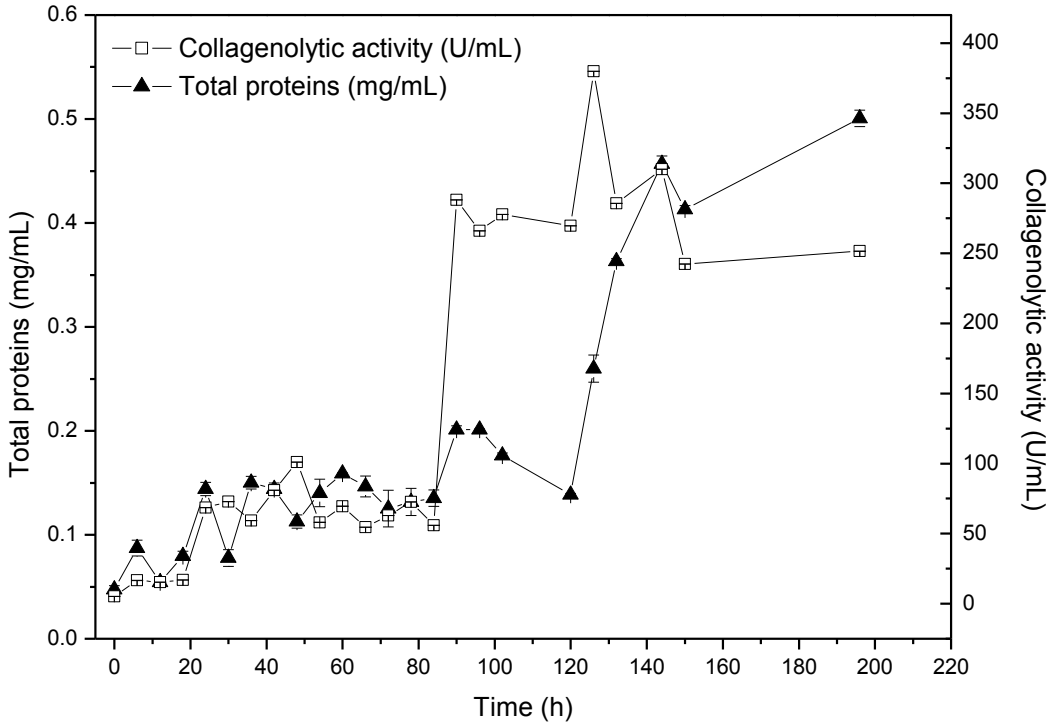
Figure 2. (A) Effect of pH on the activity of extracellular collagenase produced by *Penicillium* sp. UCP 1286 isolated from Caatinga, expressed as percentage of the maximum one obtained in 0.05 M Tris-HCl buffer (pH 9.0). (B) Effect of pH on the stability of extracellular collagenase produced by *Penicillium* sp. UCP 1286 isolated from Caatinga, expressed as the residual activity with respect to that at the beginning. Each value is the average of results of three experiments, and the error bars show the standard deviations.

Figure 3. (A) Effect of temperature on the activity of extracellular collagenase produced by *Penicillium* sp. UCP 1286 isolated from Caatinga, expressed as percentage of the maximum one obtained at 37°C. (B) Effect of temperature on the stability of extracellular collagenase produced by *Penicillium* sp. UCP 1286 isolated from Caatinga, expressed as the residual activity with respect to that at the beginning. Each value is the average of the results of three experiments, and the error bars show the standard deviations.

Figure 4. Substrate specificity of *Penicillium* sp. UCP 1286 and Commercial Collagenase (produced by *Clostridium hystolyticum*). The enzyme´s activity towards azocoll was assumed as 100%.

Figure 5. (A) SDS-PAGE patterns of *Penicillium* UCP 1286 crude extract (Cr) and 60-80% fraction obtained from precipitation with ammonium sulfate (F). MM: molecular mass. (B) Zymogram analysis of collagenase.

Figures



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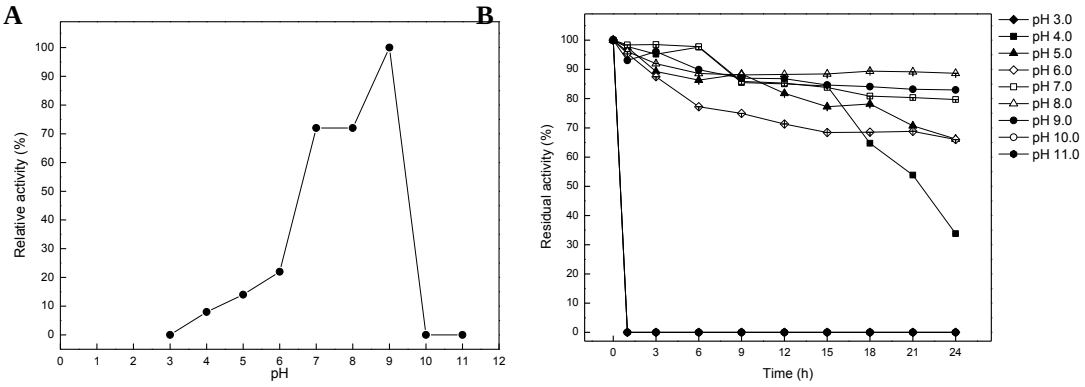

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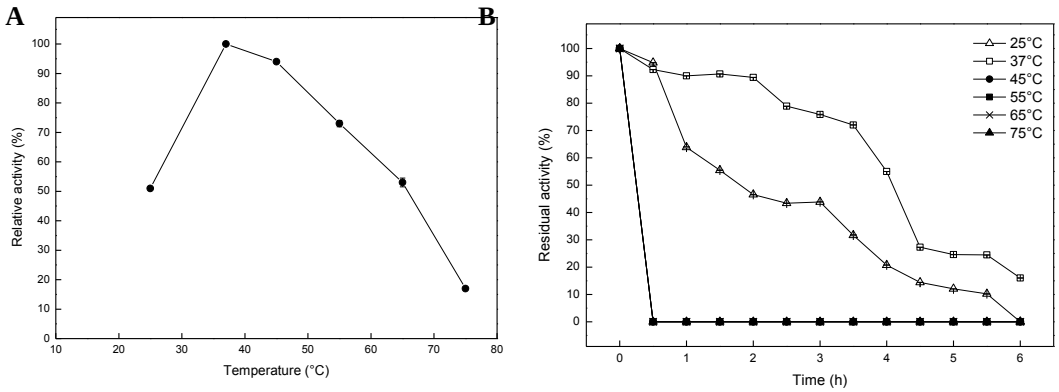

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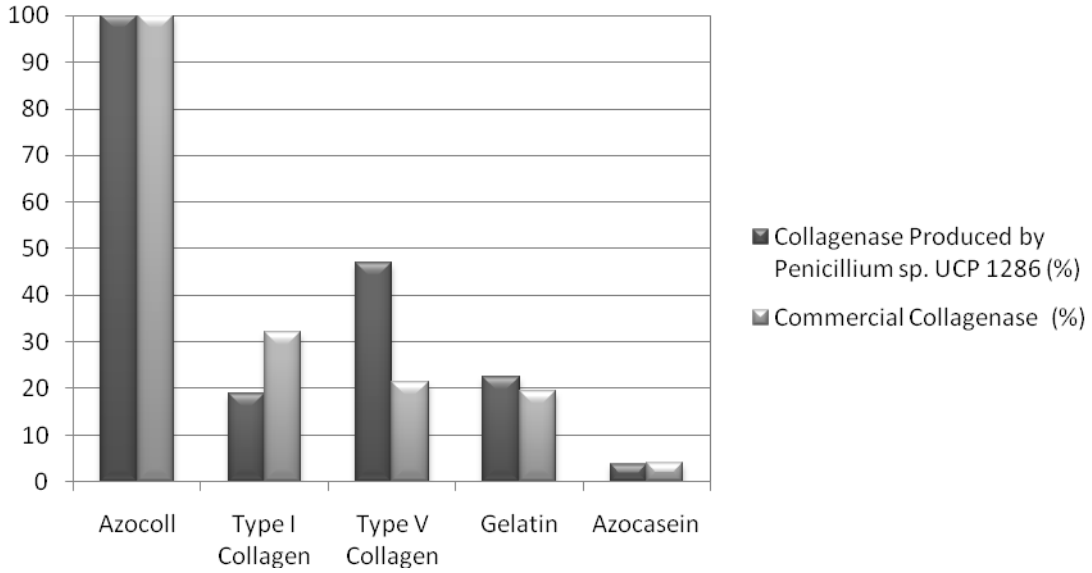

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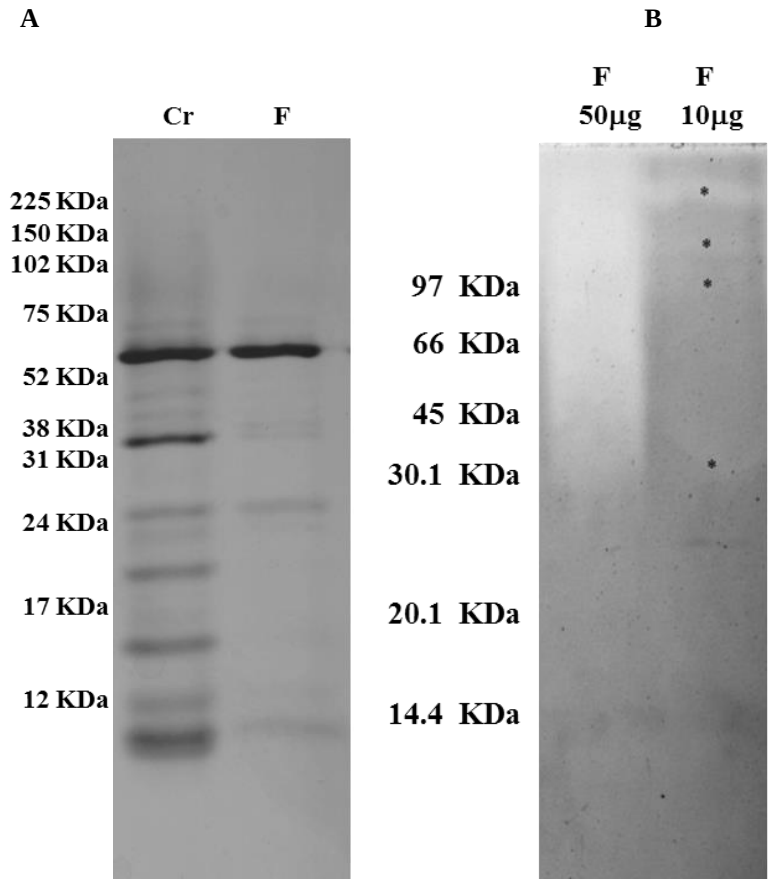

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Tables

Table 1. Factors levels used in 2^4 design to investigate the production of collagenolytic enzyme by *Penicillium* sp. isolated from Caatinga soil

Factors	Level		
	Lowest (–1)	Central (0)	Highest (+1)
pH	6.0	7.0	8.0
Gelatin concentration (%, w/v)	0.25	0.50	0.75
Temperature (°C)	24	28	32
Orbital agitation speed (rpm)	100	150	200

Table 2. Conditions and results of fermentations conducted according to the 2^4 factorial design

Run	pH	S_o (% w/v)	T (°C)	Agitation (rpm)	X (g/L)	TP (mg/mL)	A_c (U/mL)	a_c (U/mg)
1	6	0.25	24	100	0.48	0.15	531.55	3603.73
2	8	0.25	24	100	0.36	0.18	481.95	2744.20
3	6	0.75	24	100	0.80	0.25	447.65	1786.13
4	8	0.75	24	100	0.92	0.24	481.70	1996.68
5	6	0.25	32	100	0.53	0.13	560.20	4351.07
6	8	0.25	32	100	0.17	0.24	177.70	746.25
7	6	0.75	32	100	0.62	0.26	396.65	1544.14
8	8	0.75	32	100	0.72	0.19	349.50	1798.07
9	**6**	**0.25**	**24**	**200**	**0.45**	**0.16**	**632.70**	**3954.38**
10	8	0.25	24	200	0.30	0.16	471.20	2888.58
11	6	0.75	24	200	0.98	0.36	475.10	1319.72
12	8	0.75	24	200	0.40	0.36	424.15	1188.51
13	6	0.25	32	200	0.48	0.12	468.05	3920.84
14	8	0.25	32	200	0.51	0.15	424.10	2815.60
15	6	0.75	32	200	0.53	0.32	405.95	1271.08
16	8	0.75	32	200	0.38	0.20	338.65	1662.09
17	7	0.5	28	150	0.77	0.22	427.95	1923.37
18	7	0.5	28	150	0.75	0.23	424.85	1882.99
19	7	0.5	28	150	0.72	0.24	432.90	1842.13
20	7	0.5	28	150	0.74	0.23	428.80	1874.54

Results related to 126 hours of fermentation. pH = initial pH of the medium; S_o = initial concentration of gelatin; T = temperature; X = biomass concentration; TP = total protein; A_c = volumetric collagenolytic activity; a_c = specific collagenolytic activity.

Table 3. Statistically significant main effects and interactions estimated from the collagenolytic activity and biomass concentration values listed in Table 2

Factors	Effects on collagenolytic activity	Effects on biomass concentration
(1) pH	-96.11*	-0.14
(2) Gelatin concentration	-53.51	0.25*
(3) Temperature	-103.15*	-0.09
(4) Agitation	26.62	-0.07
1 by 2	63.27	0.01
1 by 3	-39.11	0.04
1 by 4	15.19	-0.07
2 by 3	18.70	-0.12
2 by 4	-34.54	-0.12
3 by 4	11.55	0.04

*Significant

Table 4. Description of work reported in the literature relating to the production of collagenolytic enzymes

Microrganism	Culture Medium	Time (h)	A_c (U/mL)	References
Aspergillus flavus	Collagen-dependent-medium	144	82.95	Mahmoud et al. (2007)
Bacillus cereus	Glycerol and gelatin	24	23.07	Suphatharaprateep et al. (2011)
Bacillus licheniformis	Yeast extract and collagen	N.I.	3.10	Baehaki et al. (2012)
Bacillus licheniformis	Luria Broth and collagen	35	0.546*	Baehaki et al. (2014)
Bacillus pumilus	Gelatin and Peptone	24	35.97	Wu et al. (2010)
Bacillus subtilis	Yeast extract and gelatin	14	3.07	Tran and Nagano (2002)
Candida albicans	Malt extract and gelatin	72	5.00	Lima et al. (2009)
Klebsiella pneumoniae	Glycerol and gelatin	24	10.53	Suphatharaprateep et al. (2011)
Penicillium aurantiogriseum	Soybean flour	72	164.00	Lima et al. (2011a)
Penicillium aurantiogriseum	Soybean flour	72	231.00	Lima et al. (2011b)
Rhizoctonia solani	Sabouraud, glucose and collagen	108	212.33	Hamdy (2008)
Streptomyces exfoliatus	Soybean flour	120	43.50	Jain and Jain (2010)
Zygosaccharomyces rouxii	Yeast extract, peptone and glucose	50	70.4	Ok and Hashinaga (1996)
Penicillium sp.	Gelatin	126	632.70	Present work

A_c = collagenolytic activity (U/mL), *Specific activity (U/mg), N.I. = not informed

7 CAPÍTULO IV

Extraction and partial characterization of a collagenase from *Penicillium* sp. UCP 1286 in poly(ethylene glycol)-phosphate aqueous two-phase system

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Extraction and partial characterization of a collagenase from *Penicillium* sp. UCP 1286 in poly(ethylene glycol)-phosphate aqueous two-phase system

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Extraction and partial characterization of a collagenase from *Penicillium* sp. UCP 1286 in poly(ethylene glycol)-phosphate aqueous two-phase system

Abstract

Collagenases are proteolytic enzymes capable of degrading both the native and denatured collagen and have many applications in industry, medicine and biotechnology. The liquid-liquid extraction using two-phase systems aqueous (ATPS) is one of the most promising processes bioseparation which can be used in the initial stages of the purification process, substituting the difficult solid-liquid separations, or even in steps of subsequent purification. ATPS offer many advantages including low-processing time, low-cost material, and low-energy consumption. The collagenase produced by *Penicillium* sp. UCP 1286 showed a stronger affinity for the bottom phase, where the highest collagenolytic activity values were observed at runs on center point, using PEG 3350 g/mol at 15.0% (w/w) concentration, and phosphate at pH 7.0 and concentration 12.5% (w/w). Enzyme was characterized stable to pH and temperature, and SDS/PAGE showed that purification was efficient.The enzyme was seemed to be a serine protease, having the optimal collagenolytic activity at 37 °C and pH 9.0.

Keywords: collagenolytic enzyme, collagen, ATPS, purification

1 Introduction

Collagen is composed of three peptide chains connected by a triple helical structure, found in connective tissues of animals and composing about 30% of the protein in the human body (Di Lullo et al. 2002; Müller 2003). Collagenases are proteolytic enzymes capable of degrading both the native and denatured collagen, producing small fragments (Tran e Nagano 2002; Hamdy 2008; Wu et al. 2010). Collagenases are related to physiological and pathological processes and have many applications in industry, medicine and biotechnology (Ravanti e Kahari 2000; Wu et al. 2010; Lima et al. 2011a; Lima et al. 2013).

There are several methods described in the literature for separation and purification of proteases. Ultrafiltration, precipitation and chromatography are common methods (Li et al. 2006). However, for industrial scale, such procedures are considered viable due to high cost and long processing time (McMaster 2007). Thus, the search for alternative manufacturing methods of purification of proteases has become increasingly common (Yavari et al. 2013). The liquid-liquid extraction using two-phase systems aqueous (ATPS) is one of the most promising processes bioseparation which can be used in the initial stages of the purification process, substituting the difficult solid-liquid separations, or even in steps of subsequent purification (Rosso et al. 2012).

The ATPS were one of the potential alternatives for employment in industry. ATPS comprise a mixture of two polymers or a polymer and a salt, which is separated into two phases in an aqueous medium. The advantages of these systems include the processing time, low material cost and low power consumption (Yavari et al. 2013). ATPS have been used to develop bioprocesses for the recovery and purification of many biological products including proteins, genetic material, bionanoparticles, cells and organelles (Asenjo and Andrews 2011).

ATPS have been used in partitioning and recovering a several molecules as human antibodies (Azevedo et al. 2009), catalase (Kavakçioğlu e Tarhan 2013), polygalacturonases (Maciel et al. 2014), citrinin (Pimentel et al. 2013), xylanase (Rahimpour et al. 2007), lipase (Ramakrishnan et al. 2016), elastase (Xu et al. 2005), alkaline protease (Yavari et al. 2013), fibrinolytic proteases (Silva et al. 2013), and others. The ATPS formed by PEG and phosphate has been described in collagenase extraction due to the fact that PEG have favorable physical properties, particularly as

regards viscosity and density difference between the phases (Rosso et al. 2012; Lima et al. 2013).

The aim of this work was to extract and purify collagenase produced by *Penicillium* sp. UCP 1286, isolated from the soil of Caatinga (Pernambuco – Brazil), and determine the best conditions for the purification of the enzyme using ATPS. For this purpose, a 2^4 full factorial design was applied to the process to characterize the optimal levels of PEG molar mass, pH, phosphate and PEG concentrations. Furthermore, collagenase extracted was characterized.

2 Material and methods

2.1 Microorganism

The *Penicillium* sp. strain (UCP 1286) isolated from Serra Talhada city soil, in Caatinga biome (Pernambuco – Brazil), were obtained from UCP - the Catholic University of Pernambuco Collection, UNICAP.

2.2 Culture medium

The maintenance medium used was malt extract agar containing: malt extract (0.5%), peptone (0.1%), glucose (2%) agar 1.5 (%). The culture medium used for enzyme production is composed of: gelatin (0.5% w/v), $MgSO_4 \cdot 7H_2O$ (0.025 w/v), K_2HPO_4 (1.5 w/v), $FeSO_4 \cdot 7H_2O$ (0.015 w/v), $CaCl_2$ (0.025 w/v) and mineral solution (1% v/v), according Lima et al. (2011b) modified. The mineral solution was prepared by adding, per 100 mL of distilled water, 100 mg $FeSO_4 \cdot 7H_2O$, 100 mg $MnCl_2 \cdot 4H_2O$, 100 mg $ZnSO_4 \cdot H_2O$, and 100 mg $CaCl_2 \cdot H_2O$, and it was used 1% from total volume of fermentation. Both medium were sterilized in autoclave at 121 °C for 15 min.

2.3 Azocoll assay for collagenolytic enzyme activity determination

The Azo dye-impregnated collagen (Azocoll; Sigma Chemical Co., St Louis, MO) assay was carried out according to a modified version of the method developed by Chavira et al. (Chavira et al. 1984). Azocoll was washed and suspended in 0.05 M Tris-HCl buffer (pH 7.2) containing 1 mM $CaCl_2$ up to a final concentration of 0.5% (w/v). Subsequently, 150 µL of cell-free filtrate 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 °C in a water bath under agitation. After 18 h of incubation, the samples were chilled in ice for 5 min to stop the reaction and centrifuged at 10,000 × *g* and 4 °C for 20 min (model KR-20000T; Kubota Seisakusho, Tokyo, Japan). The absorbance of the supernatant was measured at 520 nm by a UV-Vis spectrophotometer (model B582; Micronal, São Paulo, Brazil). One unit of enzyme activity (U) was defined as the amount of enzyme, per millilitre, that led, after 18 h of incubation, to an absorbance increase of 0.1 at 520 nm, because of the formation of azo dye-linked soluble peptides.

2.4 Protein determination

Protein concentration was determined by the method of Bradford (1976) modified using the dye as "Coomassie Blue Bright G-250" to detect protein in enzymatic samples. The calibration curve was obtained from stock solutions of bovine serum albumin (BSA).

2.5 Aqueous two-phase systems

A 40% (w/w) phosphate buffer solution was prepared according Lima et al. (2013), at room temperature (25 ± 1 °C), by mixing the amounts of dibasic and monobasic sodium phosphates needed to reach the desired pH. The required amounts of this solution were mixed with 60% (w/w) PEG solutions with different molar masses, specifically 1500, 3350 and 8000 g/mol. These solutions were then added to 15 mL graduated tubes. Aliquots of the fermented broth corresponding to 20 % (w/w) of the total mass were later added, along with enough water to give a 10 g system. After 1.0 min vortex shaking, the two phases were left to separate by settling for 120 min. The phase volumes were then measured, and the protein concentration and collagenase activity were determined in each phase. To avoid any PEG or phosphate interference, all the samples were analyzed against protein-free standard solutions having the same phase composition.

Table 1. Factor levels of the 2^4-full factorial design used to investigate collagenase partition and purification by ATPS

Factor	Level		
	Low (-1)	Center (0)	High (+1)
PEG molar mass (M_{PEG})	1500	3350	8000
PEG concentration (C_{PEG})	12.5	15.0	17.5
Phosphate concentration (C_{PHOS})	10.0	12.5	15.0
pH	6.0	7.0	8.0

2.5 Determinations of the partition coefficient, activity yield and purification factor

The collagenase partition coefficient was determined as the ratio of the collagenase activity in the top phase (A_T) to that in the bottom phase (A_B):

$$K = \frac{A_T}{A_B}$$

The activity yield was defined as the ratio of A_B to the initial activity in the fermentation broth (A_F) and expressed as a percentage:

$$Y = \left(\frac{A_B}{A_F}\right) x\ 100$$

The purification factor was calculated as the ratio of the specific activity in the bottom phase (A_B/C_B) to the initial specific activity in the fermentation broth before partition (A_F/C_F):

$$PF = \frac{A_B/C_B}{A_F/C_F}$$

where C_B and C_F are the protein concentrations, expressed in mg/mL, in the bottom phase and the fermentation broth, respectively.

2.6 Characterization of extracted collagenase

2.6.1 Effects of pH and temperature on collagenolytic enzyme activity and stability

To evaluate pH effect on collagenolytic enzyme activity, the pH of the reaction mixture containing 0.5% (w/v) of azocoll was varied over the range 3.0 ~ 11.0. The buffers used were 0.05 M citrate (pH 3.0 ~ 6.0), 0.05 M Tris-HCl (pH 7.0 ~ 9.0), and 0.05 M carbonate-bicarbonate (pH 10.0 ~ 11.0). For stability tests, the culture filtrated was incubated at 4 ºC (selected as a cold storage reference temperature at which the enzyme thermo inactivation is expected to be very low) in the above buffers at different pH values. The incubation time of samples varied from 1 to 24 h.

An analogous study was done for the effect of temperature in the same azocoll-buffer solution. To determine optimum temperature for enzyme activity, the reaction mixture was incubated at the selected temperature (from 25 to 70 ºC), while for thermo stability tests the enzyme was pre-incubated for 15 ~ 180 min at the same temperatures.

The residual activity was calculated as the ratio between the enzymatic activity, observed at the end of each incubation run, and that at the beginning, and expressed as percentage (%).

2.6.2 Substrate specificity

To test substrate specificity, the proteolytic activity of extracellular collagenolytic enzyme produced by *Penicillium* sp. UCP 1286 and commercial collagenase was also assayed on insoluble collagen (type I and type V), gelatin and azocasein. Azocasein was used as comparison substrate to check on enzyme specificity.

The activity was assayed on insoluble collagen according to Endo et al. (1987). The standard reaction mixture, containing 25 mg collagen (type I and V, from bovine Achilles tendon) in 5 mL of 0.05 M Tris-HCl buffer (pH 7.0), was incubated with 1 mL enzyme samples at 37ºC. The amount of free amino groups released was measured by the ninhydrin method of Rosen (1975). One activity unit (U) was defined as the number of µmol of L-leucine released as a result of the action of 1 mL culture filtrate containing collagenolytic enzyme, after 18 h at 37ºC.

Collagenolytic enzyme activity on gelatin was assayed by the method of Moore and Stein (1948), slightly modified. Reaction was carried out at 37ºC for 18 h after the addition of 0.1 mL of the enzyme solution to 1.0 mL of a solution containing 2 mg gelatin in 0.05 M Tris-HCl buffer (pH 7.5). The reaction was stopped by the addition of 0.1 mL of 10% (w/v) trichloroacetic acid. The medium was centrifuged at 10,000 x g for 10 min. The supernatant (0.2 mL) was mixed with 0.5 mL of ninhydrin solution, heated at 100ºC for 10 min, cooled in ice water for 5 min, and the mixture was then diluted with 2.5 mL of 50% (v/v) 1-propanol. After centrifugation at 12,000 x g for 10 min, the absorbance of the mixture was measured at 570 nm. One unit (U) of enzyme activity was expressed as µmol of L-leucine equivalents released per min.

The enzyme activity on azocasein was determined according to Leighton et al. (1973), with 1% (w/v) azocasein in a 0.1 M Tris-HCl buffer (pH 7.2). One unit (U) of protease activity was defined as the amount of enzyme required to raise the optical density at 440 nm by one unit after 1 h.

2.6.3 Effect of inhibitors

Protease inhibitors effect was investigated following the procedures of the manufacturer's guide of inhibitors. The inhibitors tested were: phenylmethylsulphonyl fluoride (PMSF) for serine proteases, ethylenediaminetetraacetic acid (EDTA) for metalloproteases, and iodoacetic acid (IAA) for cysteine proteases, at the concentration of 10 mM. For sensitivity determination, the enzyme was pre-incubated

for 30 min at 37ºC with the inhibitors. The residual activity was determined as the percentage of the proteolytic activity in an inhibitor-free control sample. After all, proceeded collagenolytic activity using the azocoll method.

2.6.4 Polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were analyzed by SDS-PAGE in a 15% polyacrylamide gel, according to Laemmli (1970). The protein molecular markers were phosphorylase b (97.0 kDa), bovine serum albumin (66.0 kDa), ovalbumin (54.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). The gel was loaded with 20 μL of concentrated enzyme by lyophilization, and subject to electrophoresis at a constant current of 100 V. Gel was stained with 0.25% (w/v) Comassie Brilliant Blue R-250 in methanol-acetic acid- water (45:10:45) and distained in the same solution without dye.

3 Results and Discussion

Table 2 shows the results of ATPS. The responses observed were collagenolytic activities of top (A_T) and bottom (A_B) phases, partition coefficient (K), activity yield (Y) and purification factor (PF) of bottom phases. It should be noticed that the run 5 did not form any biphasic system. The collagenase produced by *Penicillium* sp. UCP 1286 showed a stronger affinity for the bottom phase, where the highest collagenolytic activity values were observed at runs on center point, using PEG 3350 g/mol at 15.0% (w/w) concentration, and phosphate at pH 7.0 and concentration 12.5% (w/w). The partition coefficient, which is used to evaluate the separation of biomolecules in ATPS, was higher than 1 in all runs.

Table 2. Factor level combinations and results of the 2^4 factorial design employed to investigate the extraction of collagenase from *Penicillium* sp. UCP 1286 by PEG/phosphate ATPS. No biphasic system was observed in the run 5.

Run	M_{PEG}[a] (g/mol)	C_{PEG}[b] (% w/w)	pH	C_{PHOS}[c] (%. w/w)	A_B[d] (U/ml)	A_T[e] (U/ml)	K[f]	Y[g] (%)	PF[h]
1	1500	12.5	6	10	8.15	458.00	0.02	46.81	5.65
2	8000	12.5	6	10	0.86	545.00	0.01	55.71	12.26
3	1500	17.5	6	10	5.56	377.50	0.01	38.59	4.02
4	8000	17.5	6	10	2.65	405.00	0.01	41.40	12.23
5	1500	12.5	8	10	-	-	-	-	-
6	8000	12.5	8	10	1.37	411.00	0.01	42.01	10.03
7	1500	17.5	8	10	8.20	373.50	0.02	38.18	2.74
8	8000	17.5	8	10	0.90	332.00	0.01	33.94	7.29
9	1500	12.5	6	15	3.24	400.00	0.01	40.89	6.63
10	8000	12.5	6	15	3.45	332.50	0.01	33.99	12.02
11	1500	17.5	6	15	7.03	504.00	0.01	51.52	7.81
12	8000	17.5	6	15	3.62	305.50	0.01	31.23	10.78
13	1500	12.5	8	15	8.00	553.50	0.01	56.58	5.56
14	8000	12.5	8	15	2.80	348.50	0.01	35.62	9.69
15	1500	17.5	8	15	6.50	362.00	0.02	37.00	2.98
16	8000	17.5	8	15	5.06	264.50	0.02	27.04	4.73
17(C)	3350	15	7	12.5	1.15	797.00	0.01	81.47	25.23
18(C)	3350	15	7	12.5	1.06	797.00	0.01	81.47	24.55
19(C)	3350	15	7	12.5	1.02	799.50	0.01	81.72	25.31
20(C)	3350	15	7	12.5	1.11	812.00	0.01	83.00	27.61

a PEG molar mass. b PEG concentration. c Phosphate concentration. d Collagenolytic activity of bottom phase. e Collagenolytic activity of top phase. f Partition coefficient. g Activity yield. h Purification factor.

Lima et al. (2013) performed a 2^4 factorial design for purifying collagenase produced by *Penicillium aurantiogriseum* and found that the best results enzyme in the top phase, using PEG 1500 g/mol at 17.5% (w/w) of concentration, and phosphate pH 6.0, at 15.0% (w/w) of concentration. That would be difficult the

recovery step of final product, since the top phase is composed of PEG. According Rosso et al. (2012), that used ATPS for collagenase purification produced by *P. aurantiogriseum,* they observed an almost equal partition of the selected protein between the two phases, as the likely result of a salting out effect nearly coincident to the volume exclusion one, using PEG molar mass 550 g/mol and concentration of 20% (w/w) and potassium phosphate concentration of 17.5% (w/w).

The yields observed in this study averaged 82% in runs from the center point, and is associated with higher purification factor values (around 26). Lima et al. (2013) published lower yields, being the highest value obtained on the run 15 (61.68%), using PEG 8000 (g/mol), 17.5% (w/w) concentration and phosphate with pH 8.0 at concentration of 15% (w/w). But this run was not presented the highest factor of purification, reaching 2.65. Rosso et al. (2012) showed high efficiency values, being the highest value found in the top phase (376.8%) of the run 3, which used PEG 550 g/mol and 20% (w/w), with phosphate at a concentration of 12.5% (w/w). However, this was not the run that showed the highest factor of purification, reaching only 14.7. The run number 7, which obtained 23.5 purification factor, showed a yield of top phase equal to 242.0%, using PEG 550, with 20% (w/w) and phosphate concentration 17.5% (w/w).

Figure 1(A) shows the pH dependence of collagenolytic activity in azocoll. The enzyme was found to be very active between pH 7.0 and 9.0. At pH 6.0, only 25% of the activity remained, and the results were even worse for the most acidic conditions (pH 3.0 to 5.0) and more basic (pH 10 and 11). Figure 1(B) shows the stability at pH between 7.0 and 9.0, during 24 hours of incubation at 4 °C, the selected storage temperature. The enzyme was quite stable between pH 7.0 and 9.0, keeping about 85 to 90% of stability, after 24 hours.

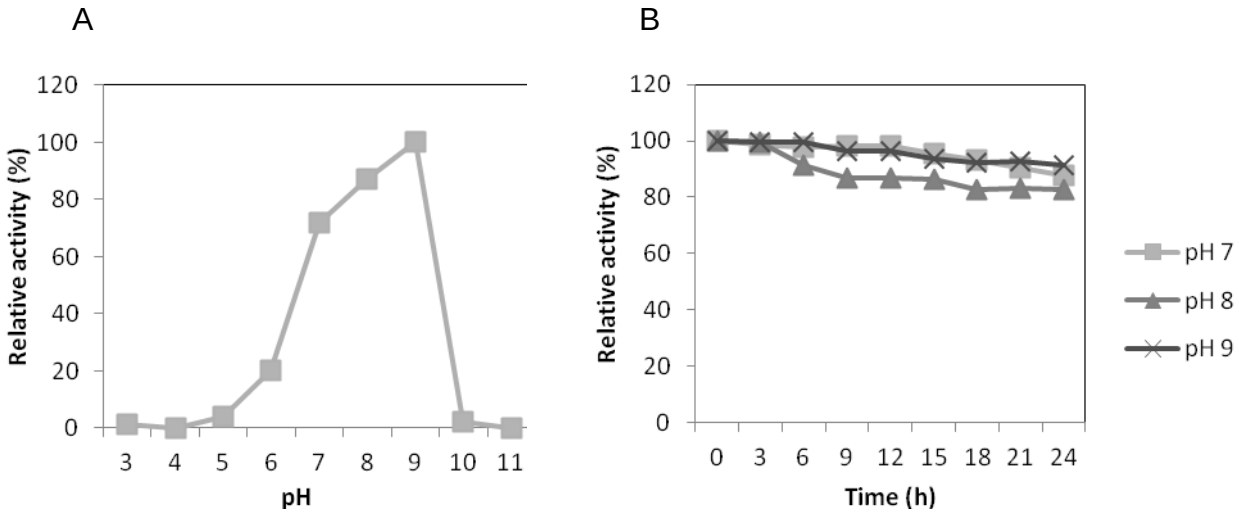

Figure 1. (A) Effect of pH on the activity of collagenase from by *Penicillium* sp. UCP 1286 purified by ATPS, expressed as percentage of the maximum one obtained in 0.05 M Tris-HCl buffer (pH 9.0). (B) Effect of pH stability of collagenase from by *Penicillium* sp. UCP 1286 purified by ATPS, expressed as the residual activity with respect to that at the beginning. Each value is the average of results of two experiments, and the error bars show the standard deviations.

To find optimum temperature for enzyme action, collagenolytic activity was measured at different temperatures between 25 and 75°C. Figure 2(A) shows that the highest value is observed at 37 °C. Above 45°C, enzymatic activity had a gradual decrease occurs until all activity is lost at 55, 65 and 75 °C. Figure 2(B) shows the results of enzyme stability over 6 hours at the different temperatures evaluated. The enzyme stability at two temperatures tested was considerable, maintaining approximately 90% of the activity after 6 hours on test.

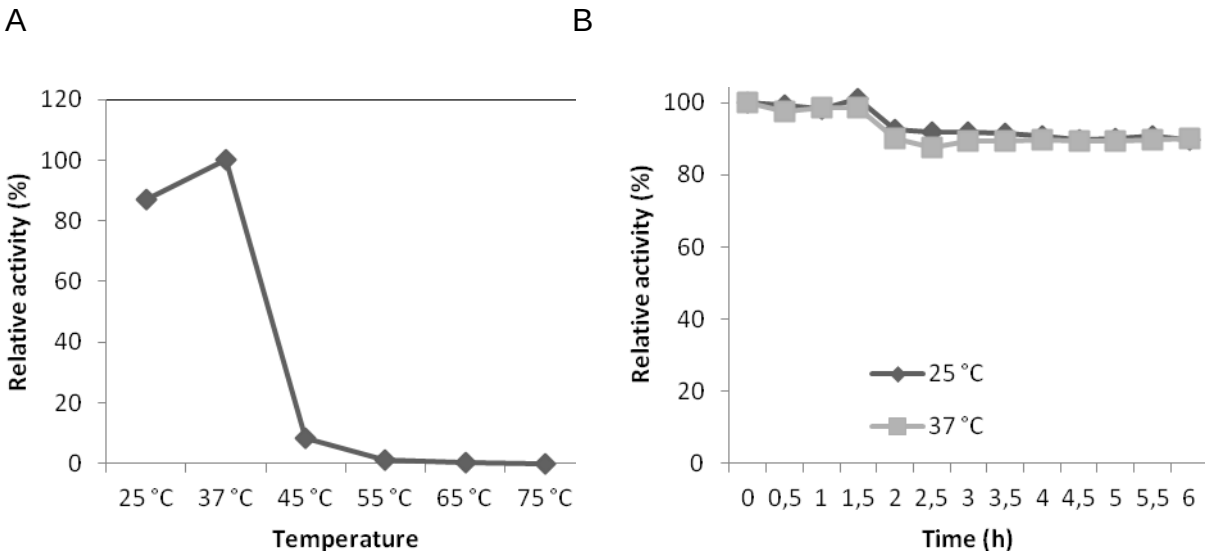

Figure 2. (A) Effect of temperature on the activity of collagenase from by *Penicillium* sp. UCP 1286 purified by ATPS, expressed as percentage of the maximum one obtained in 37 °C. (B) Effect of temperature stability of collagenase from by *Penicillium* sp. UCP 1286 purified by ATPS, expressed as the residual activity with respect to that at the beginning. Each value is the average of results of two experiments, and the error bars show the standard deviations.

The substrate specificity of the enzyme was evaluated using azocoll, collagen (types I and V), gelatin and azocasein. The highest activity was obtained with azocoll (700 U/mL), and it was assumed as 100%. The relative activity with the other substrates was lower, reaching about 20% with gelatin and type I collagen, 52% using type V collagen and 2% with azocasein.

The collagenase from by *Penicillium* sp. UCP 1286 purified by ATPS was subjected to inhibition by phenylmethylsulfonyl fluoride (PMSF), which is a method to establish whether or not one enzyme is a serine protease. After 30 minutes of incubation at 37 °C in the presence of 10 mM of PMSF, the enzyme activity was reduced to 0, whereas in the presence of 10 mM iodoacetic acid (that inhibits cysteine proteases) there was maintenance of a residual activity of 80.22%. The collagenolytic enzyme was slightly inhibited by EDTA, known to inhibit metalloproteinases (a loss of only about 5% of activity was observed). Although further testing is required, the enzyme seems to belong to the class of serine proteases.

The electrophoresis of collagenase from by *Penicillium* sp. UCP 1286 purified by ATPS presented an only band, corresponding to a molecular mass (MM) of approximately 28 kDa (Figure 3). The crude extract showed multiples bands.

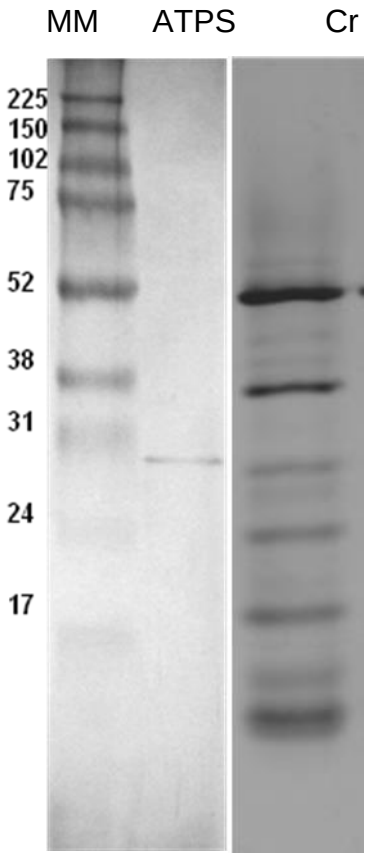

Figure 3. SDS-PAGE patterns of collagenase from by *Penicillium* sp. UCP 1286 purified by ATPS and crude extract (Cr) MM: molecular mass.

4 Conclusions

The purification technique via aqueous two-phase system (ATPS) was effective for the partition and extraction of collagenase produced by *Penicillium* sp. UCP 1286 isolated from Caatinga. The yield values and partition coefficient were satisfactory compared to those in the literature relating ATPS to purify collagenase. The run with better values of yield and partition coefficient were at runs on center point, using PEG 3350 g/mol at 15.0% (w/w) concentration, and phosphate at pH 7.0 and concentration 12.5% (w/w). Enzyme was characterized stable to pH and temperature, and SDS/PAGE showed that purification was efficient.The enzyme was

seemed to be a serine protease, having the optimal collagenolytic activity at 37 °C and pH 9.0.

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Ethical Statement/Conflict of Interest

The authors are aware of the ethical responsibilities and the manuscript has no conflict of interest.

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ANEXOS

7.1 Normas para a redação de artigos para a revista "Brazilian Journal of Microbiology"

Scope of the journal

As from 01/01/2015, the Brazilian Journal of Microbiology will be accepting texts annoncing new publically available genomes in our new "Genome Announcements" section. The purpose of this section is to allow authors of a genome submission to inform the readers of BJM that a novel complete genome sequence of a microbiological organism is now publically available and of interest to the scientific community. The genome announcement does not preclude future publication of a detailed full scientific paper later in BJM or elsewhere. The scope for Genome Announcements in BJM is outlined below:

- The authors of the Genome Announcement must be the same (or mostly the same) authors as the genome sequence deposit;
- The genome sequence must be publically available at the time of Genome Announcement submission in DDBJ/EMBL/NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank). A valid GenBank accession number for the genome must be clearly stated in the manuscript;
- Communication of complete genomes of viruses, phages, bacteria, archaea, fungi and protozoa will be considered;
- Complete sequences of gapless circular plasmids will also be accepted;
- The nucleotide sequences referred to must cover at least 95% of the predicted genome size for the organism;
- GenBank deposits pertaining complete (gapless) chromosomes, gapped (scaffolded) chromosomes and genome assemblies will be considered. It is desirable for these deposits to contain functional annotation;
- The manuscript for the Genome Announcement must contain up to 500 words in the body text plus a 150 word abstract;
- The authors must clearly state the origin of the strain, the importance of having sequenced the genome and the advantages the availability of the sequence will have to the field of microbiology;
- The text must contain sequencing methodology, including number and size of reads generated, assembly methods used, steps taken for

scaffolding and genome finishing, when applicable, and methods used for feature annotation and curation if carried out;

Acceptability for publication will be dependent on the same peer-review system currently used for other types of publication in BJM, so having a working GenBank accession number is no guarantee of publication in BJM.

Brazilian Journal of Microbiology, published by the Brazilian Society of Microbiology, publishes original research papers, and reviews, covering all aspects of Microbiology. The publication is free of charge.

The following categories of papers are acceptable for publication in Brazilian Journal of Microbiology:

- ***Research paper*:** the research paper reports results of original research, which has not been published elsewhere.
- ***Mini-review*:** Review articles should deal with microbiological subjects of broad interest.

Your manuscript must be written in clear, comprehensible English.

If you have concerns about the level of English in your submission, you may choose to have your manuscript professionally edited by a native English speaker or a scientific editing service prior to submission to improve the English. All services are to be arranged and paid for by the author, and use of one of these services does not guarantee acceptance or preference for publication. In the case of the author being a native English speaker, please replace the certificate of English editing service by a justification letter.

The text of articles accepted for publication, should be submitted to a review of the English language by American Journal Experts. To publish, you must send to BJM the certificate issued by them.

• American Journal Experts: http://www.JournalExperts.com?rcode=BSM1

SECTIONS

Industrial Microbiology:

Bacterial Fermentation

- biosynthesis and bioconversion of natural products, including antibiotics, xenobiotics, and macromolecules produced by bacteria.
- molecular aspects of bacterial biotechnology

Fungal Fermentation

- biosynthesis and bioconversion of natural products, including antibiotics, xenobiotics, and macromolecules produced by fungi
- molecular aspects of fungal biotechnology

Food Microbiology:

Food Technology

- applications of microorganisms (bacteria and fungi) for food production

Food Safety and Quality

- food borne diseases
- food spoilage
- microbial ecology in foods

Medical Microbiology:

Bacterial Pathogenesis

- genetic, biochemical, and structural basis of bacterial pathogenesis

Fungal Pathogenesis

- genetic, biochemical, and structural basis of pathogenesis of fungi

Clinical Microbiology:

Micology

- studies of medically-important fungi

Bacteriology

- studies of medically-important bacteria

Virology

- studies of medically-important virus

Environmental Microbiology:

Microbial Ecology

- ecology of natural microbial assemblages, microbial diversity of natural environments such as water, soil, sediments and higher organisms
- microbial interactions

Biotechnology

- environmental aspects of public health
- biodegradation
- bioremediation
- environmental considerations for genetically engineered microorganisms

Fungal Physiology

- fungal biochemistry, biophysics, metabolism, cell structure, stress response, growth, differentiation and other related process

Bacterial Physiology

- bacterial biochemistry, biophysics, metabolism, cell structure, stress response, growth, differentiation and other related process

Genetics and Molecular Biology of Fungi

- fungal genetics, molecular biology, gene regulation, DNA replication and repair, genomics, proteomics, transcriptomics

Genetics and Molecular Biology of Bacteria

- bacterial genetics, molecular biology, gene regulation, DNA replication and repair, genomics, proteomics, transcriptomics

Genetics and Molecular Biology of Viruses

- viral genetics, molecular biology, gene regulation, DNA replication and repair, genomics, proteomics, transcriptomics

Veterinary Microbiology

- diseases of animals
- control and/or treatment of animals
- animal pathogen diagnostics
- veterinary or zoonotic pathogens

Education in Microbiology

- Teaching strategies in microbiology
- New teaching tools in microbiology

Submission of a manuscript

Submission of a manuscript to Brazilian Journal of Microbiology is understood to imply that it has not previously been published (except in an abstract form) and that it is not being considered for publication elsewhere.

Upon receipt of a manuscript all authors will receive an electronic message acknowledging the receipt.

Responsibility for the accuracy of the manuscript content lies entirely with the authors.

Publication of a manuscript

Manuscripts are accepted for publication after having been critically reviewed by at least two referees, indicated by the Editors.

The suggestions and recommendations of the reviewers and Editors will be forwarded electronically to the corresponding author, who should return the reviewed manuscript to the Editors within the stipulated date, via online system. Whenever applicable, the corresponding author should explain or comment each modification introduced in the text.

The corresponding author will receive an electronic message whenever the manuscript moves from one status to the next.

Membership in Brazilian Society for Microbiology is not a pre requisite for submission of a manuscript for publication.

Nonmember scientists from Brazil and other countries are invited to submit papers for analysis.

ETHICS:

When the study, described in the manuscript, is related to experiments carried out with human beings and/or animals, author(s) must inform, within the text, if the research project has been approved by the Research Ethics Committee of their institution, according to the Declaration of Helsinki (http://www.ufrgs.br/HCPA/gppg/helsin5.htm). Experimental studies involving animals should follow the guidelines established by the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, National Academy of Sciences, Washington, D. C. 1996), and the *Princípios Éticos na Experimentação Animal do Colégio Brasileiro de Experimentação Animal* (COBEA) (Ethical Principles for Animal Experimentation of the Brazilian College of Animal Experimentation - http://www.cobea.org.br/index.php?pg=Principios%20Eticos).

Preparation of a manuscript

The manuscript should be submitted as **one single WORD file**. This single file

should include: the whole text, figures, tables, etc. Only manuscripts written in English will be considered.

For **research papers**, the **WORD** file should contain:

- Title
- Authors and Affiliations
- Abstract (200 to 250 words)
- Three to five key-words
- Introduction
- Materials and Methods
- Results
- Discussion
- Acknowledgements (optional)
- References

For **mini-reviews**, the **WORD** file should contain:

- Title
- Authors and Affiliations
- Abstract (200 to 250 words)
- Three to five key-words
- Text
- Acknowledgements (optional)
- References

All manuscripts should be typed double-spaced with 3 cm margins and pages should be numbered sequentially. The lines in each page of the manuscript should be numbered too. The Editors recommend that a manuscript should be critically read by someone fluent in English before submission.

Manuscripts written in poor English will not be accepted.

Research papers and *mini-reviews* consist of 20 pages, including references, tables and figures.

Abbreviations of terms and symbols should follow the recommendations of IUPAC-IUB Commission (*Comission on Biochemical Nomenclature, Amendments and Corrections*) and the units are to be used according to SI (*International Systems of Units*).

SUGGESTED REVIEWERS

Authors may submit suggestions of reviewers to evaluate the manuscripts. The following information must be provided: reviewer name, e.mail address, and the home institution.

USE OF PLANT EXTRACTS IN MICROBIOLOGICAL EXPERIMENTS

Articles that present studies with plant extracts, or other complex substances, will be accepted only after identification of compounds.

Authors may need, or wish, to use professional language editing services to improve papers in English and, therefore, overall quality. This assistance is suggested either before an article is submitted for peer review or before it is accepted for publication. Non-native English speakers and international authors who would like assistance with their writing, may likely consider the following options:

- American Journal Experts, English Editing: http://www.JournalExperts.com?rcode=BSM1
- Joanne Roberts: joroberts@uol.com.br
- ATO Traduções: www.atotraining.com.br
- Prof. Julian D. Gross, University of Oxford, Oxford Biomedical Editors: julian.gross@pharm.ox.ac.uk
- BioMed Proofreading LLC: http://www.biomedproofreading.com

ORGANIZATION

The **Title** should be a brief as possible, contain no abbreviations and be truly indicative of the subject of the paper.

Expressions like "Effects of", "Influence of", "Study on", etc, should be avoided. Care should be exercised in preparing the title since it is used in literature retrieval systems.

The **Abstract** should summarize the basic content of the paper. The abstract should be meaningful without reference to the text. An abstract should not contain references, tables or unusual abbreviations. Abstracts are reprinted by abstracting journals and therefore will be read by persons who do not have access to the entire paper.

The **Introduction** should provide the reader with sufficient information so that the results reported in the paper can be properly evaluated without referring to the literature. However, the introduction should not be an extensive review of the literature. The introduction should also give the rationale for and objectives of the study that is being reported.

The **Materials and Methods** section should provide enough information for other investigators to repeat the work.

Repetition of details of procedures which have already been published elsewhere should be avoided. If a published method is modified, such modification(s) must be described in the paper. Sources of reagents, culture media and equipment (company, city, state, country) should be mentioned in the text. Names that are registered trade marks should be so indicated. Subheading often makes this section easier to read and understand.

The **Results** section should, by means of text, tables and/or figures, give the results of the experiments. If a *Discussion* section is to be included, avoid extensive interpretation of results but do so in the *Discussion* section. If *Results* and *Discussion* are combined, then results should be discussed where, in the text, is the more appropriate. Tables and figures should be numbered using Arabic numerals. All tables and figures must be mentioned in the text.

The approximate location of tables and figures in the text should be indicated.

The **Discussion** section should discuss the results in relation to the literature cited.

The **References s**hould be in alphabetical order, by last name of the first author. All authors must be cited. The citations in the text have to be written by the last name(s) of the author(s), followed by the year of publication. As an example, see below: "...while Silva and Pereira (1987) observed that resistance depended on soil density" or "It was observed that resistance depended on soil density (Silva and Pereira, 1987)." For two or more papers by the same author(s) in a citation, list them chronologically, with the years separated by commas (example: Freire-Maia *et al*., 1966a, 1966b, 2000; Hene 2010; Padonou *et al*., 2012). Journal names should be abbreviated according to the style of *BIOSIS*. All references given in the list should be cited in the text and all references mentioned in the text should be included in the list.

Examples:

a. **Journal article**

Brito DVD, Oliveira EJ, Darini ALC, Abdalla VOS, Gontijo-Filho PP (2006) Outbreaks associated to bloodstream infections with *Staphylococcus aureus* and coagulase-negative *Staphylococcus* spp in premature neonates in a university hospital from Brazil. Braz J Microbiol37:101-107.

b. **Paper or chapter in a book**

Franco BDGM, Landgraf M, Destro MT, Gelli DS, (2003) Foodborne diseases in Southern South America. *In*: Miliotis, M.D., Bier, J.W.(eds). International Handbook of Foodborne Pathogens. Marcel Dekker, New York, USA, 733-743.

c. **Book**

Montville TJ, Matthews KR (2005) Food Microbiology - an introduction. ASM Press, Washington, D.C.

d. **Patent**

Hussong RV, Marth EH, Vakaleris DG. January 1964. Manufacture of cottage cheese. U.S. Pat. 3, 117, 870.

e. **Thesis and Dissertations**

Santos MVB (2005) O papel dos anticorpos contra os componentes da parede celular de Paracoccidioides brasiliensis na evolução da doença experimental. São Paulo, Brasil, 110p. (M.Sc. Dissertation. Instituto de Ciências Biomédicas. USP).

f. **Communications in events (Symposia, Conferences, etc)**

Silveira TS, Martins JL, Abreu FA, Rosado AS, Lins UGC (2005) Ecology of magnetotatic multicelular organisms in microcosms. XXIII Congresso Brasileiro de Microbiologia, Santos, SP, p. 272.

g. **Publication in the web**

Abdullah MAF, Valaitis AP, Dean DH (2006) Identification of a *Bacillus thuringiensis* Cry11 Ba toxin-binding aminopeptidase from the mosquito *Anopheles quadrimaculatus*. *BMC Biochemistry*. http://www.biomedcentral.com/1471-2091/7/16

h. **Webpage**

U.S. Food and Drud Administration. 2006. Enjoying Homemade Ice Cream without the Risk of *Salmonella* Infection. Available at:

http://www.cfsan.fda.gov/~dms/fs-eggs5.html. Accessed 26 May 2006.

References citing "personal communication" or "unpublished data" are discouraged, although it is recognized that sometimes they must be used. In these cases, they should be cited in the text and not in the list of references. References consisting of papers that are "accepted for publication" or "in press" are acceptable. However, references of papers that are "submitted" or "in preparation" are not acceptable.

ACKNOWLEDGMENTS: This section is optional. It acknowledges financial and personal assistance.

TABLES: should be inserted in the text according to which they are cited, and numbered sequentially in Arabic number. The title of a table should be placed in the top of it and should be brief but fully descriptive of the information contained. Headings and subheadings should be concise with columns and rows of data carefully centered below them. Should be of sufficient quality to ensure good reproduction. Please, open the following link to see the requirements to obtain the adequate resolution. (http://www.ncbi.nlm.nih.gov/pmc/about/image_quality_table.html)

FIGURES: should be inserted in the text according to which they are cited, and numbered sequentially in Arabic number. Data presented in the tables should not be repeated in the figures. The legend of the figures should be placed at their bottom. Should be of sufficient quality to ensure good reproduction. Please, open the following link to see the requirements to obtain the adequate resolution. (http://www.ncbi.nlm.nih.gov/pmc/about/image_quality_table.html)

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Conflicts of Interest

It is Brazilian Journal of Microbiology policy that everyone involved in the publication process (authors, reviewers, editorial board members, and editorial staff) must be free from conflicts of interest that could adversely influence their judgment, objectivity or loyalty to the article and assignments. The BJM recognizes that any potential conflict of interest raised must be disclosed promptly to Editor. Conflicts of interest in publishing can be defined as conditions in which an individual holds conflicting or competing interests that could bias editorial decisions. Conflicts of interest may be

only potential or perceived, or they may be factual. Personal, political, financial, academic, or religious considerations can affect objectivity in numerous ways.

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Upon receipt of the galley proofs for approval, authors of approved manuscripts should fax or email the Author's Copyright Statement to the BJM (55-11-3037-7095, bjm@sbmicrobiologia.org.br). The statement (see text below) must be signed by at least one of the authors (who agrees to inform the other authors, if any).

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7.2 Normas para a redação de artigos para a revista "World Journal of Microbiology and Biotechnology"

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

Permissions

Authors wishing to include figures, tables, or text passages that have already been published elsewhere are required to obtain permission from the copyright owner(s) for both the print and online format and to include evidence that such permission has been granted when submitting their papers. Any material received without such evidence will be assumed to originate from the authors.

Online Submission

Please follow the hyperlink “Submit online” on the right and upload all of your manuscript files following the instructions given on the screen.

Important note:

A copy of the **author checklist**, appropriately checked, must accompany every submission.

Author Checklist (docx, 83 kB)

Title Page

The title page should include:

The name(s) of the author(s)

A concise and informative title

The affiliation(s) and address(es) of the author(s)

The e-mail address, telephone and fax numbers of the corresponding author

Abstract

Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

Additional request Title page

Graphical Abstracts

You are welcome to submit a graphical abstract consisting of an image (figure, scheme) representing the contents of the article graphically. The use of color is strongly encouraged here.

Format

Manuscripts should be divided into the following sections:

• Title page
• Abstract
• Introduction
• Materials and methods
• Results
• Discussion; the Discussion section must not recapitulate the Results
• Acknowledgements
• References
• Figure legends
• Figures
• Tables

Results and Discussion should be separated.

Text

Text Formatting

Manuscripts should be submitted in Word.

Use a normal, plain font (e.g., 10-point Times Roman) for text.

Use italics for emphasis.

Use the automatic page numbering function to number the pages.

Do not use field functions.

Use tab stops or other commands for indents, not the space bar.

Use the table function, not spreadsheets, to make tables.

Use the equation editor or MathType for equations.

Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX.

LaTeX macro package (zip, 182 kB)

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

References

Citation

Cite references in the text by name and year in parentheses. Some examples:

Negotiation research spans many disciplines (Thompson 1990).

This result was later contradicted by Becker and Seligman (1996).

This effect has been widely studied (Abbott 1991; Barakat et al. 1995a, b; Kelso and Smith 1998; Medvec et al. 1999, 2000).

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

Reference list entries should be alphabetized by the last names of the first author of each work. Order multi-author publications of the same first author alphabetically with respect to second, third, etc. author. Publications of exactly the same author(s) must be ordered chronologically.

Journal article

Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in

prepubescent children. Eur J Appl Physiol 105:731-738. doi: 10.1007/s00421-008-0955-8

Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

Article by DOI

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086

Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

Dissertation

Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal’s name according to the ISSN List of Title Word Abbreviations, see

ISSN LTWA

If you are unsure, please use the full journal title.

For authors using EndNote, Springer provides an output style that supports the formatting of in-text citations and reference list.

EndNote style (zip, 2 kB)

Tables

All tables are to be numbered using Arabic numerals.

Tables should always be cited in text in consecutive numerical order.

For each table, please supply a table caption (title) explaining the components of the table.

Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.

Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

Artwork and Illustrations Guidelines

Electronic Figure Submission

Supply all figures electronically.

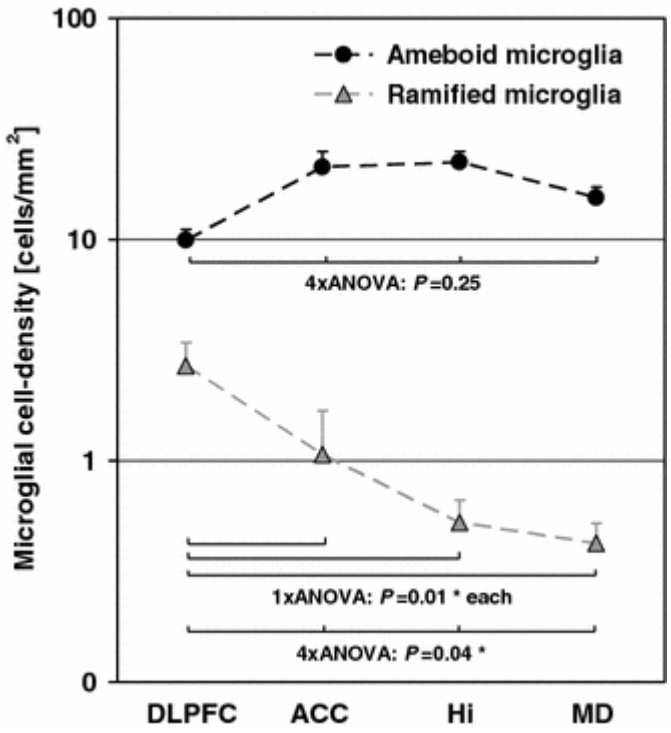
Indicate what graphics program was used to create the artwork.

For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable.

Vector graphics containing fonts must have the fonts embedded in the files.

Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

Line Art



Definition: Black and white graphic with no shading.

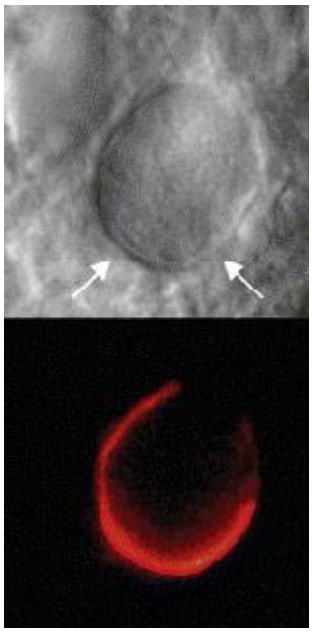
Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.

All lines should be at least 0.1 mm (0.3 pt) wide.

Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.

Vector graphics containing fonts must have the fonts embedded in the files.

Halftone Art



Definition: Photographs, drawings, or paintings with fine shading, etc.

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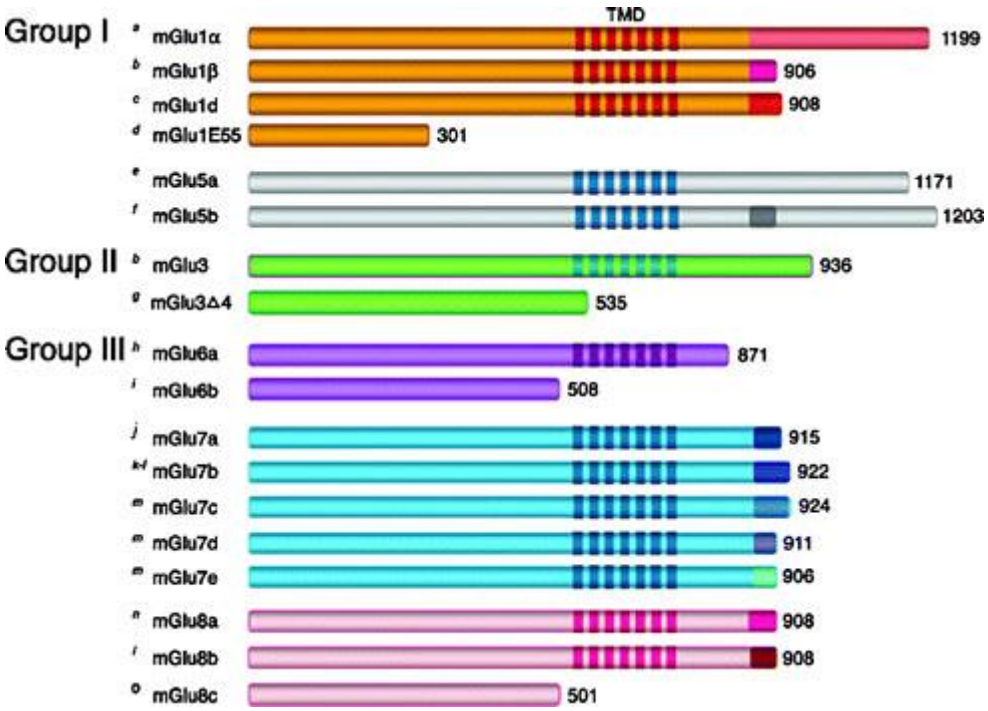

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7.3 Produção 2012 – 2016

7.3.1 Artigos completos publicados em periódicos

- WANDERLEY, M. C. A.; SOARES, M. L.; GOUVEIA, E. R. . Selection of inoculum size and Saccharomyces cerevisiae strain for ethanol production in simultaneous saccharification and fermentation (SSF) of sugarcane bagasse. *African Journal of Biotechnology* JCR, v. 13, p. 2762-2765, 2014.

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BIOMASSAS, 2015, Fortaleza. Anais do XX Simpósio Nacional de Bioprocessos, 2015.

7.3.3 Apresentações de trabalho

- WANDERLEY, M. C. A.; LIMA, C. A. ; TEIXEIRA, J. A. C. ; PORTO, A. L. F. . Collagenase production by Penicillium sp. isolated from Caatinga. 2014. V Simpósio Internacional em Diagnóstico e Terapêutica e VIII Jornada Científica do LIKA.

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