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PRODUÇÃO, CARACTERIZAÇÃO E PURIFICAÇÃO DA COLAGENASE DO *Penicillium aurantiogriseum* URM-4622, VISANDO SUA APLICAÇÃO NA PRODUÇÃO DE PEPTÍDEOS DO COLÁGENO

CAROLINA DE ALBUQUERQUE LIMA DUARTE

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco como parte dos requisitos exigidos para obtenção do título de Doutor

Orientadora: Prof[a] Dra. Ana Lúcia Figueiredo Porto

Co-orientadora: Prof[a] Dra. Maria das Graças Carneiro da Cunha

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RESUMO

Colagenases são enzimas proteolíticas capazes de degradar a região helicoidal do colágeno em pequenos fragmentos. Em contraste com as colagenases de mamíferos, que clivam a hélice do colágeno em um único ponto, as colagenases microbianas atacam múltiplos sítios ao longo da hélice e por este motivo vem sendo largamente aplicadas na obtenção de peptídeos bioativos do colágeno. Devido ao uso potencial das colagenases microbianas na produção de peptídeos bioativos, existe um interesse em encontrar novas linhagens de fungos capazes de produzir estas enzimas com novas características e em um meio de produção com baixo custo industrial. O presente trabalho teve como objetivo otimizar a produção da colagenase do *Penicillium aurantiogriseum* utilizando um meio de produção econômico a base de farinha de soja, caracterizar a enzima obtida a partir das condições de cultivo mais favoráveis, aplicar o sistema de duas fases aquosas (SDFA) polietileno glicol (PEG)/fosfato para pré-purificar a colagenase do meio de fermentação e utilizar a cologanase na forma pré-purificada para a produção de peptídeos bioativos do colágeno bovino tipo I. Três planejamentos experimentais (2^4 e 2^3 fatorial completo e um 2^2 central composto) foram empregados para otimizar a produção da colagenase. Os resultados do planejamento completo 2^4 indicaram que as variáveis mais significativas para a produção da colagenase foram a concentração da farinha de soja e o pH inicial do meio de cultura que exerceram efeitos positivos, e a temperatura que exerceu efeito negativo. Os resultados do planejamento completo 2^3 indicaram que o pH inicial do meio de cultura e a temperatura exerceram efeitos negativos significativos, enquanto que a concentração da farinha de soja apresentou efeito positivo na produção da colagenase. A produção enzimática máxima (283,36 ± 1,33 U) foi obtida a 1,645% (m/v) de farinha de soja, pH 7,21 e 24 ºC, conforme predita pela superfície de resposta. A enzima apresentou uma atividade máxima a pH 9,0 e 37 ºC, foi estável em uma ampla faixa de pH (6,0 a 10,0) e temperatura (25 a 45 ºC) e foi fortemente inibida por 10 mM de fenil metil sulfonil fluoreto. A energia de ativação e a entalpia de ativação da enzima foram 107,4 kJ/mol e 104,7 kJ/mol, respectivamente. A análise dos resultados do planejamento fatorial 2^4 utilizado para estudar a influência da massa molar do PEG (MM_{PEG}), da concentração do PEG (C_{PEG}), da concentração do fosfato (C_{FOSF}) e do pH na extração da colagenase no SDFA indicou que o fator de purificação (FP), o coeficiente de partição (K) e o rendimento em atividade (Y) da colagenase na fase superior aumentaram com o aumento da C_{PEG} e da C_{FOSF} e com a diminuição da MM_{PEG} e do pH. O FP máximo (5,23) foi obtido com MM_{PEG} de 1500 g/mol, C_{PEG} de 17,5% (m/m), C_{FOSF} de 15% (m/m) e pH 6,0, enquanto que o Y máximo (167,01%) foi obtido com MM_{PEG} de 1500 g/mol, C_{PEG} de 17,5% (m/m), C_{FOSF} de 10% (m/m) e pH 8,0. A eficiência do SDFA foi confirmada pelo estudo eletroforético, que revelou a eliminação de proteínas contaminantes. Os resultados do planejamento fatorial completo 2^3 utilizado para identificar as condições mais favoráveis de hidrólise do colágeno bovino tipo I, a partir da colagenase pré-purificada do *P. aurantiogriseum*, indicaram que o pH e a concentração do colágeno exerceram efeitos positivos sobre o grau de hidrólise, enquanto que o efeito da temperatura foi negativo. O grau de hidrólise máximo (4,65 µmol/mL) foi obtido utilizando uma concentração de colágeno de 7,5 mg/mL, pH 8,0 e 25 ºC. O perfil de peptídeos obtido por espectrometria de massa mostrou a presença de peptídeos com massas molares inferiores a 11 kDa e inúmeros peptídeos com massas molares menores de 2 kDa. Estes peptídeos apresentaram atividade antimicrobiana contra *Escherichia coli* (CIM = 0,625 mg/mL), *Bacillus subtilis* (CIM = 5 mg/mL) e *Staphylococcus aureus* (CIM = 0,55 mg/mL) e atividade antioxidante de 84,7 ± 0,24% (50 mg/mL). Os resultados do presente trabalho demonstram que *P. aurantiogriseum* é produtor de colagenase, a qual é uma alternativa tecnologicamente viável para a produção de peptídeos bioativos do colágeno.

Palavras chaves: *Penicillium aurantiogriseum*, colagenase, sistema de duas fases aquosas, peptídeos.

ABSTRACT

Collagenases are proteolytic enzymes responsible for the degradation of the helical region of native collagen in small fragments. In contrast to mammalian collagenases, which cleave the collagen helix at a single site, microbial collagenases attack multiple sites along the helix, for this reason has been widely applied to produce collagen bioactive peptides. Due to the potential use of microbial collagenase in the production of bioactive peptides, there is an interest in finding new strains of fungi capable of producing these enzymes with new features and using a cost-effective industrial medium. The present work aimed to optimize the production of *Penicillium aurantiogriseum* collagenase using a cost-effective medium composed of soybean flour, to characterize the enzyme obtained under the most favorable conditions, to applied the aqueous two-phase system (ATPS) PEG/phosphate to pre-purified the collagenase from the fermented broth and the use of pre-purified collagenase for the collagen bioactive peptides production. Three experimental designs (2^4 and 2^3 full factorial, and 2^2 central composite designs) were employed to optimize collagenase production. The 2^4 design results indicated that the most significant variables for collagenase production were soybean flour concentration and initial medium pH that had positive main effects, and temperature that had a negative one. The 2^3 design results indicated that the initial medium pH and the temperature had significant negative main effects, whereas the substrate concentration had a positive effect on the collagenase production. The maximum collagenolytic activity (283,36 ± 1,33 U) was obtained with 1.645% soybean flour, pH 7.21 and 24 ºC, predicted by the fitted response surface. The enzyme showed an optimum activity at 37ºC and pH 9.0, was stable over wide ranges of pH (6.0 -10.0) and temperature (25-45ºC) and was strongly inhibited by 10 mM phenyl methyl sulphonyl fluoride. The activation energy and the activation enthalpy were 107.4 kJ/mol and ΔH^*_d = 104.7 kJ/mol, respectively. The analysis of the 2^4 factorial results used to identify the influence of PEG molar mass (M_{PEG}), PEG concentration (C_{PEG}), phosphate concentration (C_{PHOS}) and pH on the extraction of the colagenase using ATPS indicated that purification factor (*PF)*, partition coefficient (*K)* and activity yield (*Y)* of collagenase in the top phase were increased with increasing C_{PEG} and C_{PHOS} and decreasing M_{PEG} and pH. The best *PF* value (5.23) was obtained with 1500 g/mol M_{PEG}, 17.5% (w/w) C_{PEG}, 15% (w/w) C_{PHOS} and pH 6.0. The highest *Y* value (167.01), in contrast, was obtained with the lower level of C_{PHOS} and the higher level pH 8.0. The electrophoretic study revealed that some protein contaminants were removed after the aqueous two-phase systems (ATPS). The results of the 2^3 full factorial design used to identify the most favorable conditions for type I bovine collagen hydrolysis by pre-purified collagenase from *Penicillium aurantiogriseum* showed that pH and collagen concentration had significant positive effects, whereas temperature had a negative effect, on the degree of hydrolysis. The maximum degree of hydrolysis (4.65 µg/mL) was achieved at 7.5 mg/mL of collagen, pH 8.0, and 25 °C. The peptide profile obtained under these conditions showed peptides with molecular weights less than 11 kDa, with several peptides having molecular weights of less than 2 kDa. These peptides exhibited antibacterial activity against *Escherichia coli* (MIC 0.625 mg/mL), *Bacillus subtilis* (MIC 5 mg/mL), *and Staphylococcus aureus* (MIC 0.55 mg/mL) and an antioxidant activity of 84.7% ± 0.24% (50 mg/mL). The results of this study demonstrate that *P. aurantiogriseum* is a collagenase producer, which is a technological viable alternative for the production of collagen bioactive peptides.

Key words: *Penicillium aurantiogriseum*, collagenase, aqueous two-phase system, peptides.

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1. INTRODUÇÃO

Colágenos constituem a família mais prevalente de proteínas do corpo humano, e da matriz extracelular do tecido conjuntivo em particular. Atualmente, 29 moléculas distintas de colágenos já foram identificadas (Nerenberg & Stultz, 2008). Uma característica estrutural de todos os colágenos é a presença de um domínio da proteína em conformação em tripla hélice que oferece estabilidade a estas moléculas, o que proporciona a integridade dos tecidos conjuntivos (Chung & Uitto, 2010). Todos os membros da família de colágeno são caracterizados por domínios com repetições do tripeptídeo Gli-X-Y, onde as posições de X e Y são freqüentemente ocupadas por uma prolina e uma hidroxiprolina, respectivamente (Uitto et al., 2008).

O colágeno é um biomaterial com boa compatibilidade e degradabilidade biológicas; apresenta uma ampla gama de aplicações nas indústrias de couro e filme, em materiais cosméticos e biomédicos, e como alimentos (Singh et al. 2011). Na indústria farmacêutica, o colágeno é usado para a produção de curativos, implantes vítreos e como carreador para a entrega de drogas. Além disso, o colágeno é uma fonte de peptídeos biologicamente ativos que podem ser utilizados como conservantes não alérgicos para medicamentos, como ingredientes para alimentação parenteral (Nakayama et al. 2000), no tratamento de doenças como a artrite induzida por colágeno (Zhu et al., 2007) e diabetes (Zhu et al. 2010), além de apresentarem atividade antioxidante, antimicrobiana e anti-hipertensiva (Gómez-Guillén et al. 2011).

A forma mais comum de produzir peptídeos biologicamente ativos é através da hidrólise enzimática de moléculas da proteína inteira. A bioatividade dos peptídeos gerados parece ser inerente ao tamanho e as sequências específicas de aminoácidos (Korhonen, 2009). Enzimas colagenolíticas de várias fontes (animal, vegetal e microbiana) foram empregadas com sucesso na produção de peptídeos (Korhonen & Pihlanto, 2006). Entretanto, considerando que os microorganismos poderem ser cultivados através de métodos bem estabelecidos, produzindo grandes quantidades de enzimas, as colagenases de origem microbiana aparecem como biocatalisadores atraentes para obtenção de peptídeos bioativos do colágeno em escala comercial (Reddy et al., 2008; Haddar et al., 2010).

Até agora, as colagenases microbianas têm sido purificadas através da utilização de métodos tradicionais que necessitam de muitas etapas e requerem um longo período de tempo para a sua execução, o que induz a um aumento da desnaturação da macromolécula, com a perda da sua atividade biológica, o que pode levar a um baixo rendimento de todo o processo

(Spelzini et al., 2005; Kang et al., 2005; Petrova et al., 2006; Sakurai et al., 2009; Wu et al., 2010). Devido à necessidade de se manter a atividade biológica das enzimas produzidas por via microbiana e ao fato de cerca de 50 a 90% dos custos de produção de produtos biológicos serem destinados às estratégias de purificação existe a necessidade do desenvolvimento de técnicas alternativas de separação e purificação de proteínas (Spelzini et al., 2005; Tubio et al., 2009). Assim, os sistemas de duas fases aquosas (SDFA) tornaram-se uma tecnologia de separação atraente devido às suas condições experimentais suaves e à facilidade de operarem à escala industrial (Tubio et al., 2009).

A extração líquido-líquido utilizando o SDFA é um processo de bioseparação que permite um elevado grau de recuperação e purificação de materiais biológicos. Esta técnica pode ser utilizada nas etapas iniciais de um processo de purificação, pois permite a remoção de contaminantes por um processo simples e econômico em substituição de técnicas complexas de separação sólido-líquido (Spelzini et al., 2005; Malpiedi et al., 2008).

Devido às inúmeras aplicações industriais, terapêuticas e biotecnológicas dos peptídeos do colágeno, a pesquisa por novas enzimas colagenolíticas capazes de degradar eficientemente o colágeno tem crescido nos últimos anos. Neste contexto, a colagenase produzida pelo *Penicillium aurantiogriseum* URM 4622 representa uma fonte alternativa desta enzima, considerando-se a sua obtenção com alto rendimento através da manipulação das condições de cultivo e da utilização do SDFA que permite a bioseparação desta enzima com elevado grau de purificação a baixo custo.

2. OBJETIVOS

2.1. Objetivo Geral

Produzir, caracterizar parcialmente e pré-purificar a colagenase do *Penicillium aurantiogriseum* URM-4622, com o intuito de aplicá-la na produção de peptídeos bioativos do colágeno bovino tipo I.

2.2. Objetivos Específicos

- Selecionar os parâmetros fermentativos mais significantes para a produção da colagenase pelo *Penicilium aurantiogriseum* URM 4622 através de um planejamento fatorial completo 2^4, no qual foi avaliada a influência das variáveis: temperatura, pH inicial do meio de cultura, velocidade de agitação orbital e concentração do substrato;
- Otimizar a produção da colagenase através de um planejamento fatorial completo 2^3, no qual foi avaliada a influência da temperatura, pH inicial do meio de cultura e concentração do substrato e de um planejamento central composto 2^2, no qual foi avaliada a influência do pH inicial do meio de cultura e da concentração do substrato;
- Caracterizar a colagenase no extrato bruto quanto aos aspectos físico-químicos, tais como: pH ótimo, temperatura ótima, estabilidade ao pH e à temperatura, ação de inibidores, especificidade a substratos e avaliação dos parâmetros termodinâmicos da enzima;
- Avaliar a influência das variáveis: massa molar do PEG (MM_{PEG}), concentração do PEG (C_{PEG}), concentração de fosfato (C_{FOSF}) e pH sobre a extração da colagenase utilizando o SDFA com auxílio de um planejamento fatorial (2^4), tendo o coeficiente de partição (K), a recuperação em atividade (Y) e o fator de purificação (FP) como as variáveis resposta;
- Estabelecer as melhores condições de purificação da colagenase utilizando o SDFA, após análise dos resultados do planejamento fatorial;
- Avaliar a influência das variáveis: pH, concentração de colágeno e temperatura sobre o grau de hidrólise do colágeno utilizando a colagenase pré-purificada através do SDFA;
- Identificar o perfil de peptídeos, obtidos sob as condições mais favoráveis de hidrólise, através da especrometria de massa;
- Avaliar a atividade antimicrobiana e antioxidante do poll de peptídeos.

3. CAPÍTULO I

1. REVISÃO DA LITERATURA

1.1. Colágeno

Os colágenos são os principais componentes da matriz extracelular na maioria dos tecidos dos vertebrados (Uitto et al., 2008). Compõem uma superfamília de proteínas fibrosas estruturais e insolúveis presentes em todos os organismos multicelulares e podem ser encontrados, por exemplo, na pele, nos ossos, nos tendões, nos dentes e nos vasos sanguíneos (Ravanti & Kähäri, 2000, Uito et al., 2008, Chung & Uitto, 2010).

Existem vários tipos de colágenos e estas macromoléculas são as proteínas mais abundantes no corpo humano. Quase 3% das proteínas totais encontradas nos mamíferos são colágenos. Estão presentes em todo o corpo e envolvidos em vários processos bioquímicos, fisiológicos e patológicos, como, por exemplo, no desenvolvimento ósseo fetal, no desenvolvimento embrionário, na reparação de feridas, na invasão de tumores malígnos, nas ulcerações intestinais, na inflamação periodontal crônica, na artrite reumatóide e na invasão de microrganismos patogênicos (Gelse et al., 2003; Watanabe, 2004; Nerenberg & Stultz, 2008).

Um total de 29 moléculas de colágenos geneticamente distintas têm sido até agora descritas nos tecidos de vertebrados e designadas por algarismos romanos de I a XXIX, de acordo com a ordem da sua descoberta (Myllyharju & Kivirikko, 2004, Söderhäll et al., 2007, Chung & Uitto, 2010).

Estruturalmente, as moléculas de colágenos são formadas por três cadeias polipeptídicas helicoidais, chamadas de cadeia α, superenoveladas. Enquanto que alguns colágenos são homotrímeros, formados por três cadeias α idênticas, outros podem ser heterotrímeros contendo duas ou mesmo três subunidades polipeptídicas geneticamente distintas. Conseqüentemente, existem muito mais do que 40 genes nos tecidos dos vertebrados que codificam as subunidades polipeptídicas diferentes (Myllyharju & Kivirikko, 2004; Watanabe, 2004; Uito et al., 2008).

Uma característica estrutural de todos os colágenos é a presença de um domínio da proteína em conformação em tripla hélice que oferece estabilidade a estas moléculas para servir como blocos estruturais de construção, proporcionando a integridade dos tecidos conjuntivos (Chung & Uitto, 2010). A unidade estrutural básica do colágeno é chamada de tropocolágeno, o qual possui uma massa molar em torno de 285 kDa e é constituído de três

cadeias polipeptídicas do mesmo tamanho que variam de acordo com o tipo de colágeno. A molécula do colágeno tipo I, por exemplo, é composta por duas cadeias polipeptídicas $\alpha_{1,}$ com massa molar de 100 kDa, e uma cadeia polipeptídica $\alpha_{2,}$ com massa molar de 95 kDa que são superenoveladas umas com as outras formando uma tripla hélice (Duarte et al., 2005).

O enovelamento das cadeias α individuais numa conformação de tripla hélice é baseado na característica da seqüência primária, que consiste numa seqüência tripeptídica repetitiva Gli-X-Y, onde as posições de X e Y são freqüentemente ocupadas por uma prolina e pela sua modificação pós-transducional a 4-hidroxiprolina, respectivamente (Uitto et al., 2008).

Em colágenos maduros, a glicina e a prolina, representam cada uma, 20% dos aminoácidos presentes. Este alto teor de prolina não é comum em relação aos montantes observados em outras proteínas naturais. Além disto, a prolina é um aminoácido peculiar devido à estrutura do anel da pirrolidina, que impõe restrições rígidas sobre a rotação N-Cα. Assim, a ligação peptídica da prolina constitui uma das mais difíceis de ser hidrolisada e resiste à proteólise inespecífica, tais como a digestão com a pepsina. Devido à sua estrutura rígida, a degradação enzimática do colágeno é restrita a um limitado número de proteases e é obtida utilizando as colagenases, uma classe de proteinases altamente específica para o colágeno (Watanabe, 2004; Uitto et al., 2008; Chung & Uitto, 2010).

Em alguns colágenos, como no colágeno tipo I, o mais abundante, encontrado na pele e nos ossos, seu domínio central de cada uma das três cadeias, contém um segmento de repetição ininterrupta Gli-X-Y abrangendo cerca de 1000 aminoácidos. Em outros colágenos, como no tipo IV (o colágeno da membrana basal) e tipo VII (o colágeno de ancoragem das fibrilas), a seqüência de repetições Gli-X-Y contém imperfeições que interrompem a conformação em tripla hélice (Uitto et al., 2008). Estas interrupções podem proporcionar flexibilidade às moléculas de colágeno e fornecem também locais suscetíveis à clivagem proteolítica inespecífica da seqüência primária (Chung & Uitto, 2010).

Em função da sua arquitetura fibrilar, nos tecidos, as moléculas de colágenos geneticamente distintas podem agrupar-se em diferentes subgrupos (Uitto et al., 2008). Os colágenos tipo I, II, III, V e X alinham-se em grandes fibrilas extracellular e são designadas de colágenos fibrilares. O colágeno tipo IV é organizado em uma rede entrelaçada dentro da membrana basal, enquanto que o colágeno tipo VI forma microfibrilas distintas e o tipo VII forma fibrilas de ancoragem. Os colágenos associados às fibrilas com tripla hélice interrompidas incluem os tipos IX, XII, XIV, XIX, XX e XXI. Vários destes tipos de

colágenos associam-se com as fibras de colágeno maior e servem como pontes moleculares, estabilizando a organização da matriz extracelular (Olsen, 1995; Chung & Uitto, 2010).

Os principais colágenos da pele humana são do tipo I e do tipo III e representam cerca de 80% e 10% do volume total de colágeno, respectivamente (Tabela 1). Estes dois colágenos associam-se para formar grandes fibras extracelulares, característica da derme humana. O colágeno tipo V está presente na maioria dos tecidos conjuntivos, incluindo a derme, onde representa menos de 5% do colágeno total. Na derme, o colágeno tipo V está localizado na superfície das fibras de colágeno formadas por grandes colágenos (tipo I e III), e regula o crescimento lateral destas fibras. Outro colágeno presente na pele sob a forma de grandes fibras, na junção dermo-epidérmica e na membrana basal vascular, é o colágeno tipo IV (Chung & Uitto, 2010).

Tabela 1. Heterogeneidade genética do colágeno humano.

Tipo de Colágeno	**Composição da Cadeia**	**Estrutura supramolecular**	**Distribuição tecidual[b]**
I	$[\alpha_1(I)_2\ \alpha_2(I)$	Fibrilar	Derme, ossos e tendões
III	$[\alpha_1(III)]_3$	Fibrilar	Derme fetal, vasos sanguíneos trato gastrointestinal
IV	$[\alpha_1(IV)]_2\alpha_2(IV)$[a]	Membrana Basal	Geral
V	$[\alpha_1(V)]_2\alpha_2(V)$[a]	Fibrilar	Geral
VI	$\alpha_1(VI)\alpha_2(VI)_3(VI)$[a]	Microfibrilas	Geral
VII	$[\alpha_1(VII)]_3$	Fibrilas de Ancoragem	Fibras de Ancoragem
VIII	$[\alpha_1(VIII)]_3$	Formas em rede	Endotélio
XIII	$[\alpha_1(XIII)]_3$	Transmembrana	Geral incluindo epiderme
XIV	$[\alpha_1(XIV)]_3$	FACIT	Pele e córnea
XV	$[\alpha_1(XV)]_3$	Membrana Basal	Geral
XVII	$[\alpha_1(XVII)]_3$	Transmembrana	Hemidesmossomos na pele, córnea, e membranas mucosas
XXIX	Não conhecida	Não conhecida	Epiderme

[a] Cadeias adicionais identificadas;

[b] Menores quantidades podem estar presentes em outros tecidos. Fonte: Chung & Uitto, 2010.

O colágeno tipo II é predominantemente encontrado na cartilagem articular e é a proteína mais abundante neste local, representando cerca de 15 a 25% do peso úmido, 50% do peso seco e mais de 90% do conteúdo de colágeno total (Goldring, 2008). O colágeno tipo II, com a sua estrutura molecular específica é utilizado em diversas aplicações alimentares (por exemplo, como agente de clarificação e emulsificante). A sua utilização estende-se ainda a outras aplicações industriais (xampus e batons) e farmacêuticas (microencapsulação e revestimento de comprimidos). Hoje, há uma demanda crescente pelo colágeno tipo II já que as pesquisas sugerem que este colágeno pode suprimir a artrite reumatóide (AR) e promover articulações saudáveis (Cao & Xu, 2008).

Os colágenos são biomateriais com potencialidade para aplicações biotecnológicas devido ao seu significativo envolvimento em muitos eventos celulares. Consequentemente as aplicações comerciais do colágeno estão em rápido crescimento. Por exemplo, o colágeno vem sendo adicionado regularmente como ingrediente na preparação de medicamentos, bebidas, alimentos, cosméticos, biomateriais e em uma variedade de produtos para a saúde (Watanabe, 2004; Uesugi et al., 2008).

1.1.1. Peptídeos do colágeno

Os produtos resultantes da degradação do colágeno, os peptídeos do colágeno, apresentam diversas atividades biológicas de interesse médico e industrial, levando ao estabelecimento de um amplo campo de aplicações em rápido crescimento (Uesugi et al. 2008). Na medicina, estes peptídeos são utilizados como agentes para a osteoporose (Uesugi et al., 2008), agentes imunoterápicos (Goshev et al., 2005) e no tratamento de úlceras gástricas (Uesugi et al., 2008) e da hipertensão (McAlindon et al., 2011). Na indústria farmacêutica eles são componetes de cremes hidratantes para a pele, ingredientes para alimentação parenteral e são também utilizados como conservantes não alérgicos em medicamentos (Nakayama et al., 2000).

Pesquisas recentes têm demonstrado que os peptídeos do colágeno são eficazes no tratamento da artrite induzida pelo colágeno (Zhu et al. 2007), apresentam efeitos benéficos contra a diabetes e a hipertensão (Zhu et al. 2010), facilitam a aprendizagem e melhoraram a memória por reduzirem os danos oxidativos ao cérebro (Pei et al. 2010), são capazes de inibir a atividade da enzima conversora da angiotensina I (ECA) (Wen-hang et al. 2007) e apresentam atividade anti-oxidante (Li et al. 2007) e antimicrobiana (Gómez-Guillén et al. 2011).

Para a preparação de peptídeos de colágeno, o método de clivagem química que é comumente utilizado requer reações altamente reativas que utilizam o brometo de cianogênio (CNBr) como catalisador. Portanto, as enzimas colagenolíticas, que apresentam elevada atividade e especificidade e são estáveis, são as preferidas para a produção de peptídeos do colágeno com aplicações médicas e industriais (Watanabe, 2004; Uesugi et al. 2008).

1.2. Colagenases

1.2.1. Definição, classificação e fontes

As colagenases são definidas como proteases capazes de degradar a tripla hélice do colágeno nativo e desnaturado e estão envolvidas em vários processos fisiológicos e patológicos, tais como o desenvolvimento ósseo fetal, desenvolvimento embrionário, cicatrização, artrite reumatóide, invasão de tumor maligno, ulceração intestinal e a inflamação periodontal crônica (Kang et al., 2005; Jansen et al., 2007; Gutierrez-Fernandez et al., 2007; Wu et al., 2010).

As proteases colagenolíticas são classificadas em dois grandes grupos: as metalocolagenases e as serinocolagenases. A hidrólise do colágeno pelas metaloproteases ocorre principalmente entre a ligação peptídica do resíduo X e Gli-Pro (Kang et al., 2005). As metalocolagenases foram inicialmente descobertas em explantes de tecidos de girinos e são enzimas que contêm zinco, mas que geralmente também necessitam de cálcio para apresentarem uma melhor atividade e estabilidade (Gross & Lapiere, 1962; Peterkofsky, 1982), e estão envolvidas no remodelamento da matriz extracelular (Kang et al., 2005; Parks, 2007; Gutierrez-Fernandez et al., 2007).

Por outro lado, as serinocolagenases foram primeiramente isoladas do hepatopâncreas de um caranguejo (*Uca pugilator*) e estão provavelmente envolvidas na digestão dos alimentos ao invés da morfogênese (Eisen et al., 1973), bem como na produção de hormônios e de peptídeos farmacologicamente ativos, e em várias outras funções celulares, tais como na digestão de proteínas, na fibrinólise, na coagulação do sangue e na fertilização (Kang et al., 2005).

As colagenases são produzidas por animais (tecidos de vertebrados, caranguejos, larvas de moscas e insetos) (Goshev et al., 2005; Wu et al., 2010), vegetais (Kim et al., 2007) e microrganismos (Wu et al., 2010). No entanto, os microrganismos são uma fonte preferida destas proteases por causa de sua ampla diversidade bioquímica e da sua susceptibilidade à manipulação genética (Sandhya et al., 2005).

O modo de ação destas enzimas parece ser dependente da fonte da qual é obtida. As colagenases bacterianas clivam a ligação peptídica entre X-Gli (onde X é freqüentemente um aminoácido neutro) da sequência Gli-Pro-X-Gli-Pro-X na região não polar da molécula do colágeno. As colagenases produzidas pelo *Clostridium histolyticum* clivam o colágeno em múltiplos sítios, enquanto que as colagenases produzidas pelos fibroblastos humanos clivam a molécula de colágeno em um único sítio (Leu-ILeu) produzindo dois fragmentos de ¼ e ¾ (Kanth et al., 2008, Huebner et al., 2010) (Figura 1). Kim et al. (2007) identificaram duas colagenases extraídas do rizoma de gengibre (*Zingiber officinale*) que foram capazes de clivar o colágeno tipo I em múltiplos sítios, os quais são encontrados no interior da tripla hélice desta molécula.

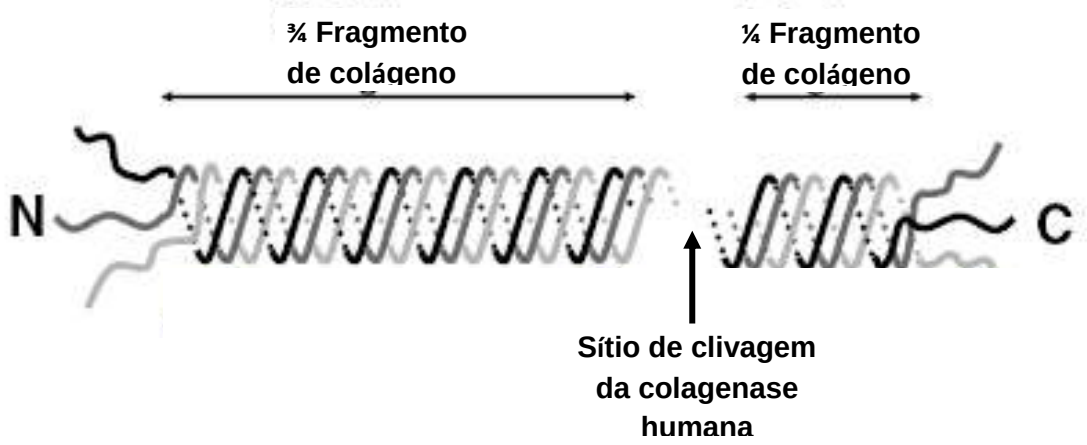


Figura 1. Representação esquemática do sítio de clivagem da colagenase humana. Fonte: Huebner et al., 2010.

1.2.2. Colagenases de origem microbiana

No passado, era dada muita atenção ao isolamento de colagenases de tecidos de origem animal. Entretanto, nos últimos anos, vem sendo enfatizada a obtenção de colagenases de origem microbiana, já que estas enzimas apresentam vantagens quando comparadas às de origem animal. A disponibilidade de substratos de baixo custo, as inesgotáveis fontes naturais de microrganismos e a necessidade de procedimentos simples de isolamento e purificação podem ser citadas como algumas dessas vantagens (Suhosyrova et al., 2003; Wu et al., 2010).

Outra vantagem das colagenases de origem microbiana em relação às de origem animal diz respeito à forma como hidrolisam o colágeno. As colagenases de origem animal possuem a capacidade de clivar somente o colágeno nativo em um único sítio, isto é, são enzimas que clivam a cadeia helicoidal do colágeno resultando em dois fragmentos (Figura

2), acessíveis a poucas proteases específicas, o TCA (tropocolágeno A) e o TCB (tropocolágeno B). Diferentemente das anteriores, as colagenases de microrganismos possuem a capacidade de hidrolisar tanto o colágeno nativo como o colágeno desnaturado. Têm afinidade por vários sítios ao longo da cadeia, representando assim, uma fonte promissora para pesquisas e aplicações biotecnológicas (Jung & Winter, 1998; Hamdy, 2008).

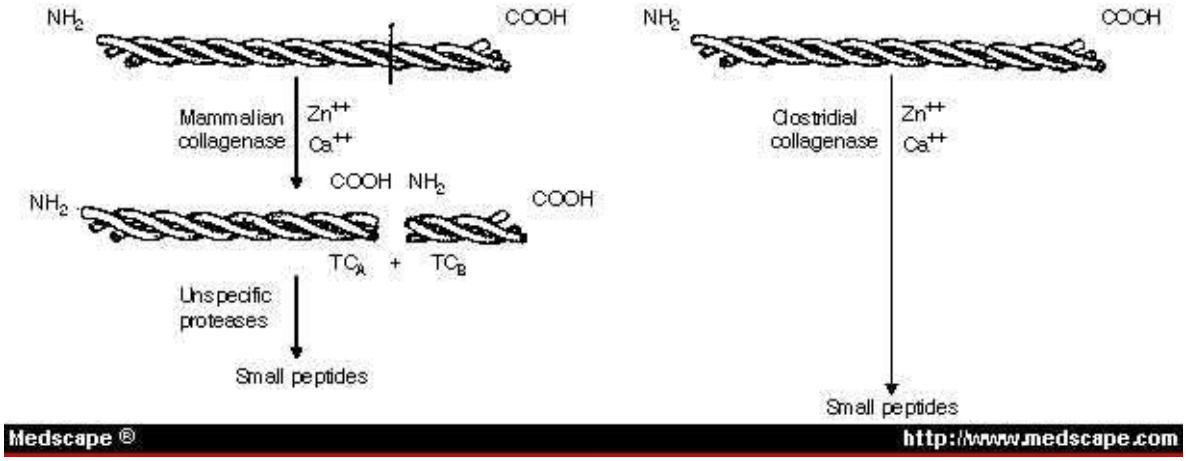


Figura 2. Ação das colagenases de origem animal x colagenases de *Clostridium* sp. Fonte: Watanabe, 2004.

Atualmente, a literatura sobre a produção de colagenases microbianas é bastante vasta. Estas apresentam diferenças de acordo com o grupo de microrganismos produtores, substratos utilizados na produção, características bioquímicas e físico-químicas, especificidade a substratos e estrutura molecular (Suhosyrova et al., 2003; Tsuruoka et al., 2003; Kang et al. 2005; Petrova et al., 2006; Lima et al., 2009; Sakurai et al., 2009; Wu et al., 2010).

A metalocolagenase produzida pelo *Aspergillus Flavus* em meio contendo 2% de colágeno apresentou pH e temperatura ótimos de 7,4 e 30 ºC, respectivamente. Esta enzima foi inibida pelo EDTA e apresentou uma massa molar de 24 kDa (Suhosyrova et al., 2003). Quanto à serinocolagenase produzida pelo *Alicyclobacillus sendaiensis* em meio contendo batata apresentou atividade ótima a pH ácido (pH 3,9) e à temperatura de 60 ºC. Esta enzima foi capaz de degradar o colágeno tipo I e o azocoll e apresentou um peso molecular de 37 kDa (Tsuruoka et al., 2003).

Kang et al. (2005) identificaram uma colagenase com massa molar de 35 kDa produzida pelo *Vibrio vulnificus* em meio contendo 4% de gelatina que apresentou uma atividade máxima a pH 7,5 e a 35 ºC e que foi inibida pela 10-fenantrolina e pelo EDTA. Petrova et al (2006) isolaram do solo da Bulgária uma linhagem de *Streptomyces* sp. que

produziu duas colagenases, com pesos moleculares de 116 kDa e 97 kDa, após a fase exponencial de crescimento em meio contendo amido e ambas apresentaram atividade ótima a pH 7,5 e à temperatura de 37 ºC.

Lima et al. (2009) identificaram uma colagenase produzida pela *Candida albicans* em meio contendo 2% de gelatina que apresentou uma atividade máxima a pH 8,2 e à temperatura de 45 ºC. Uma colagenase produzida pelo *Streptomyces parvulus* apresentou pH ótimo em torno de 9,0 e a temperatura ótima de 37 ºC. Esta enzima foi fortemente inibida por agentes quelantes e apresentou uma massa molecular de 52 kDa (Sakurai et al. 2009). Wu et al. (2010) isolaram um linhagem do *Bacillus pumilus* das águas residuais de uma indústria do couro capaz de produzir uma colagenase em meio contendo 2% de gelatina e que apresentou massa molecular de 58,6 kDa e atividade máxima a pH 7,5 e 45 ºC de temperatura. Esta enzima mostrou uma atividade altamente específica para o colágeno nativo da pele.

Entre os microrganismos, os fungos filamentosos apresentam vantagens como produtores de enzimas, tais como: o baixo custo dos meios utilizados associados a uma alta produtividade. Além disto, as suas enzimas hidrolíticas são liberadas extracelularmente, o que torna a sua recuperação a partir de caldo de fermentação particularmente fácil (Vishwanatha et al., 2010). Na literatura, já existem trabalhos que relatam a produção de colagenases por fungos pertencentes aos gêneros *Aspergillus* (Suhosyrova et al. 2003), *Cladosporium, Alternaria* e *Penicillium* (Yakovleva et al., 2006).

1.3. *Penicilium aurantiogriseum*

O *Penicillium* é um gênero complexo de fungos que apresentam mais de 225 espécies em conformidade com determinados critérios morfológicos (Clemmensen et al., 2007). Muitas espécies deste gênero são utilizadas para a produção de medicamentos importantes, como, por exemplo, a penicilina, enquanto que outros são utilizados para a fermentação de alimentos, como o *Penicillium roqueforti,* o bolor dos queijos azuis, o *P. camemberti*, o bolor dos queijos brancos, e o *P. nalgiovense*, o bolor de salames fermentados (Frisvad & Samson, 2004). Muitas outras espécies podem causar a deterioração dos alimentos e a deterioração de outros materiais armazenados (Samson et al., 2004; Rand et al., 2005). Várias espécies do gênero *Penicillium* podem, além disso, produzir enzimas hidrolíticas como xilanases (Meshram et al., 2008), lipases (Yang et al., 2010) e proteases (Boutrou et al., 2006).

Entre as espécies do gênero, o *Penicillium aurantiogriseum* é um fungo filamentoso encontrado como típico contaminante de cereais e seus produtos derivados. Apresenta, em geral, temperatura mínima de crescimento perto de 21ºC e máxima em 30ºC, sendo a

temperatura ótima de 23ºC (Zardetto et. al, 2005). O seu crescimento pode ser estimulado com a concentração de 10% de CO_2 e segue crescendo com até 30% de dióxido de carbono, embora nestas condições, ocorra um aumento da fase lag pela diminuição do ritmo de crescimento (Zardetto et. al, 2004).

Vários trabalhos têm demonstrado que espécies do gênero *Penicillium* são produtores potenciais de proteases. Germano et al. (2003) e Agarwal et al. (2004), relataram o uso do *Penicillium sp.* utilizando a soja como fonte de carbono e nitrogênio para a produção de proteases, enquanto que Yakovleva et al. (2006) identificaram a produção de enzimas com atividade colagenolítica em três espécies de *Penicillium*: *Penicillium janthinellum, Penicillium chrysogenum e Penicillium citrinum.*

1.4. Aplicações das colagenases

As colagenases microbianas têm apresentado aplicações práticas em todo o mundo e são alternativas viáveis nos laboratórios de pesquisas científicas, na medicina, e nas indústrias farmacêuticas, cosmecêuticas, alimentícia e do couro. As aplicações destas enzimas podem ser divididas em duas categorias: (1) aquelas em que as colagenases são utilizadas diretamente, e (2) aquelas em que são utilizados os produtos resultantes da sua ação (Watanabe, 2004).

Várias formulações de colagenases produzidas pelo *Clostridium histolyticum* (Ch-Col) estão disponíveis comercialmente. Apesar das preparações comerciais da Ch-Col ainda não figurarem entre os maiores volumes de vendas de produtos enzimáticos (Van Wart 2004), uma crescente busca por estas preparações está em curso devido às suas inúmeras aplicações clínicas (Tamai et al., 2008).

Na medicina, as colagenases microbianas têm sido diretamente utilizadas em tratamentos clínicos de diversas doenças. A sua utilização terapêutica direta abrange:

(1) O tratamento da Doença de Peyronie (Jordan 2008) e da Doença de Dupuytren (Watt et al. 2010).

(2) O tratamento de feridas necrosadas, escaras, cicatrizes pós-operatórias, e o tratamento de psoríase e pediculoses (Erdeve et al., 2007; Markovich, 2008), em cicatrizes de queimaduras de crianças (Özcan et al., 2002), em lesões de mamilos de mulheres em aleitamento (Kuşcu et al., 2002) e o tratamento de cicatrizes hipertróficas (Cheng et al., 1999);

(3) Estudos terapêuticos pré-clínicos em diferentes tipos de fibroses destrutivas, como na cirrose hepática (Jin et al. 2005), oclusões crônicas arteriais (Segev et al. 2005) e aderência abdominal pós-operatória (Tander al. 2007);

(4) O isolamento e transplante de células, como células da pele (Wang et al. 2004), hepatócitos (Ouji et al. 2010), células das ilhotas pancreáticas (Shimoda et al. 2010), e cardiomiócitos (Neumann et al. 2007) e,

(5) O tratamento da hérnia de disco intervertebral (Chu, 1987), o tratamento da retenção de placenta (Eiler e Hopkins, 1993; Fecteau et al. 1998), e como pré-tratamento para melhorar a terapia gênica no câncer mediado pelo adenovírus (Kuriyama et al. 2000).

Giuliano et al. (2010) realizaram um estudo no qual avaliaram as características do semem tratado e não tratado com colagenase para sua posterior aplicação em técnicas de fertilização assistida. Os resultados do referido estudo mostraram que o tratamento do sêmen com 0,1% de colagenase melhorou as suas propriedades reológicas, como também facilitou a separação dos espermatozóides do plasma seminal, promoveu uma progressiva motilidade espermática, mantendo a funcionalidade e integridade da membrana dos espermatozóides. Os autores deste trabalho concluiram que o protocolo utilizado nesta pesquisa pode ser usado para produção de embriões *in vitro*.

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

Kuşcu et al. (2002), desenvolveram um trabalho utilizando a colagenase do *Clostridium histolyticum* na prevenção e tratamento de lesões do mamilo de mulheres em período de aleitamento, as quais se desenvolvem imediatamente após o parto, podendo provocar dores intensas no mamilo, impedindo até a continuidade do aleitamento. Os resultados foram semelhantes aos obtidos com os tratamentos convencionais mostrando assim a ação eficaz da colagenase neste tipo de lesão.

Segundo Jung & Winter (1998) existem inúmeras vantagens na utilização de colagenases de microrganismos no tratamento de feridas:

a) Removem o tecido necrosado com maior eficiência pela sua capacidade de hidrolisar vários tipos de colágeno;
b) São indolores e não hemorrágicos;
c) Podem ser utilizadas por longos períodos de tempo e também, em associação com outros medicamentos;
d) Atraem macrófagos e fibroblastos para o local da ferida;
e) Aumentam a formação de tecidos de granulação e estimulam o próprio organismo a promover a cicatrização.

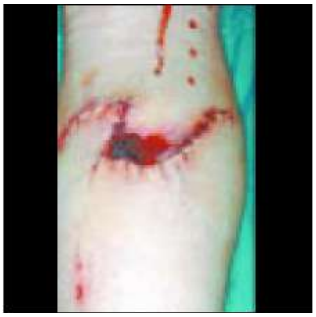
Alguns exemplos de sucesso da utilização de pomadas contendo colagenase estão ilustrados na Figura 3.

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

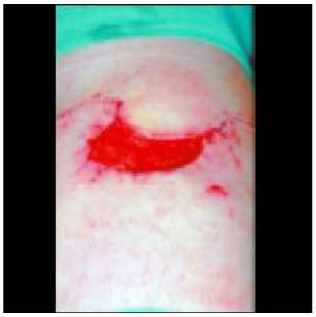
Em laboratório, as proteases colagenolíticas têm sido utilizadas na preparação de células isoladas de fígado de rato e na cisão de peptídeos de colágeno em proteínas de fusão (Watanabe 2004). Nishi et al., (1998) observaram que ao fundir proteínas ou peptídeos (por exemplo, fatores de crescimento) ao domínio de ligação do colágeno da colagenase do *C. histolyticum* (ColH) ocorria a formação de sistemas de entrega dessas proteínas, que não são difusíveis e apresentaram longa duração *in vivo*. As colagenases microbianas também são utilizadas para hidrolisar a cartilagem da traquéia bovina que permite a preparação de células intactas de mamíferos em cultura e na limpeza de células sanguíneas para melhorar o rastreamento em diagnósticos médicos (Kim et al., 2007). Matsushita et al (2001) realizaram um estudo onde utilizaram colagenases para ancorar moléculas de sinalização em tecidos contendo colágeno, apresentando um grande potencial como sistema de entrega de drogas contra a artrite e o câncer.

Proteases com atividade colagenolítica vêm sendo utilizadas na indústria de alimentos no processo de amaciamento de carnes o que resulta na produção de produtos derivados de carne com melhor sabor e textura (Benito et al., 2002).

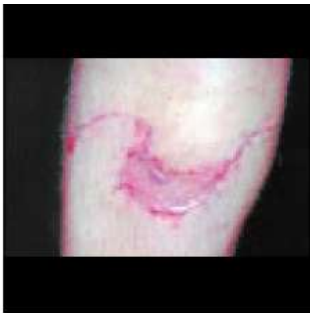
Caso 1. Paciente com 40 anos de idade com uma cirurgia devido a um câncer no membro inferior.



a. Necrose parcial

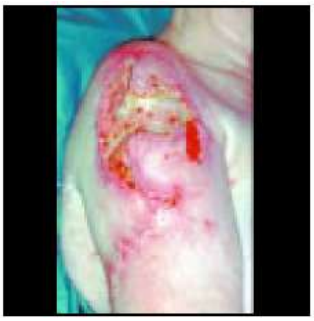


b. 2 semanas após. necrotomia e subseqüente aplicação da pomada com colagenase.

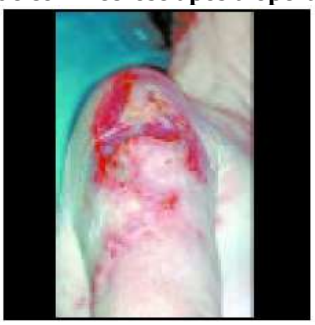


c. Após 4 semanas. Cicatrização quase completa.

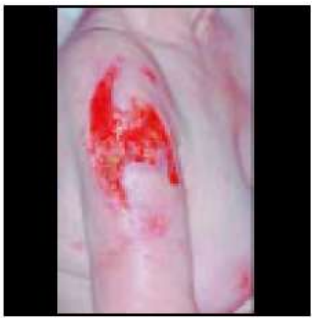
Caso 2. Paciente com 71 anos de idade com necrose após a operação de um tumor no ombro direito.



a. Necrose parcial da pele uma semana após a operação. Presença de ulceração profunda.

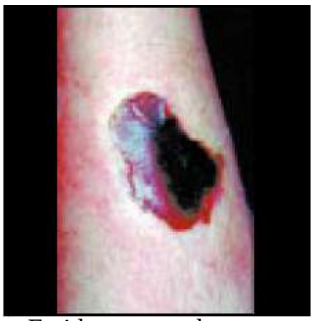


b. Início do desbridamento enzimático com pomada de colagenase após necrotomia, e oclusão com curativo de poliuretano.

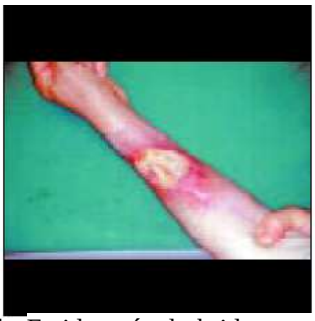


c. Após 10 dias de tratamento com pomada de colagenase. O tecido de granulação está bem estabelecido e o ferimento está pronto para enxertia.

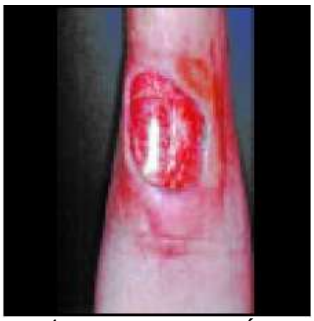
Caso 3. Paciente com 65 anos de idade sob tratamento citostático.



a. Ferida necrosada no ante-braço.



b. Ferida após desbridamento cirúrgico. Antes do início do tratamento com a pomada de colagenase.



c. 4 semanas após o tratamento com a pomada de colagenase. A ferida está pronta para o enxerto.

Figura 3. Casos clínicos ilustrando o sucesso da utilização de pomadas contendo colagenase.

Fonte: Watanabe, 2004.

Trabalhos recentes têm demonstrado que as colagenases podem ser utilizadas como uma alternativa viável no processo de tingimento empregado pela indústria do couro, devido ao fato de serem biocatalisadores atóxicos e ecologicamente compatíveis. Este processo emprega muitos corantes sintéticos que podem sofrer exaustão incompleta com a consequente eliminação de corantes através dos efluentes. Desta forma, trabalhos têm sido realizados no intuito de aumentar a absorção desses corantes pelo couro durante o seu processamento o que acarreta em uma menor eliminação de corantes para o meio ambiente. Através da utilização de colagenases durante o tingimento do couro é possível obter quase 100% de absorção do corante devido à abertura das fibras de colágeno. Além disto, características como maciez, suavidade e aparência foram melhoradas após o tratamento enzimático. A utilização de colagenases neste processo fornece assim um novo horizonte para o processo de tingimento ecológico (Kanth et al. 2008).

1.5. Fatores que influenciam a produção de proteases microbianas

A otimização de processos é um assunto de importância central nos processos de produção industrial. Nomeadamente, no que diz respeito aos processos de produção biotecnológicos, em que mesmo pequenas melhorias podem ser decisivas para o sucesso comercial, o processo de otimização é atualmente uma componente indiscutível da agenda de qualquer interesse comercial. Na tecnologia da fermentação, a melhoria da produtividade do metabolito microbiano é realizada, em geral, através da manipulação de parâmetros nutricionais e físico-químicos dos meios de cultura e por melhorias nas linhagens microbianas como resultado da seleção por mutação. Estas ações podem alterar significativamente o rendimento do produto (Reddy et al., 2008).

A seleção de microrganismos e a otimização da composição dos meios de cultura possibilitam a obtenção de elevados rendimentos e enzimas com propriedades e especificidades bem determinadas (Rodrigues & Santánna, 2001). É bem conhecido que a produção de protease extracelular por microrganismos é fortemente influenciada por componentes do meio, especialmente fontes de carbono e nitrogênio, fatores físico-químicos, tais como pH, temperatura, velocidade de agitação orbital e tempo de incubação. Além disso, a produção também pode ser influenciada pelo tamanho do inoculo (Lima et al., 2009). No entanto, não existe nenhum meio estabelecido para a produção máxima de proteases de diferentes fontes microbianas, pois cada microrganismo apresenta características bioquímicas e fisiológicas próprias e requer diferentes condições de cultivo para a produção enzimática máxima (Rao et al., 2006).

1.5.1. Concentração do substrato

O custo do meio de produção representa cerca de 30-40% do custo da produção industrial de enzimas. Considerando-se este fato, a utilização de meios de cultivo econômicos para a produção de proteases microbianas é especialmente importante. Para este efeito, a farinha de soja tem sido reconhecida como um ingrediente potencialmente útil e rentável, já que é produzida em grande parte como um subproduto durante a extração do óleo de soja. Além disso, a análise química deste substrato mostrou que é composto por aproximadamente 40% de proteína e é rico em outros componentes orgânicos e inorgânicos, sugerindo-o como um bom candidato para meios de cultura (Joo et al., 2002).

As fontes de carbono e nitrogênio fazem parte da composição dos meios de cultura e são nutrientes essenciais para o crescimento dos microrganismos. Com base na literatura, um dos parâmetros mais importantes que influenciam os processos fermentativos é a concentração de substrato ou fontes de nitrogênio e de carbono utilizados para o crescimento do microrganismo (Elibol et al., 2005). O efeito da concentração do substrato como indutor para a produção de protease varia de um microrganismo para outro, entretanto, fontes de nitrogênio complexas são geralmente preferidas. Entre estas, a utilização de farinha de soja como fonte de nitrogênio tem sido uma alternativa economicamente atraente para a produção industrial destas enzimas, já que é um substrato com custo menor e prontamente disponível composto de aproximadamente 40% de proteína (Chellappan et al., 2006; Wang et al., 2008; Lima et al., 2009).

Vários trabalhos têm reportado a influência da concentração do substrato na produção de proteases por microrganismos. Laxman et al. (2005) observaram um aumento na produção de protease pelo *Conidiobolus coronatus* quando a concentração da farinha de soja foi aumentada de 1% para 2%. Por outro lado, a produção máxima de protease a partir do *Aspergillus flavus* (Srinivasan & Dhar, 1990), *Bacillus subtilis* (Chu, 2007) e do *Conidiobolus coronatus* (Sutar et al., 1992) foi obtida utilizando 0,8-1,2, 1,5 e 4% de soja, respectivamente.

1.5.2. pH inicial do meio de cultura

O pH inicial do meio de cultura é um parâmetro físico-químico que influencia muitos processos bioquímicos tais como, a expressão e secreção de enzimas e o transporte de substâncias através da membrana celular (Anandan et al., 2007). Devido a este fato, muitos trabalhos vêm sendo realizados para estudar a influência do pH inicial do meio de cultura na produção de proteases. Lima et al. (2009) observaram um aumento na produção de uma colagenase produzida pela *Candida albicans* com o aumento do pH inicial do meio de cultura

de 5,0 para 7,0. Da mesma forma, Patel et al. (2005) observaram uma melhoria progressiva da produção de uma protease extracelular produzida pelo *Bacillus* sp., com o aumento do pH inicial do meio de cultura obtendo uma produção máxima de 185 U/mL a pH 9.0.

Chi et al. (2007) também observaram que o pH inicial do meio de cultura influenciou a produção de uma protease produzida por uma levedura marinha, *Aureobasidium pullulans*, e obtiveram a maior produção desta enzima a pH 6.0. Finalmente, *Roseobacter* sp. MMD040 produziu uma maior concentração de protease a pH 7,0, e manteve quase o mesmo rendimento a pH 9,0 (Shanmughapriya et al., 2008). Em conjunto, estes resultados confirmam a importância do pH inicial do meio de cultura para a produção de proteases microbianas, entretanto o valor ideal desse parâmetro é dependente da espécie microbiana e do sistema de fermentação utilizado.

1.5.3. Temperatura

A temperatura é um dos parâmetros mais críticos que precisa ser controlado no processo fermentativo (Chi & Zhao, 2003). Na verdade, a temperatura de incubação tem um profundo efeito sobre a duração da fase de síntese da enzima e sobre o fluxo de metabólitos (Chellappan et al., 2006, Wang et al., 2008). Além disso, a temperatura não regula apenas a síntese das enzimas, mas possivelmente a secreção destas enzimas, alterando as propriedades da membrana celular (Anandan et al., 2007).

Vários autores têm realizado estudos variando a temperatura de incubação no processo fermentativo com o objetivo de otimizar a produção de proteases microbianas. Para dar apenas alguns exemplos, Laxman et al. (2005) investigaram o efeito da temperatura (25-50 ºC) na produção de uma protease por um fungo filamentoso (*Conidiobolus coronatus*) e observaram que a produção máxima foi obtida a 28 ºC e baixos níveis de atividade foram produzidos a 37 ºC. A 50 ºC, nenhum crescimento foi observado e consequentemente não ocorreu produção da enzima.

Abidi et al., (2008) observaram que a produção máxima de uma protease alcalina de *Botrytis cinera* foi encontrada a 28 ºC. A 38 ºC, nenhum crescimento de *Botrytis cinera* foi observado e por isso a atividade foi muito baixa.

Wang et al., (2008) realizaram um estudo de otimização da produção de uma protease pele bactéria *Colwellia* sp. utilizando a metodologia de superfície de resposta, a qual indicou que a temperatura apresentou o maior efeito na produção da protease. A produção máxima ocorreu a temperaturas entre 16 a 20 ºC. Segundo estes autores, possivelmente, temperaturas

mais baixas promoveram um adequado fluxo de metabólitos o que resultou em uma maior secreção de proteases, o que foi confirmado pela máxima utilização de substrato protéico.

1.5.4. Velocidade de agitação orbital

A velocidade de agitação orbital é outro componente físico importante nos processos fermentativos devido à influência que exerce na síntese microbiana de proteases extracelulares. Na fermentação aeróbia, a transferência de oxigênio é uma variável-chave e é uma função da aeração e da velocidade de agitação. Portanto, é necessário estabelecer uma combinação ótima entre o fluxo de ar e a agitação. A agitação influencia não só a transferência de massa de oxigênio como também a força de cisalhamento sobre os microrganismos (Portumarthi et al., 2007).

Vários trabalhos têm demonstrado que a produção de proteases pode ser otimizada com o aumento da velocidade de agitação (Portumarthi et al., 2007; Rao et al., 2006; Lima et al., 2009). Entretanto, altas velocidades devem ser evitadas uma vez que um aumento drástico deste parâmetro pode ter conseqüências negativas, como a ruptura das células, alterações morfológicas e autólise o que leva a uma diminuição da produtividade (Rao et al., 2006).

1.6. Utilização de planejamentos experimentais como ferramenta para a otimização da produção de proteases microbianas

O custo da produção de enzimas é um grande obstáculo para o sucesso da sua aplicação industrial. Por este motivo existe uma busca crescente por microrganismos que produzam proteases com altos rendimentos. A seleção do microrganismo e a otimização da composição do meio de cultura possibilitam a obtenção de elevados rendimentos. As abordagens estatísticas representam uma ferramenta útil para estudos de processos de produção em biotecnologia devido à variedade de vantagens reconhecidas com a sua utilização (Reddy et al., 2008).

A otimização das condições de cultivo através de métodos clássicos envolve a mudança de apenas uma variável de cada vez mantendo os outros fatores constantes. Estes métodos envolvem assim muito tempo e por este motivo são dispendiosos e incapazes de detectar a verdadeira condição ideal, principalmente devido à incapacidade de detectarem as interações entre os fatores em estudo. Em contra partida, os métodos estatísticos de otimização necessitam de menos tempo e de um menor número de dados experimentais e ainda são capazes de detectar as interações das variáveis estudadas. Além disso, os

procedimentos estatísticos têm vantagens devido à utilização dos princípios fundamentais da estatística: randomização, replicação e duplicação (Wang et al., 2008; Oskouie et al., 2008).

A Metodologia de Superfície de Resposta (MSR) é um dos procedimentos de otimização comumente utilizado, desenvolvido com base em um planejamento fatorial completo do tipo central composto. A MSR é uma coleção de técnicas matemáticas e estatísticas que são úteis para modelagem e análise onde uma resposta de interesse é influenciada por várias variáveis e o objetivo é otimizar esta resposta. O primeiro passo na MSR é encontrar uma aproximação adequada para a verdadeira relação entre a resposta e as variáveis independentes. Normalmente um polinômio de ordem baixa em alguma região das variáveis independentes é empregado para a modelagem. Se a resposta é bem modelada por uma função linear das variáveis independentes, então a função de aproximação é de primeira ordem. Se existir uma curvatura no sistema, então um polinômio de grau mais elevado deve ser utilizado, tal como o modelo de segunda ordem. Esta metodologia ajuda a identificar as variáveis que influenciam as respostas e as interações entre as variáveis, permite ainda selecionar as melhores condições, quantificar a relação entre uma ou mais respostas medidas e os fatores mais importantes em número limitado de experimentos (Oskouie et al., 2008; Reddy et al., 2008; Singh & Chhatpar, 2010).

Há uma crescente aceitação para a utilização de abordagens estatísticas experimentais em biotecnologia. Muitos trabalhos têm reportado a otimização satisfatória da produção de proteases de origem microbiana utilizando a abordagem estatística (Wang et al., 2008; Oskouie et al., 2008; Reddy et al., 2008; Oskouie et al., 2008; Raí & Mukherjee, 2010). A aplicação do planejamento estatístico para a seleção e otimização das condições de cultivo permite uma rápida identificação dos fatores importantes e as interações entre eles (Raí & Mukherjee, 2010).

Oskouie et al. (2008) realizaram um planejamento central composto para otimizar a composição do meio de cultura e explicar os efeitos combinados de três composições diferentes de meios para a produção de uma protease alcalina produzida pelo *Bacillus claussi*. A produção máxima de protease alcalina foi de 1.520 U/mL após 42 h de incubação representando um aumento de 1,5 vezes na produção de protease sobre o ponto central e um aumento global de 6 vezes sobre o meio basal.

Wang et al., (2008) otimizaram as condições de cultura para a produção de uma protease extracelular da bactéria *Colwellia* sp.. A metodologia de superfície de resposta foi aplicada para os parâmetros mais significativos de fermentação (caseína, citrato de sódio, temperatura e Tween-80) identificados anteriormente pela abordagem clássica (uma variável

de cada vez). Um planejamento central composto 2^4 foi utilizado para determinar a produção máxima da protease. Utilizando esta metodologia foram determinadas as melhores condições (5,18 g/L caseína, 3,84 g/L de citrato de sódio, temperatura de 7,96 ºC, 0,23 g/L de Tween-80) para a produção máxima desta protease (183,21 U/mL).

Thys et al. (2006) realizaram um planejamento fatorial 2^3 com o objetivo de otimizar a produção da protease por uma estirpe da *Microbacterium* sp.. A produção da protease foi testada em diferentes fontes de nitrogênio (caseína, peptona, extrato de levedura, gelatina, proteína de soja, farinha de penas e soro de queijo). A farinha de penas foi o substrato selecionado para testar o efeito de três variáveis (temperatura, pH inicial e concentração da farinha de penas) sobre a produção da protease. A análise estatística dos resultados mostrou que, nos intervalos estudados, somente o pH não teve um efeito significativo sobre a produção da protease enquanto a interação entre pH e concentração de farinha de penas foi significativa. As condições ótimas foram 25 ºC, pH inicial de 7,0 e 12,5 g/l de farinha de penas que permitiram uma atividade máxima de 202,7 U/ml.

No trabalho de Lima et al. (2009), as condições de cultura (pH, tempo de incubação, temperatura, tamanho do inóculo, velocidade de agitação e concentração de substrato), para a produção de uma colagenase extracelular produzida pela *Candida albicans*, foram estudadas utilizando três planejamentos experimentais (um planejamento fatorial fracionário (2^{6-2}) e dois planejamentos fatoriais completos (2^3)). A análise dos dados do planejamento fracionário indicou que a velocidade de agitação e a concentração de substrato apresentaram um maior efeito sobre a produção da colagenase. Com base nestes resultados, dois planejamentos fatoriais completos sucessivos (2^3) foram executados em que os efeitos da concentração do substrato, velocidade de agitação e pH foram estudados. A análise desses planejamentos mostrou que todas as variáveis escolhidas foram significativas para a produção da enzima e a produção máxima da colagenase (6,8 ± 0.4 U) foi obtida a pH 7,0, com uma velocidade de agitação de 160 rpm e concentração de substrato (gelatina) de 2%.

1.7. Purificação de colagenases microbianas

As colagenases microbianas apresentam inúmeras aplicações nas indústrias químicas e farmacêuticas, na medicina e em trabalhos experimentais. Além disso, estas enzimas também são úteis na tecnologia de alimentos. Devido às inúmeras aplicações que possuem, faz-se necessária a utilização de métodos para isolamento e purificação de enzimas com atividade colagenolítica produzidas por microrganismos, já que estas enzimas estão presentes em meios complexos constituídos por diversos componentes. Além disso, a purificação é uma etapa

necessária para esclarecer as suas características bioquímicas e estruturais (Sukhosyrova et al., 2003; Sakurai et al., 2009; Wu et al., 2010).

Atualmente, as colagenases microbianas têm sido purificadas através da utilização de métodos tradicionais de isolamento e purificação que incluem: a precipitação com sulfato de amônio, ultrafiltração e sucessivas etapas cromatográficas, como, por exemplo, cromatografia de afinidade, troca iônica, exclusão molecular e interação hidrofóbica (Tabela 2) (Kang et al., 2005; Petrova et al., 2006; Sakurai et al., 2009; Wu et al., 2010).

Tsuruoka et al. (2003) purificaram uma serinocolagenase produzida pelo *Alicyclobacillus sendaiensis* com um rendimento em atividade de 52% e fator de purificação de 18,3. Para obter a enzima pura foram utilizadas quatro etapas cromatográficas sucessivas: troca aniônica (90,4%), interação hidrofóbica (80,1%), troca iônica (62,0%) e cromatografia de hidroxiapatita (52,4%).

Uma enzima colagenolítica produzida pelo *Vibrio vulnificus* foi purificada utilizando quatro etapas cromatográficas e após a etapa final de purificação, a enzima apresentou uma atividade específica de 12.638,8 U/mg com um fator de purificação e rendimento de 13,2 e 11,4%, respectivamente (Kang et al., 2005)

Sakurai et al. (2009) purificaram uma colagenase a partir do extrato bruto do *Streptomyces parvulus* através da utilização de etapas cromatográficas sucessivas (Q-Sepharose, Sephacryl S-200 e Butil-Toyopearl). Ao final destas etapas, a enzima foi purificada a partir da amostra bruta com um rendimento de 55% e um fator de purificação de 96,0.

Uma nova colagenase produzida pelo *Bacillus pumilus* foi isolada do meio de cultura utilizando três métodos tradicionais de purificação que incluíram a precipitação com sulfato de amônio, a cromatografia por exclusão molecular e troca iônica. Ao final do processo de purificação foi obtido um rendimento de 7% e um fator de purificação de 31,5 (Wu et al., 2010).

Os métodos tradicionais de isolamento e purificação de proteínas envolvem muitas etapas: precipitação com sulfato de amônio, etapas cromatografias sucessivas, diálise e concentração final do produto, que requerem um longo período para a sua execução, o que pode induzir, em alguns casos, a um aumento da desnaturação da macromolécula, com perda da sua atividade biológica o que pode levar a um baixo rendimento de todo o processo. O processo de purificação é um problema de grande importância, devido à complexidade das misturas de proteínas e a necessidade de manter as atividades biológicas. Além disto, os métodos tradicionais de purificação apresentam um alto custo (Spelzini et al., 2005).

Tabela 2. Etapas utilizadas para a purificação de colagenases microbianas.

Microrganismos	**Métodos de Purificação**	**FP[a]**	**R[b]**	**Referências**
Steptomyces parvulus	Q-sepharose	9,9	72	Sakurai et al. (2009)
	Sephacryl S-200	10,7	45	
	Butil-Toyopearl	95,7	55	
Rhizoctonia solani	Sulfato de Amônio (40%)	1,8	84,1	Hamdy (2008)
	DEAE-Celulose	9,1	62,8	
	Sephadex G_{150}	18,7	60,5	
Streptomyces sp.	Ultrafiltração	1,65	79,46	Petrova et al. (2006)
	Sulfato de Amônio (70%)	11,7	65,62	
	DEAE Sephadex G_{25}	18,9	36,86	
	Mono Q	27,3	15,1	
Vibrio vulnificus	Q-Sepharose 1st	2,8	58,0	Kang et al. (2005)
	Q-Sepharose 2nd	3,3	19,8	
	Superdex 200 1st	7,0	14,4	
	Superdex 200 2nd	13,2	11,4	
Bacillus sp.	DEAE Celulose	3,2	3,2	Okamoto et al. (2001)
	Sephacril S 300 HR	7,1	7,1	
Bacillus subtilis	Ultrafiltração	4,8	34,4	Nagano & To (1999)
	DEAE Sepharose	6,4	17,3	
	CM cellulose	6,9	5,2	
	Butil-Toyopearl	13,7	3,4	
	Sephadex G_{75}	16,5	1,9	

[a] Fator de Purificação; [b] Rendimento (%).

Cerca de 50-90% dos custos de produção de produtos biológicos são destinados às estratégias de purificação e, portanto, o processo de refino de material biológico exige técnicas de recuperação e de purificação efetivas e de baixo custo. Para este efeito, o desenvolvimento de técnicas de separação e purificação de proteínas tem sido um requisito importante para muitos dos avanços na indústria da biotecnologia (Malpiedi et al., 2009).

1.8. Sistema de Duas Fases Aquosas

Recentemente, os sistemas de duas fases aquosas (SDFA) tornaram-se uma tecnologia de separação atraente devido às condições experimentais suaves e a facilidade de trabalhar em escala industrial. Estratégias práticas para elaboração dos processos de extração utilizando os SDFA são necessárias para superar a má compreensão dos mecanismos moleculares que governam o comportamento dos solutos no SDFA (Túbio et al., 2009).

A extração líquido-líquido utilizando o sistema de duas fases aquosas (SDFA) é um processo de bioseparação que permite um elevado grau de purificação e recuperação de materiais biológicos. Esta técnica pode ser utilizada nas etapas iniciais de um processo de purificação (por exemplo, separando proteínas dos restos celulares), pois permite a remoção de contaminantes por um processo simples e econômico, e na substituição de técnicas complexas de separação sólido-líquido (Spelzini et al., 2005; Malpiedi et al., 2008).

O SDFA apresenta uma série de vantagens em relação aos métodos convencionais para o isolamento e purificação de proteínas: (a) o equilíbrio de partição é atingido muito rapidamente, (b) o alto teor de água das duas fases (70-80% v/v), o que significa biocompatibilidade e baixa tensão interfacial e minimização da degradação de biomoléculas, (c) a facilidade de extração em grande escala; (d) a utilização de material de baixo custo, (e) a possibilidade da reciclagem dos polímeros; (f) ausência de adição de solventes orgânicos na extração e (g) diversos anions inorgânicos podem ser utilizados como agentes de extração solúveis em água (Spelzini et al., 2005; Malpiedi et al., 2008; Bulgariu et al., 2008).

1.8.1. Composição do sistema de duas fases aquosas (SDFA)

Em 1896, Beijerinck observou que quando soluções aquosas de amido e gelatina ou ágar e gelatina eram misturadas, um sistema composto por duas fases aquosas era formado, com a parte inferior rica em amido (ou ágar) e a superior rica em gelatina. Em 1947, Dobry e Boyer-Kawenoki testaram inúmeros pares de polímeros em concentrações variadas e observaram a separação de fases na maioria deles (Carvalho et al., 2008).

Foi Albertsson, no entanto, quem em 1958 propôs a aplicação do sistema de duas fases aquosas (SDFA) como ferramenta de biosseparação. Ele utilizou sistemas constituídos por polietileno glicol (PEG) e dextrana para separar, com sucesso, várias biomoléculas. Desde então, diversos estudos vêm sendo realizados expandindo a utilização de SDFA na partição de células, organelas, enzimas, metais, esporos, ácidos nucléicos e proteínas.

Na mesma década, Albertsson reintroduziu a idéia da separação de fases de polietileno glicol (PEG) com soluções de eletrólitos de fosfato ou sulfato. Ele também observou que (i),

além da separação de fases, a maioria do sistema (% p/p) era à base de água, e (ii) as biomoléculas foram capazes de se distribuírem de forma desigual entre as fases. Após o estudo de Albertsson, outros pesquisadores têm sugerido que sistemas PEG/sal oferecem um ambiente ameno para células, organelas biológicas e proteínas e são sistemas interessantes para promover a pré-concentração, extração e purificação de biomoléculas e biomateriais (Mazzola et al., 2008).

Para formar as duas fases, soluções aquosas de dois polímeros, geralmente polietileno glicol (PEG) e dextrana ou de um polímero (PEG) e um sal inorgânico (fosfato, citrato e outros) são necessários. Entretanto, apenas acima de certa concentração crítica, um sistema bifásico se forma espontaneamente. Para fins industriais, sistemas formados por polímero/fosfato de potássio são os mais comumente utilizados (Túbio et al., 2007; Malpiedi et al., 2008).

1.8.2. Diagrama de Fases do Sistema de duas Fases Aquosas

Para a utilização do SDFA é necessário o conhecimento do comportamento das fases nos sistemas. Para isto são obtidos os diagramas de fases para os componentes, nos quais as composições dos constituintes para a separação das fases são determinadas. Apenas acima de certa concentração crítica, um sistema bifásico se forma espontaneamente (Malpiedi et al., 2008).

As concentrações críticas dos componentes de um sistema bifásico são estabelecidas pelas curvas binodais. Na Figura 4 está representada uma curva binodal para um sistema polímero/sal. A curva que divide a região em duas fases é chamada de curva binodal ou curva de equilíbrio. A região acima da curva binodal é chamada de bifásica e a região abaixo, monofásica (Mazzola et al. 2008).

Convencionalmente, os componentes presentes em maior quantidade nas fases inferior e superior são representados no eixo das abscissas e das ordenadas, respectivamente. A quantidade de água é calculada por diferença. Também são representadas as linhas de amarração (“Tie-line”), que são retas que ligam os pontos no diagrama que representa a composição das duas fases em equilíbrio. Qualquer conjunto de pontos que pertençam à região bifásica e que estejam sobre a mesma linha de amarração fornecerá as fases superiores que possuirão propriedades termodinâmicas iguais (densidade, volume molar, entalpia molar, etc), entretanto, serão distintas as suas variáveis termodinâmicas extensivas (massa, volume, etc). Aplica-se o mesmo raciocínio para as fases inferiores formadas a partir de composições globais localizadas sobre uma mesma linha de amarração (Da Silva e Loh, 2006).

Outra particularidade de um diagrama de fases é o ponto crítico (C). O ponto crítico é aquele no qual as propriedades físico-químicas (composição e volume, dentre outras) das duas fases são teoricamente iguais (Albertsson, 1986). Quanto mais a composição do sistema se aproxima do ponto crítico menor é a diferença entre as fases, ou seja, no ponto crítico as composições e os volumes entre as fases são teoricamente iguais. No entanto, nas proximidades do ponto crítico, pequenas alterações na composição dos sistemas provocam mudanças drásticas, levando a mudança do sistema de uma para duas fases e vice-versa (Albertsson, 1986; Kaul, 2002).

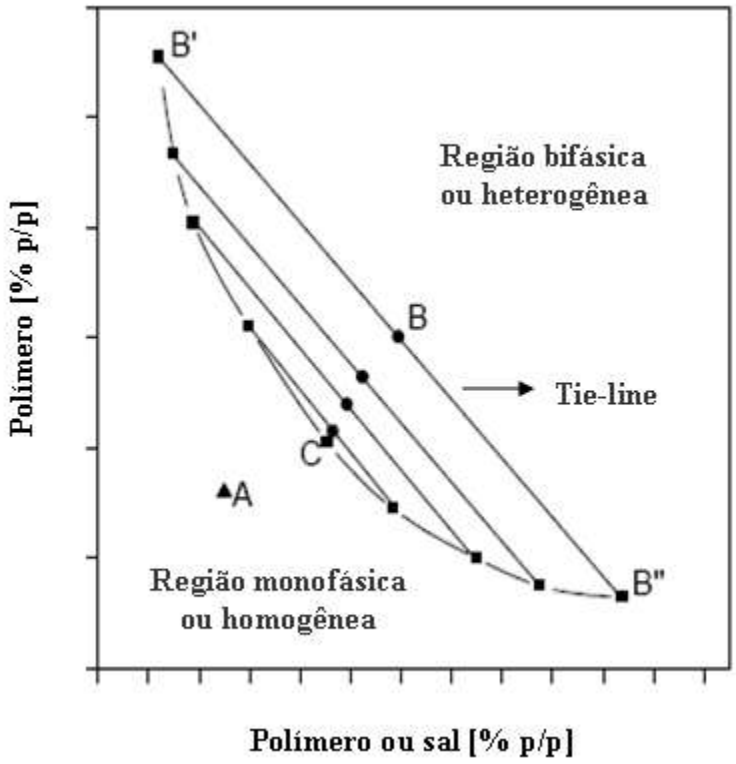


Figura 4. Diagrama de fases para um sistema de duas fases aquosas. Binodal (−■−), linha de amarração ou "tie line" (−●−), região monofásica (A), região de duas fases (B) onde B' e B'' são as composições das fases superior e inferior do sistema e (C) o ponto crítico. Fonte: Pereira, 2005.

As curvas binodais podem ser obtidas por três métodos diferentes. O primeiro deles consiste em determinar a composição das fases que formam o sistema através da cromatografia HPLC. O segundo método baseia-se em um processo de titulação entre os componentes que formam os sistemas (PEG, sais e água). A grande vantagem deste sistema de titulação é a simplicidade e rapidez, porém como o método baseia-se na observação visual de mudança de fase tem-se um determinado grau de imprecisão quando as fases se alternam

(Vernau & Kula, 1990). O terceiro método que pode ser utilizado consiste na combinação entre os dois métodos citados anteriormente. Este método baseia-se na preparação de vários sistemas de fases com composições diferentes. Em seguida, adiciona-se água em cada sistema sob agitação até as soluções se tornarem transparentes. Finalmente determina-se a composição dos novos sistemas monofásicos e com isto obtêm-se os pontos da curva binodal (Ribeiro, 2001).

O diagrama de fases pode ser influenciado por uma série de fatores incluindo a concentração e peso molecular do polímero, temperatura, adição de sais ao sistema e pH (Mazzola et al., 2008). De um modo geral, os diagramas de fases para sistemas polímero-polímero e polímero-sal são tanto mais assimétricos quanto maior for a diferença de peso molecular entre os dois componentes (Albertsson, 1985). Além disto, também se verifica um aumento do declive da "tie-line". Por outro lado, quanto maior for a massa molar de um composto, menor será a concentração necessária para a formação das duas fases (Diamond e Hsu, 1992).

1.8.3. Fatores que influenciam a partição de biomoléculas no sistema de duas fases aquosas (SDFA)

As propriedades gerais dos sistemas de duas fases aquosas têm sido estudadas por vários pesquisadores. No entanto, os mecanismos que regem a partição de materiais biológicos ainda não estão bem entendidos. Ao adicionar um composto ou uma partícula a um sistema de duas fases aquosas, observa-se a sua distribuição diferente entre as duas fases. Esta resulta das diferentes características físico-químicas das fases e, conseqüentemente da diferença de afinidade do componente adicionado para cada uma delas (Waziri et al., 2004; Malpiedi et al., 2008).

Quantitativamente a diferença de afinidade é expressa em termos de um parâmetro denominado coeficiente de partição (K) que é obtido pela razão de concentrações ($C_{S\ e\ I}$) ou atividades ($A_{S\ e\ I}$) nas fases superior e inferior. Quanto maior ou menor do que 1 for o coeficiente de partição, mais o componente tenderá a se concentrar na fase superior ou na fase inferior, respectivamente. O coeficiente de partição observado (Kp) é resultado das forças de Van der Waals, interações hidrofóbicas, ligações de hidrogênio e interações iônicas das biomoléculas com a fase circundante (Waziri et al., 2004; Malpiedi et al., 2008).

Deve-se levar em consideração que a partição de uma biomolécula alvo em sistema de duas fases aquosas depende de muitos fatores, tanto intrínsecos quanto extrínsecos. As propriedades intrínsecas incluem o tamanho da molécula, a sua natureza eletroquímica e a

hidrofobicidade superficial, conformação molecular e bioespecificidade, enquanto que as propriedades extrínsecas incluem a massa molar e a concentração dos componentes das fases, força iônica, pH, tipo de tampão, temperatura, entre outros (Azevedo et al, 2009; Saravanan et al., 2008). Pouco se pode prever do comportamento de uma molécula sob diversos fatores, sabe-se, no entanto, por exemplo, que proteínas altamente hidrofóbicas tendem a migrar preferencialmente para a fase superior, rica em polímero e que, manipulando sistematicamente os fatores extrínsecos é possível modificar o comportamento da partição da proteína alvo e os seus contaminantes (Salgado et al, 2008).

Para facilitar o desenvolvimento do processo de extração nos SDFA, diversas etapas básicas devem ser seguidas. Estas etapas estão apresentadas na Figura 5 (Rito-Palomares, 2004). Em primeiro lugar, a seleção do tipo de SDFA, uma vez que pode ser um SDFA polímero-polímero ou um sistema polímero-sal. Esta seleção é muitas vezes regulada por considerações econômicas. Devido ao baixo custo dos produtos químicos que formam as fases, os sistemas polímero-sal têm sido preferidos. Estes sistemas também são atraentes por causa da rápida separação das fases e a disponibilidade para separações comerciais, que permitem uma rápida e contínua separação de proteínas. O sal inorgânico mais comumente empregado é o fosfato de potássio, entretanto a alta concentração de sal necessária representa um problema no seu descarte, levando a problemas ambientais (Tubio et al., 2009).

Uma vez que a seleção geral do SDFA tenha sido alcançada, um diagrama de fases é necessário para definir qual composição do sistema será empregado. Finalmente, trabalhos experimentais ainda devem ser realizados para saber o comportamento da partição das proteínas desejadas no SDFA com diferentes massas molares e concentrações do polímero, tipos e concentrações de componentes ou sais adicionados, pH e temperaturas, a fim de encontrar as condições adequadas para a extração seletiva da molécula alvo (Tubio et al., 2009).

Quando os SDFA são aplicados para isolar uma proteína que está presente em uma mistura de proteínas complexas (ou seja, um homogeneizado de um produto natural ou uma proteína expressa por um microrganismo) a primeira etapa da pesquisa é determinar as características de particionamento da proteína-alvo no estado puro e o efeito das variáveis do meio no seu coeficiente de partição (Spelzini et al., 2005; Malpiedi et al., 2008).

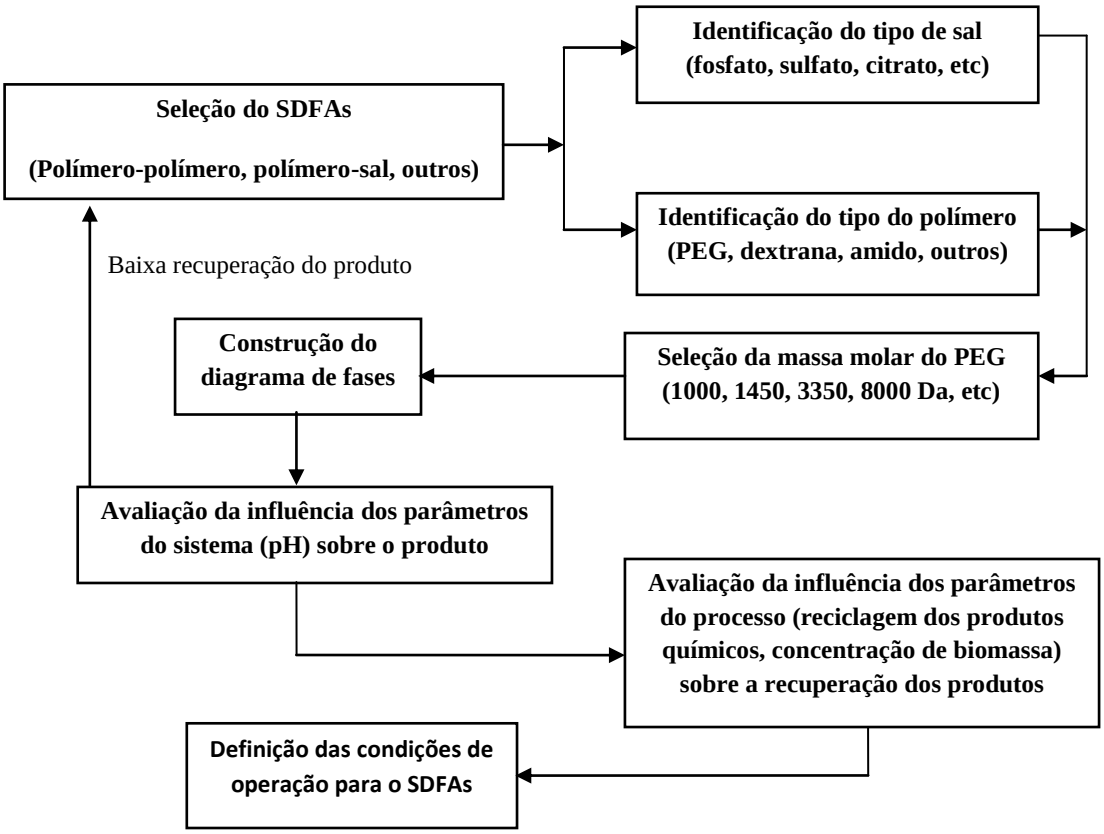


Figura 5. Estratégias para o desenvolvimento do SDFA para recuperação de produtos biológicos. Fonte: Rito-Palomares, 2004.

Embora o SDFA seja considerado como um sistema que apresenta condições brandas para a manipulação de materiais biológicos, já foram relatados trabalhos nos quais a conformação nativa das proteínas foi afetada devido a interações PEG-proteína, isto é, mudanças da estrutura terciária da proteína podem ocorrer (Lebreton & Lyddiatt, 2000). Assim, é essencial avaliar o impacto geral que a separação entre duas fases aquosas pode ter sobre a estrutura da biomolécula (Malpiedi et al., 2008).

O SDFA tem sido aplicado para a partição e a recuperação de uma variedade de proteases que incluem: bromelina (Babu et al., 2008), papaína (Nitsawang et al., 2006), e protease microbiana (Porto et al., 2008) (Tabela 3).

Tabela 3. Características gerais dos SDFA selecionados para a recuperação de proteínas.

Tipo de SDFA	Origem biológica	Molécula alvo	EE	RP	Referências
Polímero-polímero:					
PEG-dextrana	*A. niger*	B-glicosidase	1	95	Johansson & Rczey, 1998
PEG-amido	Trigo	α-amilase	1	75	Pietruszka et al., 2000
PEG-HPS	*S.cerevisiae*	Álcool desidrogenase	1	77-100	Venancio et al., 1996
Polímero-sal:					
PEG-fosfato	*A. niger*	Quimosina	2	81-95	Spelzini et al., 2005
	B. subtilis	Protease alcalina	1	96	Chouyyok et al., 2005
	T. reesei	B-glicosidase	1	92	Gautam et al., 2006
	Ananas comosus	Bromelina	1	228	Babu et al., 2008
PEG-citrato	Fonte comercial	BSA	2	92	Yan-Min et al., 2010
	Pâncreas bovino	Tripsinogênio	1	84	Malpiedi et al., 2009
	Clostridium perfringens	Protease	1	131	Porto et al., 2008
PEG-sulfato de amônio	*P.thermophila*	Xilanase	1	98,7	Yang et al., 2008
	Carica papaya latex	Papaína	1	51	Nitsawang et al., 2006

HPS, hidroxipropil amido; EE, etapas de extração; RP, Recuperação do produto (%).

1.8.3.1. Efeito da Massa Molar e da Concentração do Polímero

O estudo da influência da massa molar do polímero pode ser uma ferramenta muito útil para manipular o coeficiente de partição das proteínas nos SDFA (Tubio et al., 2009). Em geral, um aumento da massa molar do polímero do sistema de duas fases aquosas, em uma determinada composição de fases, diminui a partição da molécula alvo para a fase rica em

polímero. Quanto maior for a massa molar do polímero, menor será o volume de solvente disponível, o que implica em uma diminuição da solubilidade das proteínas na fase rica em polímero e consequentemente uma diminuição do coeficiente de partição (Yan-Min et al., 2010).

Além deste efeito, a massa molar do PEG também exerce efeito sobre as interações hidrofóbicas entre as proteínas e as moléculas do polímero. O aumento da massa molar do polímero proporciona um aumento na capacidade de interação entre suas moléculas com proteínas hidrofóbicas. Aparentemente, isto é independente do volume excluído, pois os PEGs com uma grande massa molar possuem a capacidade de formarem interações intra-moleculares, adquirindo assim uma conformação mais compacta (Tubio et al., 2004).

A concentração de PEG proporciona um efeito semelhante, já que uma alta concentração de polímero disponibiliza um número maior de unidades de polímeros envolvidos na separação da proteína e desta forma uma maior quantidade da molécula alvo particiona para a fase PEG, devido ao aumento do número de interação hidrofóbica que podem ser formadas entre as proteínas e as moléculas do polímero (Saravanan et al., 2007).

A partição de proteínas é conduzida não apenas por um mecanismo de entropia, como também pelos efeitos da entalpia (Saravanan et al., 2008). De acordo com Pico et al., (2007), sob o ponto de vista molecular, os PEGs de menor massa molar podem interagir fortemente com as proteínas, enquanto os PEGs de maior massa molar têm capacidade para formar ligações intra-moleculares. A transferência da proteína para uma das fases exige a quebra da interação dos componentes das fases para criar uma cavidade onde a proteína será incluída. Portanto, o balanço energético pode ser positivo ou negativo dependendo se as interações proteínas/polímeros são atrativas ou repulsivas, e isto, depende da massa molecular do PEG.

Segundo Saranavan et al. (2007) maiores coeficientes de partição para as proteínas podem ser obtidos com menores massas molares de PEG em comparação com PEG de maior massa molar devido ao fato de que a tensão interfacial é menor quando a massa molar do PEG é menor. É conhecido que a tensão interfacial entre as fases é um fator determinante que influencia o comportamento de partição das partículas e células (Johansson, 1985).

Segundo Spelzini et al. (2005) a massa molar dos polímeros pode influenciar nos parâmetros termodinâmicos do SDFA. Estes autores observaram uma mudança na entalpia com o aumento da massa molar do PEG, enquanto que uma diminuição deste parâmetro foi observada com o aumento da massa molar, além disso, um comportamento semelhante para a mudança de entropia foi observado. Uma vez que estas funções termodinâmicas fornecem informações sobre os mecanismos moleculares que influenciam a transferência de proteínas

da fase rica em sal para a fase rica em PEG, a grande mudança da entalpia sugere a quebra de várias ligações intermoleculares (da água estruturada no domínio da proteína e no polímero), quando essa transferência é realizada. Este efeito poderia ser devido a uma importante interação PEG-proteína o que induz o deslocamento da água do domínio da proteína para favorecer a sua interação com o PEG. A mudança observada na entropia concorda com este mecanismo, e está associado a um aumento da desordem do sistema pela capacidade de quebrar a estrutura do PEG para se ligar ao domínio da proteína.

Yan-Min et al. (2010) realizaram um estudo de extração e reextração da albumina de soro bovino (ASB) utilizando o sistema de duas fases aquosas PEG-citrato e observaram que o aumento da massa molar do PEG resultou em menos espaço disponível para a ASB na fase superior, o que levou à diminuição do coeficiente de partição (K). Este comportamento está de acordo com o efeito do volume de exclusão devido à diminuição do volume livre disponível na fase superior. Por este motivo a albumina mostrou maior afinidade para a fase superior quando se utilizou o PEG de menor massa molar. Além deste efeito, o caráter hidrofóbico do PEG aumenta com o aumento da massa molar e devido ao fato da albumina apresentar caráter hidrofílico, a mesma particionou preferencialmente para fases ricas em PEGs de menor massa molar que são menos hidrofóbicos.

1.8.3.2. Efeito da temperatura

A influência da temperatura nos SDFA é bastante complexa devido ao seu efeito na composição das fases em equilíbrio, assim como na alteração da estrutura da biomolécula e desnaturação (Sarubbo, 2000). Geralmente, para baixas temperaturas (menores do que 20°C) a curva binodal desloca-se em direção às baixas concentrações dos componentes que formam as fases, resultando no aumento do comprimento das linhas de amarração. Os sistemas de fases próximos do ponto crítico podem ser mais influenciados pela mudança de temperatura devido à sua instabilidade, quando a curva binodal é deslocada, podendo assim o sistema passar facilmente para a região monofásica (Cesar, 2005).

O efeito da temperatura varia de acordo com o tipo de sistema, polímero/polímero ou polímero/sal. Para o sistema PEG/dextrana, a formação das fases é facilitada a temperaturas baixas (menores do que a do ambiente) e para os sistemas PEG/fosfato, a situação é oposta, pois temperaturas mais altas e próximas da temperatura ambiente facilitam a separação entre as fases. O aumento da temperatura do sistema causa ainda, em um sistema PEG/sal, o aumento na concentração do PEG na fase polimérica e a redução da sua concentração na fase

salina. Este efeito é uma das razões de se trabalhar com a temperatura fixa do sistema (Cesar, 2005).

Segundo Saravanan et al. (2008), a temperatura é um fator importante na partição de proteínas no SDFA. O efeito da temperatura sobre a separação de proteínas é diferente para os diferentes sistemas, dependendo do tipo de polímero utilizado. Tem sido relatado que quando a temperatura aumenta, a estrutura do PEG torna-se mais estendida e, conseqüentemente, a sua interação com a proteína diminui o que pode levar a uma diminuição do coeficiente de partição. No trabalho realizado por estes autores, o coeficiente de partição das duas proteínas estudadas (ovoalbumina e mioglobina) diminuiu com o aumento da temperatura.

Outros estudos, entretanto, observaram efeito contrário, por exemplo, no estudo realizado por Spelzini et al., (2005) foi observado um aumento da partição das duas proteínas estudadas (quimosina e pepsina) para a fase rica em PEG com o aumento da temperatura, o que corroborou com o caráter endotérmico do particionamento. Adicionalmente, a mudança da entropia para a partição das duas proteínas foi positiva, e esta mudança foi o fator que impulsionou a partição da proteína do ponto de vista termodinâmico.

Segundo Gautam & Simo (2006) o coeficiente de partição de uma β-glicosidase produzida pela *Trichoderma reesei* aumentou com o aumento da temperatura do sistema PEG-fosfato. Para compreender este comportamento foi investigado o efeito da temperatura sobre as concentrações do PEG e dos sais de fosfatos nas duas fases aquosas. Eles observaram que a binodal moveu-se gradualmente para a origem, com o aumento da temperatura. Este resultado, que já foi observado por vários autores, indica que um aumento da temperatura aumenta a concentração do PEG e do sal de fosfato na fase superior e inferior do sistema, respectivamente. Isto é devido à aderência preferencial das moléculas de água para a superfície polar do sal ao invés do PEG, a temperaturas mais altas. Consequentemente, as moléculas de água disponíveis para a solvatação do PEG na fase inferior, diminuem à medida que aumenta a temperatura, diminuindo a solubilidade do PEG na fase inferior. Isto é conhecido como o efeito *salting-out*, que se torna mais forte com o aumento da temperatura.

1.8.3.3. Efeito do pH

O pH é um dos parâmetros mais importantes para otimizar a purificação de proteínas solúveis (Saranavam et al., 2007). De acordo com Albertsson (1986), no modelo clássico para o particionamento de proteínas (ou enzimas) no SDFA, o coeficiente de partição da proteína é separadamente influenciado em curto prazo pelas forças de Van der Waals e em longo prazo por interações eletrostáticas moleculares, da seguinte forma:

$$\ln K_p = \ln K_0^{\cdot} + \left[Z_p F \Big/ RT \right] \Delta\varphi \qquad (1)$$

onde K_p e K_0 denotam o coeficiente de partição da proteína a um dado pH e o coeficiente de partição da proteína no ponto isoelétrico (pI), respectivamente. O termo $(Z_PF/RT)\Delta\varphi$ representa a interação eletrostática que é o produto entre a carga da superfície da proteína (Z_P), a constante de Faraday (F) e a diferença de potencial eletrostático entre as duas fases ($\Delta\varphi = \varphi_{sup} - \varphi_{inf}$) (Chouyyok et al., 2005). Desta forma, o pH pode afetar a partição de proteínas, seja alterando a carga do soluto (Z_p) ou alterando a proporção das espécies carregadas presentes nas fases ($\Delta\varphi$) (Malpiedi et al., 2008; Saravanan et al., 2008).

A influência da carga da biomolécula na sua partição pode ser estudada medindo o coeficiente de partição em dversos valores de pH. Contudo, a influência da carga da biomolécula depende muito do tipo de sal presente no sistema, uma vez que diferentes sais apresentam diferentes potencias elétricos entre as fases. Alterações no pH podem induzir mudanças conformacionais na estrutura das proteínas, causando mudanças em seu comportamento de separação. Em condições extremas de pH pode ocorrer a desnaturação de proteínas. Geralmente, a partição de proteínas desnaturadas é diferente da partição das proteínas na sua forma nativa, o que pode ser atribuído não só à maior área superficial da forma desnaturada, mas também ao fato da superfície exposta desta ser muito mais hidrofóbica. Como regra geral as proteínas carregadas negativamente (nos casos em que o pH é superior ao pI) tem maior afinidade pela fase superior que é rica em PEG (Babu et al., 2008).

Em pH abaixo do ponto isoelétrico (PI) as proteínas têm uma carga líquida positiva, porque o grupamento amina ganha um próton extra e a pH alto elas têm uma carga líquida negativa, porque a carboxila perde o seu próton. O pH no qual a proteína tem uma carga líquida igual a zero é chamado de ponto isoelétrico. A valores de pH mais elevados do que o ponto isoelétrico a carga líquida da proteína é negativa, enquanto que a valores de pH menores do que o ponto isoelétrico a carga líquida da proteína é positiva e, portanto, o coeficiente de partição das proteínas aumenta com o aumento do pH, que pode ser causado pelas interações eletrostáticas entre a proteína e as unidades de PEG (Saravanan et al., 2008).

Vários autores têm relatado que acima do ponto isoelétrico, as enzimas/proteínas são poliânions que particionam preferencialmente para a fase rica em PEG no SDFA. Além disso, ocorre uma rejeição da enzima/proteína pelos poliânions inorgânicos (fase inferior) que pode ser atribuída a uma maior repulsão entre as cargas pontuais das cadeias laterais dos

aminoácidos na superfície da enzima/proteína que está na forma de poliânions e os poliânios inorgânicos da fase inferior, enquanto que as proteínas com carga positiva são atraídas para a fase inferior (Nalinanon et al., 2009).

Malpiedi et al (2008) estudaram a influência do pH no comportamento da partição do α-quimotripsinogênio (ChTRPz) e do tripsinogênio (TRPz) bovinos utilizando um sistema de duas fases aquosas PEG-citrato e observaram que a pH 5,20 e 8,20 tanto o TRPz quanto o ChTRPz estavam carregados positivamente ($Z_P > 0$), pois os seus pIs são 9,30 e 9,50, respectivamente, e $\Delta\varphi$ adotou valores positivos devido ao fato dos ânions de citrato terem sido excluídos da superfície do PEG, sendo particionados para a fase inferior. O aumento do valor do pH de 5,20 para 8,20 causou uma diminuição do Z_P, enquanto que a magnitude da $\Delta\varphi$ foi aumentada devido ao aumento no comprimento da linha de amarração, o que implica um aumento da diferença entre a concentração de citrato na fase superior e inferior. Como conseqüência, o aumento observado para ambos os Kp dos zimogênios a pH 8,20 pôde ser atribuído a um efeito predominante da diminuição da carga positiva líquida das proteínas, pois a pH menor do que o PI a carga positiva líquida aumenta com a diminuição do pH (Túbio et al., 2006).

1.8.3.4. Efeito do tipo e da concentração de sal

Os sais são freqüentemente utilizados nos SDFA para melhorar a partição das moléculas alvo entre as fases (Rawdkuena et al., 2011). A adição de sais, mesmo em concentrações milimolares, influencia fortemente a partição de materiais eletricamente carregados. Geralmente, tipos diferentes de sais apresentam íons diferentes com diferentes afinidades pelas duas fases resultando numa diferença de potencial elétrico entre as fases, que por sua vez direciona a partição de materiais biológicos carregados. A partição de proteínas modelos é realizada pelo movimento causado pelas interações e repulsões eletrostáticas entre as fases carregadas do sistema e das proteínas (Saravanan et al., 2008).

A presença de sal pode afetar a partição de duas maneiras: enfraquecendo ou fortalecendo as interações entre (1) os polímeros e as biomoléculas, ou (2) grupos ionizados com a carga líquida oposta de proteínas (Rawdkuen et al., 2011). A eficácia do sal é principalmente determinada pela natureza do ânion. Ânions com muitas cargas são os mais eficazes na seguinte ordem: $SO4^{2-} > HPO4^{2-} > CH3COO^{-} > Cl^{-}$. A ordem de cátions é geralmente dada como: $NH4^{+} > K^{+} > Na^{+} > Li+ > Mg2^{+} > Ca2^{+}$ (Roe, 2000).

A adição de sais em uma solução aquosa de PEG leva a um arranjo ordenado das moléculas de água em torno das moléculas do PEG devido à capacidade dos sais

desestabilizarem a estrutura da água. A formação de uma camada de água ao redor de cátions resulta em uma estrutura mais compacta, com um volume menor ocupado pela molécula de PEG (Nalinanon et al., 2009).

1.8.4. Comportamento do coeficiente de partição de biomoléculas em Sistemas de Duas Fases Aquosas composto de PEG/Sal

A representação esquemática do mecanismo básico de partição de biomoléculas em sistemas PEG/sal está representada na Figura 6. Em tais sistemas a partição depende do efeito do volume de exclusão na fase rica em polímero (fase superior) e do efeito *salting out* na fase rica em sal (inferior) (Figura 6a). O volume ocupado pelo polímero aumenta com o aumento da concentração (Figura 6b) e da massa molar do próprio polímero (Figura 6c), que resultam em um menor espaço para a biomolécula na fase superior, assim as biomoléculas tendem a migrar para a fase inferior, efeito chamado de volume de exclusão (Babu et al., 2008).

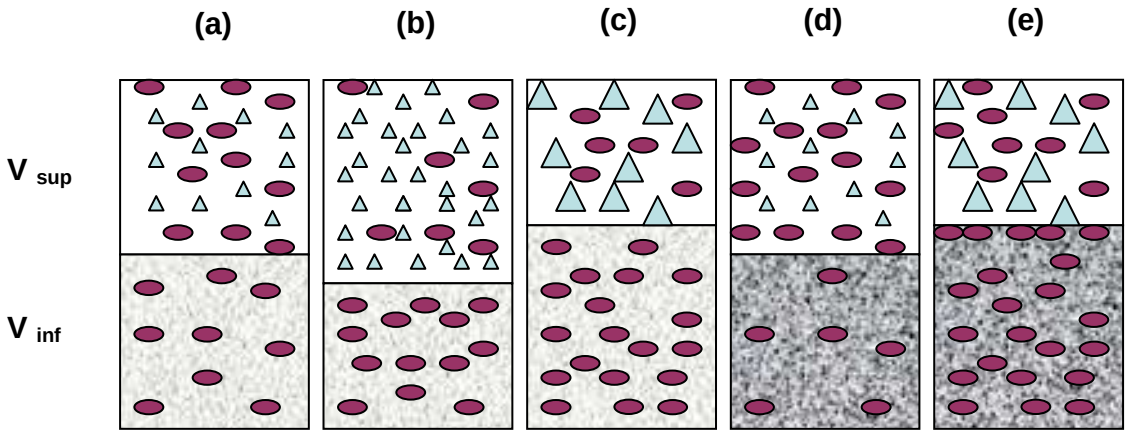


Figura 6. Representação esquemática do comportamento da partição de biomoléculas em SDFA: (a) sistema PEG/sal típico; (b) efeito do aumento da concentração do polímero; (c) efeito do aumento da massa molar do polímero; (d) efeito do aumento da concentração de sal; (e) efeito combinado do volume de exclusão e "*salting out*". (▲) Polímero; (●) enzimas/proteínas); (⁘) sal. V_{sup}: volume da fase superior; V_{inf}: volume da fase inferior. Fonte: Babu et. al, 2008.

A solubilidade das biomoléculas na fase inferior, rica em sal, diminui com o aumento da concentração do sal (Figura 6d), o que resulta em um aumento da partição de biomoléculas na fase superior e a este efeito dá-se o nome de "*salting out*" (Babu et al., 2008). Os sistemas

constituídos por polímeros com elevada concentração ou alta massa molar e com elevada concentração de sal (Figura 4e) resultam na partição de biomoléculas na interfase devido à influência de ambos os efeitos volume de exclusão e "*salting out*" (Babu et al., 2008).

1.8.5. Aplicação de Planejamentos Experimentais em SDFA

A utilização de planejamentos experimentais é uma boa ferramenta para conhecer as relações entre os principais fatores que influenciam a partição de proteínas no SDFA. Alguns dos fatores normalmente analisados, conhecidos como de primeira ordem são: massa molar do polímero, concentração do polímero e do sal, pH e temperatura. A elaboração de planejamentos experimentais tem sido de fundamental importância nestes estudos, pois reduz o número de experimentos necessários, indicando as principais variáveis que interferem significativamente no SDFA e ainda disponibiza a significancia dos efeitos das interações entre as mesmas (Porto et al., 2008; Yan-Min et al., 2010).

Um planejamento experimental ou fatorial consiste em uma série de ensaios em que a cada estudo envolve todas as possíveis combinações dos níveis e fatores a serem investigados. Qualquer experimento que possua um número *k* de fatores, cada um com apenas dois níveis (ex.: superior (+1) e inferior (-1)), é conhecido como planejamento experimental de 2 níveis (2^k). O numero de ensaios experimentais necessários para completar uma replicata de estudo é dada por $2x2x...x2=2^k$ onde *k* é o numero de fatores, dando assim o seu nome (Ahmad et. al, 2008).

Vários trabalhos têm utilizado planejamentos experimentais para avaliar quais as variáveis que influenciam o SDFA. Porto et al. (2008) otimizaram a extração da protease de *Clostridium perfrigens* utilizando três planejamentos experimentais sucessivos (um 2^4 e dois 2^3) no SDFA PEG/citrato. A massa molar do PEG (MM_{PEG}), a concentração do PEG (C_{PEG}) e do citrato (C_C) e o pH foram as variáveis independentes, enquanto que o fator de purificação, o coeficiente de partição, o rendimento em atividade e a seletividade foram selecionadas como variáveis resposta. O melhor desempenho do sistema foi obtido através da utilização das seguintes condições: MM_{PEG} = 10.000 g/mol, C_{PEG} = 22% (v/v) e C_C = 8,0% (v/v) em pH 8,5. Nestas condições, o rendimento em atividade observado foi de 131%, enquanto que o fator de purificação foi de 4,2. Através da utilização de planejamentos estatísticos estes autores conseguiram identificar de forma rápida os fatores que influenciaram a partição da protease no SDFA.

Yan-Min et al (2010) realizaram um estudo de otimização, através da utilização de um planejamento central composto, da extração da albumina de soro bovino (ASB) utilizando o

sistema de duas fases aquosas PEG-citrato. Neste trabalho, o SDFA foi examinado com relação aos efeitos da massa molar do PEG (PEG 1000, 2000, 4000 e 6000), concentração do PEG e do citrato de potássio, concentração da albumina e pH na partição da albumina. O maior rendimento da albumina (99%) foi obtido na fase superior sob as seguintes condições: 19% (v/v) de PEG 1000, 20% (v/v) citrato de potássio e 0,75 mg/g albumina a pH 7,0 e 30 ºC.

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4. CAPÍTULO II

Production and characterization of a collagenolytic serine proteinase by *Penicillium aurantiogriseum* URM 4622: A factorial study

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RESEARCH PAPER

Production and Characterization of a Collagenolytic Serine Proteinase by *Penicillium aurantiogriseum* URM 4622: A Factorial Study

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Abstract A 2^4 full factorial design was used to identify the main effects and interactions of the initial medium pH, soybean flour concentration, temperature and orbital agitation speed on extracellular collagenase production by *Penicillium aurantiogriseum* URM4622. The most significant variables for collagenase production were soybean flour concentration and initial medium pH that had positive main effects, and temperature that had a negative one. Protein concentration in soybean flour revealed to be a significant factor for the production of a collagenase serine proteinase. The most favorable production conditions were found to be 0.75% soybean flour, pH 8.0, 200 rpm, and 28°C, which led to a collagenase activity of 164 U. The enzyme showed an optimum activity at 37°C and pH 9.0, was stable over wide ranges of pH and temperature (6.0 – 10.0 and 25 – 45°C, respectively) and was strongly inhibited by 10 mM phenylmethylsulphonylfluoride. The first-order rate constants for collagenase inactivation in the crude extract, calculated from semi-log plots of the residual activity versus time, were used in Arrhenius and Eyring plots to estimate the main thermodynamic parameters of thermoinactivation (E^*_d = 107.4 kJ/mol and ΔH^*_d = 104.7 kJ/mol). The enzyme is probably an extracellular neutral serine collagenase effective on azocoll, gelatin and collagen decomposition.

Keywords: collagenase, enzyme production, *Penicillium aurantiogriseum*, submerged culture, factorial design

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1. Introduction

Proteases are a highly complex group of enzymes that differ in their substrate specificity, catalytic mechanism, and active site [1]. These enzymes, which are able to hydrolyze the peptide bond of proteins, represent one of the largest groups of industrial enzymes, with increasing market demand due to their usefulness in various industrial sectors and in basic research [2].

Collagenolytic proteases, which degrade the native triple helix of collagen, are involved in various physiological and pathological situations, such as fetal bone development, embryonic development, wound repair, rheumatoid arthritis, malignant tumor invasion, intestinal ulceration and chronic periodontal inflammation [3]. These proteases are classified into two major groups: Metallocollagenases and serine collagenases. Hydrolysis by metalloproteases takes place mostly between the peptide bond of residue X and Gly-Pro. Metallocollagenases, first discovered in tissue explants of tadpole, are zinc-containing enzymes, but usually also require calcium for their optimum activity and stability. They are involved in remodeling the extracellular matrix. On the other hand, serine collagenases were first isolated

from the hepatopancreas of the fiddler crab (*Uca pugilator*) and are probably involved in food digestion rather than morphogenesis, along with in the production of hormones and pharmacologically active peptides, besides various cellular functions, such as protein digestion, blood-clotting, fibrinolysis and fertilization [4].

The collagenolytic activity of these enzymes has industrial, biotechnological, medicinal and commercial applications. Collagenase hydrolyzes bovine trachea cartilage, allowing for the preparation of intact mammalian cells in culture and cleaning blood cells for improved screening in medical diagnostics [5]. Potential therapeutic applications include wound healing [6] and preclinical therapeutic studies on various types of destructive fibrosis, such as liver cirrhosis [7].

Collagens are the major protein constituents of extracellular matrix and the most abundant proteins in all higher organisms. The triple helix, tightly coiled, of collagen molecule assembles into water-insoluble fibers or sheets, which are cleaved only by collagenases, being resistant to other proteinases [8]. Collagen peptides produced by collagenolytic enzymes have been used in the chemical, medical, cosmetic and food industries, as well as in experimental applications of molecular biology [9]. They can be used as seasonings, non-allergic preservatives for drugs, as ingredients for dietary materials and parentally-fed products, and for the treatment of diseases such as collagen-induced arthritis [10].

Collagenolytic proteases are ubiquitously found in plants [5], animals [11] and microorganisms [12]. However, the microorganisms are the preferred sources of these proteases because of their broad biochemical diversity and their susceptibility to genetic manipulation. Among the microbes, fungi have a distinct advantage as enzyme producers, because their hydrolytic enzymes are released extracellularly, which makes their recovery from the fermented broth particularly easy [2]. Reports are available on collagenase biosynthesis by fungi belonging to the genera *Aspergillus*, *Cladosporium*, *Alternaria* and *Penicillium* [13].

It is well known that extracellular protease production in microorganisms is greatly influenced by medium components, especially carbon and nitrogen sources, physical factors such as pH, temperature, inoculum size, orbital agitation speed and incubation time [14]. However, no single medium has been established for optimal production of protease from different microbial sources, because each organism requires different conditions for maximum production [15].

Usually 30 ~ 40% of industrial production cost of enzymes is related with the cost of the growth medium, making the search for cost-effective media particularly relevant [16]. Soybean flour has been recognized as a potentially useful and cost-effective ingredient, because it is a by-product from oil extraction. It consists of approximately 40% proteins and is rich in other organic and inorganic compounds, thus being a good candidate for a culture medium [17].

Factorial statistical designs are useful to study enzyme production conditions. When conventional "one-factor-at-a-time designs are used, they usually require a large number of experimental runs. Worse still, they are likely to miss possible interaction effects between production parameters [18].

Environmental conditions can play an important role in the induction or repression of extracellular proteases [19]. The aims of this work were to study by a statistical approach the influence of soybean flour concentration, pH, temperature and agitation speed on collagenase production by *P. aurantiogriseum* URM4622 and to characterize the enzyme obtained under the most favorable conditions.

2. Material and Methods

2.1. Microorganism and culture medium

The *P. aurantiogriseum* dierchx (URM4622) strain was obtained from the Culture Collection of the Department of Mycology of the Federal University of Pernambuco (Micoteca). The fungi was isolated from the microscopy-room air of "Micoteca" and at the moment qualitatively characterized, on a solid medium, only for its ability to produce protease (data not published). The full characterization of this strain is currently under way.

The strain was maintained at 4ºC in a malt extract agar medium, consisting of 0.5% (w/v) malt extract, 0.1% (w/v) peptone, 0.5% (w/v) glucose, and 1.5% (w/v) agar.

The soybean flour medium described by Porto *et al.* [20], composed of 0.5% (w/v) filtered soybean flour (SF), 0.1% (w/v) NH_4Cl, 0.06% (w/v) $MgSO_4 \cdot 7H_2O$, 0.435% (w/v) K_2HPO_4, 0.01% (w/v) glucose, and 1.0% (v/v) mineral solution, was used for collagenase production. The mineral solution was prepared 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 $CaCl_2 \cdot H_2O$. This fermentation medium was sterilized in an autoclave at 121ºC for 20 min.

2.2. Screening of significant variables for collagenase production

The 2^4 full design mentioned above was carried out at all combinations of the levels given in Table 1. The central point was run in quadruplicate, to provide an estimate of the pure error variance in the experimental responses. From that, experimental errors of the effects were estimated [18] and used to assess the significance of the effects and interactions of the independent variables – initial medium

Table 1. Factor levels used in the 2^4 design to investigate the production of collagenase by *P. aurantiogriseum* URM4622

Factors	Level		
	Lowest (−1)	Central (0)	Highest (+1)
Soybean flour concentration (%, w/v)	0.25	0.50	0.75
Initial medium pH	6.0	7.0	8.0
Orbital agitation speed (rpm)	100	150	200
Temperature (°C)	28	32	36

pH, SF (substrate) concentration, temperature and orbital agitation speed – on the production of collagenase.

The full 2^4 model comprises a constant term, four main effects, six two-factor interaction effects, four three-factor interactions and a four-factor interaction. The goodness-of-fit of this model was evaluated by analysis of variance. The statistical significance of each effect, at 95% confidence level, was assessed by comparisons with the experimental pure error obtained from the replicate runs at the central point. All statistical and graphical analyses were carried out by the *Statistica 8.0* software (StatSoft Inc., Tulsa, OK, USA).

Inoculum spores were produced in agar 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 10^6 spores/mL, fermentations were carried out at the selected temperature in 1.0 L Erlenmeyer flasks containing 250 mL of the culture medium. Cotton caps were used to minimize water evaporation. The broth obtained at the end of fermentation (72 h) was vacuum filtered through 0.45 μm-pore diameter nitrocellulose membranes to remove the mycelium. Being the target collagenase an extracellular one, the filtrate so obtained was analyzed to determine the final protein concentration and collagenase activity and used as a source of the enzyme (referred to as crude extract). All the experiments concerning collagenase characterization were also made using such a crude extract.

2.3. 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°C for 24 h.

2.4. Azocoll assay for collagenase 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.* [21]. 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). After this, 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 3 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 means of a UV-Vis spectrophotometer, model B582 (Micronal, São Paulo, Brazil). One unit of enzyme activity (U) was defined as the amount of enzyme, per mL of crude extract that led, after 3 h of incubation, to an absorbance increase of 0.1 at 520 nm, as a result of the formation of azo dye-linked soluble peptides.

The specific activity was calculated as the ratio of the enzymatic activity to the total protein content of the sample, and expressed in U/mg.

2.5. Protein determination

Protein concentration was determined according to Smith *et al.* [22], using bovine serum albumin as standard.

2.6. Effects of pH and temperature on collagenase activity and stability

To evaluate the effect of pH on collagenase 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 enzyme was incubated at 4°C (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 28 h. The collagenolytic activity of samples was measured at 37°C, using 0.5% (w/v) azocoll in 0.05 M Tris-HCl buffer (pH 9.0).

An analogous study was done for temperature in the same azocoll-buffer solution. To determine the optimum temperature for enzyme activity, the reaction mixture was incubated at the selected temperature (from 25 to 70°C), while for the thermostability tests the enzyme was pre-incubated for 15 ~ 360 min at the same temperature, and then the collagenase activity determined at 37°C.

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 (%).

2.7. Substrate specificity

To test the substrate specificity, the proteolytic activity of extracellular collagenase was also assayed on insoluble

collagen, gelatin and azocasein.

The activity was assayed on insoluble collagen according to Endo *et al.* [23]. The standard reaction mixture, containing 25 mg collagen (type 1, bovine Achilles tendon) in 5 mL of 0.05 M Tris-HCl buffer (pH 7.0), was incubated with 1 mL enzyme samples at 37°C. The amount of free amino groups released was measured by the ninhydrin method of Rosen [24]. 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 collagenase, after 18 h at 37°C.

The collagenase activity on gelatin was assayed by the method of Moore and Stein [25], slightly modified. The reaction was carried out at 37°C for 18 h after the addition of 0.1 mL of the enzyme solution to 1.0 mL of 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 × *g* for 10 min. The supernatant (0.2 mL) was mixed with 0.5 mL of ninhydrin solution, heated at 100°C 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 × *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.* [26], 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.

2.8. Effect of inhibitors

The effect of protease inhibitors 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; pepstatin A for aspartic proteases at the concentration of 1.0 mM. For sensitivity determination, the enzyme was pre-incubated for 30 min at 37°C with the inhibitors. The residual activity was determined as the percentage of the proteolytic activity in an inhibitor-free control sample. In particular, the conditions selected for tests with PMSF were consistent with the observations of James [27], and those for tests with EDTA with most of literature reports on metalloprotease inhibition studies.

2.9. Kinetic and thermodynamic modeling

To investigate the dependence of protease activity on temperature, it was assumed that an equilibrium state is reached between the active and inactive (unfolded) enzyme structures. According to the thermodynamic approach already applied successfully to other enzymatic systems [28], the increase in the initial activity with temperature is counterbalanced by a "reversible unfolding" equilibrium, resulting in a net activity decrease. At temperatures below the optimum value ($T < T_{opt}$), it is reasonable to assume that the enzyme unfolding is negligible, and the rate constant (k) varies with temperature according to the well-known Arrhenius equation [28]. Since the enzymatic activity (A_c) is directly proportional to the rate constant, the activation energy of the catalytic event (E^*, kJ/mol) can be estimated from the slope of the straight line obtained from a plot of $\ln A_c$ against 1/T, when $T < T_{opt}$. The activation enthalpy (ΔH^*, kJ/mol) was estimated from the slope of the straight line obtained from the plot of $\ln(A_c/T)$ against 1/T, according to Eyring equation [29], derived from the transition state theory [30].

Applying the Gibbs equation to this equilibrium, we obtain:

$$K_i = B \cdot e^{-\Delta H_i^{\circ}/RT} \quad (1)$$

where K_i is the unfolding equilibrium constant, ΔH°_i the corresponding standard enthalpy change, B a pre-exponential factor and R the ideal gas constant.

Combining these equations with Michaelis-Menten equation adapted to enzyme unfolding, we obtain [28]:

$$k = \frac{A \cdot e^{-E^*/RT}}{1 + B \cdot e^{-\Delta H_i^{\circ}/RT}} \quad (2)$$

where A is the Arrhenius pre-exponential factor.

At low temperatures, the denominator tends to unit, and this equation simplifies to Arrhenius equation. At high temperatures, the contribution of the inactivated form of the enzyme becomes predominant, and Equation (2) simplifies to:

$$k = \frac{A}{B} e^{(\Delta H_i^{\circ} - E^*)/RT} \quad (3)$$

Plotting the values of $\ln A_c$ recorded at $T > T_{opt}$, a new straight line was obtained, from whose slope ΔH°_i was estimated.

As far as the long-term effect of temperature on enzyme stability is concerned, it is reasonable to assume that it is subjected to irreversible denaturation, responsible for progressive activity loss. It can be described as a temperature-dependent first-order reaction with k_d (/h) as the rate constant. The rate of activity loss due to denaturation can therefore be modeled by the equation:

$$\ln \psi = -k_d \cdot t \quad (4)$$

where ψ is the ratio of the residual activity at time t to that at $t = 0$.

The temperature dependence of k_d was modeled by Arrhenius and Eyring-like equations similar to those given above, and the related activation energy (E^*_d) and enthalpy (ΔH_d^*) were estimated from the respective slopes.

3. Results and Discussion

3.1. Factor screening for collagenase production

The goal of this part of the work was to explore the influence of the initial medium pH, soybean flour (SF) concentration, temperature and agitation speed on extracellular collagenase production by *P. aurantiogriseum* URM4622, according to the 2^4 factorial design described in Section 2.2. Although the statistical analysis was performed for all four responses, namely biomass concentration, collagenolytic activity, total protein and specific activity, the second was considered the most interesting one for industrial purposes; therefore, it was used to evaluate the influence of the four independent variables on enzyme production.

Table 2 lists the conditions and results of these fermentations, which were performed for an incubation time of 72 h, according to previous findings (results not shown). The highest values of the collagenolytic activity (164 U/mL) and biomass concentration (1.8 g/L) were obtained with run 8, carried out with 0.75% SF concentration, 200 rpm, pH 8.0 and 28ºC.

A full factorial model was fitted to 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 in Table 3. A simplified model based only on the effects of Table 3 showed no lack-of-fit, and had a coefficient of determination (R^2) of 0.998. The values of the significant effects indicate that, on average, higher activities were obtained when factors 2 (SF concentration) and 1 (initial medium pH) were selected at their highest levels, and factor 4 (temperature) at the lowest one. However, several interactions were significant: 1*4, 2*3, 2*4, and 1*2*4. In practical terms, this implies that the effect of a given factor depends on the levels of the others. It should be noted that there was a significant interaction involving factors 2 and 3 (orbital agitation speed), even though the main effect of factor 3 was not significant.

This complex situation can be better understood with the help of the cubic plot of Fig. 1, where the estimated collagenolytic activity is displayed against the level combinations of factors 1, 2, and 4. The largest effect of SF concentration is shown as a contrast between the back and the forward faces of the cube. The negative temperature

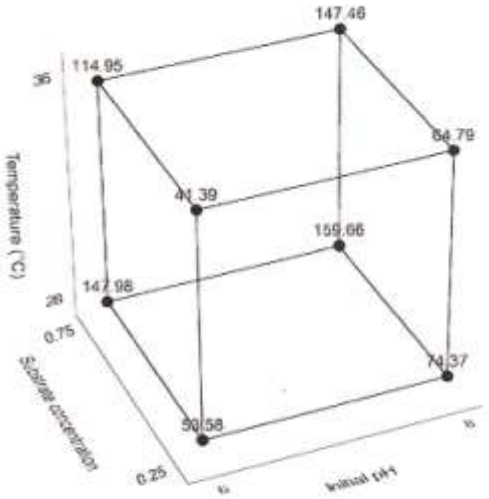

Fig. 1. Cubic plot of the collagenolytic activity values predicted from the effects of Table 3.

effect can be perceived as the decrease in the responses as one goes from the bottom to the top face. Finally, the positive pH effect is shown by the comparatively smaller increase between the left and right faces. The highest estimated response (159.66) is located on the bottom right back corner of the cube, and corresponds to 0.75% SF concentration, pH 8.0 and 28ºC, as already suggested by an examination of the data of Table 2.

It is well known that one of the most important parameters influencing the fermentation system is the amount of substrates providing the nitrogen and carbon sources [31]. It was reported that the effect of a nitrogen supplement on protease production differs from one microorganism to another, although complex nitrogen sources are usually adopted [14,19,32]. Among these, SF would be economically attractive for the industrial production of these enzymes, because it is inexpensive, readily available, and composed of approximately 40% of protein. Three different SF levels were tested in this study, namely 0.25, 0.50, and 0.75% (w/v). Consistent with literature reports on other systems [16,33], we found that SF concentration was the variable most influencing collagenase production, with a positive significant effect of 83.97 (Table 3). These results suggest that protein content of this substrate could be a significant factor for the production of a collagenolytic serine proteinase. Laxman *et al.* [33] observed a similar increase in protease production by *Conidiobolus coronatus* when SF concentration was raised from 1 to only 2%.

Since the initial pH of the culture medium influences many enzymatic processes, enzyme production, cell transport across membranes and expression of extracellular pro-

Table 2. Conditions and results of *P. aurantiogriseum* URM4622 fermentations performed according to the 2^4 full factorial design[a]

Run	pH	S_0 (%)	Agitation (rpm)	T (°C)	X (g/L)	TP (mg/mL)	A_e (U)	a_e (U/mg)
1	6.0	0.25	100	28	0.316	0.26	55.7	210
2	8.0	0.25	100	28	0.360	0.19	77.5	401
3	6.0	0.75	100	28	1.25	0.57	148.0	258
4	8.0	0.75	100	28	1.43	0.51	155.0	302
5	6.0	0.25	200	28	0.616	0.25	51.6	207
6	8.0	0.25	200	28	0.706	0.18	72.0	403
7	6.0	0.75	200	28	1.63	0.57	148.0	261
8	8.0	0.75	200	28	1.84	0.51	164.0	319
9	6.0	0.25	100	36	0.466	0.23	44.0	188
10	8.0	0.25	100	36	0.470	0.15	67.5	443
11	6.0	0.75	100	36	1.28	0.53	111.0	210
12	8.0	0.75	100	36	1.14	0.38	142.0	367
13	6.0	0.25	200	36	0.628	0.22	38.9	179
14	8.0	0.25	200	36	0.312	0.13	62.9	467
15	6.0	0.75	200	36	1.31	0.53	119.0	224
16	8.0	0.75	200	36	0.980	0.41	153.0	371
17c	7.0	0.50	150	32	1.18	0.34	99.6	289
18c	7.0	0.50	150	32	1.16	0.35	101.0	288
19c	7.0	0.50	150	32	1.21	0.34	98.5	289
20c	7.0	0.50	150	32	1.28	0.34	99.0	289

[a]Results refer to 72 h of fermentation. pH = initial pH of the medium; S_0 = soybean flour concentration; T = temperature; X = biomass concentration; TP = total protein; A_e = volumetric collagenolytic activity; a_e = 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) Initial medium pH	22.09	−1.26*
(2) Soybean flour concentration	83.97	0.87
(3) Orbital agitation speed	1.11*	0.16
(4) Temperature	−16.75	−0.20
1*4	5.86	−0.16
2*3	5.94	0.00*
2*4	−5.86	−0.16
3*4	1.42*	−0.20
1*2*4	4.55	−0.05*

*Not significant. The main effects were nevertheless retained in the models for hierarchical reasons.

teases [34], we observed, as expected, a gradual increase in collagenase production when the external pH was raised from 6 to 8. This is in agreement with the results of Lima *et al.* [14], who obtained an increase in collagenase production by *Candida albicans* from 1.8 to 3.5 U when raising the pH from 5.0 to 7.0. Similarly, Chi *et al.* [35] observed that the initial pH of the medium influenced protease production by the marine yeast *Aureobasidium pullulans*, with the highest yield of alkaline protease occurring at pH 6.0. Taken together, these results confirm the importance of the initial medium pH on protease production, although the optimum value of this parameter is microorganism-dependent.

It is also known that temperature is one of the most critical parameters in the fermentation process [36]. In fact, incubation temperature has a strong effect on the duration of the enzyme's synthesis phase [32]. Temperature regulates enzymatic synthesis, but also possibly the secretion of the enzyme by changing the properties of the cell wall [34]. In this work, temperature had a negative effect (−16.75) on protease production, the largest yield being obtained at the lowest level (28°C) (Table 3). Probably, protease secretion was improved at the lowest temperature, to maintain adequate metabolic fluxes under these stress conditions [37], *i.e.* to sustain the assimilation of proteins as the substrate.

The literature on the production of proteases by filamentous fungi is extremely rich. To provide only a few examples, the temperature of maximum protease production by *Engyodontium album* BTMFS10 was 25°C [32] and by *C. coronatus* 28°C [33]. In general, these microorganisms show optimum growth and maximum production within the range of 10 ~ 30°C, and only a few of them appear to require temperatures above 30°C [32,33].

We also found that temperature exerted a significant negative effect on cell growth, whereas SF concentration

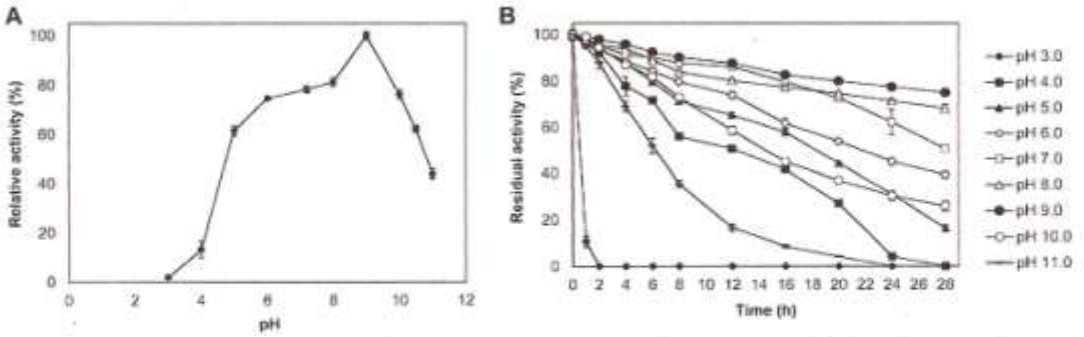

Fig. 2. (A) Effect of pH on the activity of extracellular collagenase produced by *P. aurantiogriseum* URM4622, expressed as percentage of the maximum one obtained in 0.05 M Tris-HCl buffer (pH 9.0). (B) Effect of pH on the stability of extracellular collagenase produced by *P. aurantiogriseum* URM4622, 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.

showed a significant positive one (Table 3), with the largest growth (1.84 g/L) being observed at 28ºC and 0.75% SF concentration (Table 2). This finding is in reasonable agreement with the growth temperatures reported in the literature for *P. aurantiogriseum*, *i.e.* minimum temperature of –2ºC, maximum of 30ºC, and optimum at 23ºC [38].

3.2. Effect of pH on collagenase activity and stability

Fig. 2A shows the pH-dependence of extracellular collagenase activity on azocoll. The enzyme was very active at the pH range of 8 ~ 10 and showed the highest activity at pH 9.0. This value coincides with that found by Sakurai *et al.* [39] for the collagenase from *Streptomyces parvulus*, but is higher than that (8.0) reported for *C. albicans* [14]. More than 60% of the highest activity was retained at the pH range of 5.0 ~ 10.5, whereas the worst results were obtained under more acidic conditions (pH 3.0 ~ 4.0). These results are consistent with those of known collagenases, which were reported to exhibit optimum activity values under neutral or slightly alkaline conditions [4,14, 39], but differ from those reported by Tsuruoka *et al.* [40], who identified a novel collagenolytic enzyme produced by *Alicyclobacillus sendaiensis* with an optimum at pH of 3.9.

Fig. 2B shows the collagenase stability at the pH range of 3.0 ~ 11.0 during 28 h of incubation at 4ºC, selected as a cold storage reference temperature. The enzyme was particularly stable at pH 6.0 ~ 10.0 during the first 8 h, but after 28 h its stability was restricted to the narrower pH range from 7.0 (50.7%) to 9.0 (75.1%). The collagenase produced by *P. aurantiogriseum* proved to be very stable in comparison with those produced by other microorganisms. For example, it was reported that a collagenase produced by *C. albicans* was quite stable within the pH range of 7.2 ~ 8.2 after 4 h [14] and two isolated and characterized collagenases from *Streptomyces* sp. 3B maintained little more than 50% of their maximum activities at pH 6.5 ~ 9.0 after only 30 min [41].

3.3. Effect of temperature on collagenase activity and stability

To find the optimal temperature for enzyme action, the collagenolytic activity was measured at various temperatures

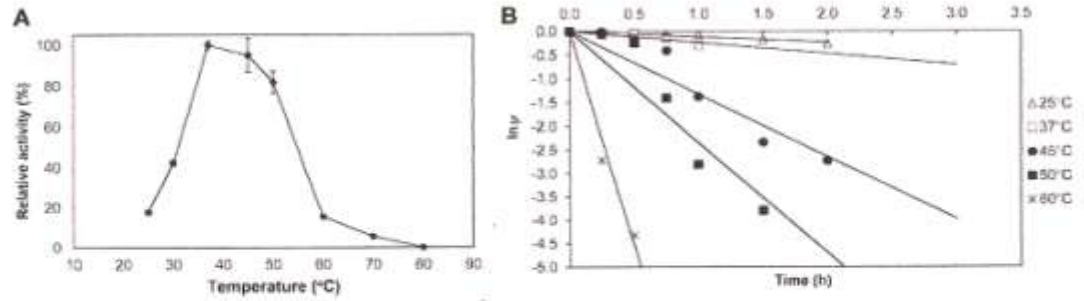

Fig. 3. (A) Effect of temperature on the activity of extracellular collagenase produced by *P. aurantiogriseum* URM4622, expressed as percentage of the maximum one obtained at 37ºC. (B) Effect of temperature on the stability of extracellular collagenase produced by *P. aurantiogriseum* URM4622, expressed as the logarithm of the residual activity with respect to that at the beginning, at each temperature. Each value is the average of the results of three experiments, and the error bars show the standard deviations.

ranging between 25 and 80℃. It was shown to increase with temperature up to 37℃ (Fig. 3A), then decreased continuously and was completely lost at 80℃. More than 80% of the activity was found in the temperature range 37 ~ 50℃.

This result coincides with that reported for two new collagenases from *Streptomyces* sp. 3B (37℃) [41] and does not appreciably differ from that (35℃) of a collagenase from the marine bacterium *Vibrio vulnificus* [4]. However, it is quite lower than that (45°C) observed for proteases from *A. pullulans* [35] and *C. albicans* [14]. Finally, the observed maximum activity found in this work was only one fifth of that recently found for an extracellular protease from *C. buinensis* [42].

Fig. 3B shows semi-log plots of collagenase thermal stability expressed as residual activities at different temperatures with respect to the initial ones. Within the tested temperature range, the activity followed the typical one-step decay of a first-order denaturation pattern, like that observed for both native and immobilized purified lipases [43] and for ascorbate oxidase in a *Cucurbita maxima* extract [44]. The enzyme was very stable after 1.5 h at the temperature range of 25 ~ 45℃, retaining 96.2, 96.3, and 81.6% of the initial activity at 25, 37, and 45℃, respectively. After 6 h, the enzyme was satisfactorily stable at the temperature range of 25 ~ 37℃ and retained 78.5 and 72.7% of the initial activities at 25 and 37℃, respectively. However, at 70℃ its activity was completely lost after only 15 min.

From the slopes of these straight lines, we estimated (with $0.81 \leq R^2 \leq 0.97$) the values of the first-order kinetic constants (k), which varied from 0.116/h at 25℃ to 9.1/h at 60℃, corresponding to half-lives ($t_{1/2}$) of 5.97 h and 4.6 min, respectively. The rate constants progressively increased with temperature as thermal inactivation became more significant, probably due to the breaking of strong electrostatic bonds [44]. Compared with an extracellular protease of *C. buinensis* ($t_{1/2}$ = 2.97 h at 25℃ and 13.9 min at 80℃) [42], our collagenase proved to be interestingly thermostable up to 45℃, but quite unstable beyond this threshold. In addition, as expected from its extracellular localization, $t_{1/2}$ was lower than that of intracellular enzymes (21 ~ 128 min), such as ascorbate oxidase from different sources, at relatively high temperatures (55 ~ 70℃) [44].

The thermal profile of extracellular *P. aurantiogriseum* collagenase was similar to the one from *S. parvulus* [39], which proved to be fairly stable up to 45℃, became remarkably unstable above 50℃, and was almost completely inactivated after 30 min. The collagenases from *Streptomyces* sp. 3B [41] and *V. vulnificus* [4] were reported to be less stable. Although the former showed good stability in reply to 1 h of incubation at 37℃, no more than 50% of its initial activity was retained at 42℃ after 40 min, and a dramatic activity loss occurred at 47℃ after only 20 min. The latter was stable up to 40℃, but at 45℃ only 10% of its initial activity remained after 1 h [4].

The comparison with other proteases revealed that the activity of a serine carboxypeptidase from *Kluyveromyces marxianus* decreased significantly at 60℃, with almost complete inactivation (> 99%) after 30 min at 55℃ [45]. Ma *et al.* [46] observed that an alkaline protease from *A. pullulans* was stable up to 20℃ but became rapidly inactive beyond 45℃, with total inactivation at 50℃ after 30 min.

Plotting the values of residual activity of collagenase after incubation at different temperatures according to Arrhenius and Eyring equations, we obtained the straight lines illustrated in Fig. 4, from which we estimated the thermodynamic parameters listed in Table 4, together with those of the initial catalytic activity.

The activation enthalpy of collagenase activity (ΔH^* = 108.0 kJ/mol) is remarkably lower than the enthalpy variation of the reversible unfolding of the enzyme (ΔH^0_i = 222.3 kJ/mol), which means that the latter equilibrium was comparatively disfavored. As a consequence, it is likely that the subsequent time-dependent thermal denaturation directly involved the active form of the enzyme, with negligible unfolding. The thermodynamic parameters of the catalytic event (E^* = 110.5 kJ/mol; ΔH^* = 108.0 kJ/mol) are higher than those reported for extracellular aspartate protease from *Phycomyces blakesleeanus* (E^* = 29.7 kJ/mol) [47] and halophilic subtilase from *Halobacillus* sp. (ΔH^* = 47 kJ/mol) [48], for pepsin and thermopepsin (E^* = 29.3 kJ/mol) [49], which is indicative of less effective hydrolytic activity.

The activation enthalpy of the irreversible inactivation exhibited a positive value (ΔH^*_d = 104.7 kJ/mol), consistent with the fact that enzyme denaturation is an endothermic process. This value and that of the activation energy (E^*_d

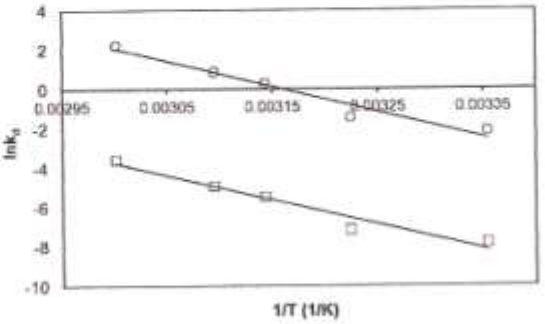

Fig. 4. Arrhenius (○) and Eyring (□) plots of the first-order rate constant for protease denaturation versus the reciprocal of the absolute temperature.

Table 4. Thermodynamic parameters of collagenase activity, reversible unfolding and irreversible denaturation

Parameter	Activity[a]	Denaturation[a]	Unfolding[b]
E (kJ/mol)	110.5	107.4	–
ΔH (kJ/mol)	108.0	104.7	222.3

[a]Activation parameters.
[b]Standard variations of equilibrium parameters.

= 107.4 kJ/mol) are 2 ~ 3-fold those estimated for the extracellular protease from *C. buinensis* (ΔH^*_d = 37.3 kJ/mol; E^*_d = 40 kJ/mol) [42] and reflect a better thermostability at relatively low temperatures. Considering that the thermal energy increases the structural fluctuation in the protein and thus weakens or disrupts noncovalent bonds, these higher values of E^*_d and ΔH^*_d indicate stronger intramolecular stabilizing forces and a less extended conformation [47]. On the other hand, as expected from the extracellular localization of the *P. aurantiogriseum* collagenase, its ΔH^*_d and E^*_d values are smaller than those reported for the irreversible denaturation of different intracellular enzymes (ΔH^*_d = 220 ~ 235 kJ/mol) [28,50], for alkaline protease from *Aspergillus oryzae* either native (ΔH^*_d = 293 kJ/mol) or with engineered twin disulphide bridges (ΔH^*_d = 324 ~ 388 kJ/mol) [51], and for thermostable proteases from the thermophilic bacteria *Sulfolobus solfataricus* (E^*_d = 494 kJ/mol) [52] and *Thermomonospora fusca* (ΔH^*_d = 386 kJ/mol) [53].

3.4. Substrate specificity

Among the protein substrates studied, azocoll, gelatin and collagen (type I) were the most suitable for extracellular *P. aurantiogriseum* URM4622 protease. The enzyme showed the highest activity towards azocoll (105.0 U and 393.0 U/mg) (Table 5). Among the different substrates tested by Tsuruoka *et al.* [40] to investigate the substrate specificity of a collagenolytic serine-carboxyl proteinase from *A. sendaiensis*, the highest specific activities were found for azocoll (258.0 U/mg) and collagen (253.0 U/mg). Nakayama *et al.* [10] obtained a collagenase from the thermophilic *Bacillus* sp. strain NTAp-1 that decomposed azocoll and collagen much more efficiently than casein, the supernatant

Table 5. Substrate specificity of *P. aurantiogriseum* URM4622 protease

Substrate	Total activity (U)	Specific activity (U/mg)	Relative activity[a] (%)
Azocoll	105.0	393.0	100.0
Collagen (type I)	14.9	55.8	14.2
Gelatin	27.1	102.0	25.8
Azocasein	5.2	19.6	5.0

[a]The enzyme's activity towards azocoll was assumed as 100%.

Table 6. Effect of inhibitors on the activity of the *P. aurantiogriseum* collagenase

Inhibitor	Concentration[a] (mM)	Residual activity (%)
PMSF	10	24.1
Iodoacetic acid	10	100
EDTA	10	93.6
Pepstatin A	1	99.1

[a]Final concentration in the assay mixture.

of whose culture contained a collagenolytic activity in azocoll assay of 22.0 U and a caseinolytic activity of 0.68 U. The collagenase produced by *P. aurantiogriseum* was much more efficient in decomposing azocoll than those of *A. sendaiensis* [40] and *Bacillus* sp. strain NTAp-1 [10].

3.5. Effect of inhibitors

The extracellular collagenase from *P. aurantiogriseum* was submitted to inhibition by phenylmethylsulfonyl fluoride (PMSF), which is one method to establish whether or not an enzyme is a serine protease [27]. It kept only about 24% of its activity after 30 min of incubation at 37°C in the presence of 10 mM PMSF, and retained 100 and 99.1% of its activity in the presence of 10 mM iodoacetic acid and 1 mM pepstatin A, respectively (Table 6). Although there would be the need for more complete characterization of the enzyme, this will be the object of the next study. Nevertheless, these preliminary results suggest that the enzyme belongs to a family of serine collagenolytic proteases since the enzyme was inhibited by the irreversible inhibitor of serine proteases. The serine collagenolytic proteases were firstly isolated from the hepatopancreas of the fiddler crab (*Uca pugilator*), but subsequently several microorganisms that produce serine collagenases were discovered. Nagano and To [54] identified a collagenolytic serine protease produced by *Bacillus subtilis* FS-2 that was strongly inhibited by diisopropyl fluorophosphate (DFP) a typical serine protease inhibitor. Okamoto *et al.* [55] found a collagenolytic serine protease from *Bacillus* sp. MO-1 that was highly sensitive to both DFP (95% inhibition) and PMSF (97% inhibition).

The collagenase under investigation was only slightly inhibited by EDTA, which inhibits metalloproteases (about 6% of activity loss), behaving like the proteases from *B. subtilis* FS-2 [54], *Bacillus* sp. MO-1 [55] and *A. sendaiensis* NTAP-1 [40]. However, it was less sensitive to EDTA than the proteases from *V. vulnificus* (about 88% of activity loss) [4], *Streptomyces* sp. 3B (100% of activity loss) [41] and *S. parvulus* (89% of activity loss) [39].

Several microbial collagenases have been characterized [4,39,41,54-58]. A direct comparison of the biochemical properties of the collagenolytic protease from *P. aurantiogriseum* URM 4622 and collagenases from different sources

Table 7. Direct comparison of biochemical properties of the collagenolytic protease from *Penicillium aurantiogriseum* URM 4622 and collagenases from different sources

Microorganism	Optimal temperature	Optimal pH	Classification of protease group	Reference
Penicillium aurantiogriseum	37°C	9.0	Serine protease	This work
Steptomyces parvulus	NA	9.0	Metalloprotease	Sakurai *et al.* [39]
Bacillus subtilis	50°C	9.0	Metalloprotease	Nagano & To [54]
Streptomyces sp.	37°C	7.5	Metalloprotease	Petrova *et al.* [41]
Vibrio vulnificus	35°C	7.5	Metalloprotease	Kang *et al.* [4]
Streptomyces omiyaensis	50°C	8.0 ~ 9.0	Serine protease	Uesugi *et al.* [56]
Streptomyces griseus	45°C	7.5 ~ 8.6	Serine protease	Uesugi *et al.* [56]
Aspergillus flavus	NA	7.4	Metalloprotease	Sukhosyrova *et al.* [57]
Bacillus pumilus	35°C	7.5	Metalloprotease	Wu *et al.* [58]
Bacillus sp.	60°C	8.2	Serine protease	Okamoto *et al.* [55]

NA = Not available.

is presented in Table 7. The characterization of the collagenolytic protease from *P. aurantiogriseum* established several similar features of this enzyme in comparison with other collagenolytic enzymes. These enzymes are generally active at neutral and alkaline conditions, with an optimum pH between 7 and 9, and their activities are irreversibly inhibited by inhibitors either of serine proteases or of metalloproteases.

4. Conclusion

The fungus *P. aurantiogriseum* URM4622 was able to produce large amount of extracellular collagenase that was stable over a wide range of pH and temperature. These results are promising for industrial applications. The most significant variables for collagenase production were singled out, along with their interactions. The collagenase production was improved when soybean flour (SF) concentration and initial medium pH were raised and the temperature was lowered. SF concentration was shown to be the single most impacting variable, which suggests that the protein content of this ingredient is a significant factor for the production of a collagenolytic serine proteinase. The use of SF as an alternative source of nitrogen would be economically attractive for the industrial production of this enzyme because this substrate is inexpensive, readily available, and contains approximately 40% of protein. The optimal collagenase activity with 0.05 M Tris-HCl buffer was found at 37°C and pH 9.0. The activation energy and enthalpy of enzyme activity estimated by a combination of the Arrhenius and Eyring models were $E^* = 110.5$ kJ/mol and $\Delta H^* = 108.0$ kJ/mol, respectively. The extracellular collagenase from *P. aurantiogriseum* is likely to be a serine collagenolytic protease that efficiently decomposes azocoll, gelatin and type I collagen. The next logical step of this research is to purify this protease and to submit it to complete characterization for possible industrial exploitation, including the identification of the catalytic serine residue.

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5. CAPÍTULO III

Fermentation medium for collagenase production by *Penicillium aurantiogriseum* URM4622

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Fermentation Medium for Collagenase Production by *Penicillium aurantiogriseum* URM4622

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Medium composition and culture conditions for maximal collagenase production by Penicillium aurantiogriseum *URM4622 were optimized using a response surface approach. A full two-level design on three factors (initial medium pH, soybean flour concentration, and temperature) was employed to identify the most significant fermentation parameters for collagenase production, and a subsequent central composite design (CCD) was used to find the optimal levels of the two most significant factors (initial medium pH and soybean flour concentration). The design results indicated that the initial medium pH and the temperature had significant negative main effects, whereas the substrate concentration had a positive effect on the collagenase production. The maximum collagenolytic activity predicted by the fitted response surface was expected to occur at pH 7.21, 1.645% soybean flour concentration and 24°C. Three replicate experiments were run at these conditions and yielded an activity response of 283.36 ± 1.33 U, which not only is the highest obtained in this study but also represents a 5-fold increase over the lowest response observed in the initial design. Since all experiments were carried out with an inexpensive substrate, the final results point out to a cost-effective medium for collagenase production with potential industrial-scale applications.* © 2011 American Institute of Chemical Engineers *Biotechnol. Prog.*, 000: 000–000, 2011
Keywords: collagenase, optimization, inexpensive media, central composite design, Penicillium aurantiogriseum

AQ3

Introduction

Collagenases [E.C. 3.4.24.3] are proteolytic enzymes responsible for the degradation of the helical region of native collagen into small fragments. In contrast to mammalian collagenases, which cleave the collagen helix at a single site, microbial collagenases attack multiple sites along the helix.[1] In the past, much attention has been given to the isolation of collagenases from animal tissues. Recently, the emphasis has shifted to obtaining collagenases from microbes, which are an advantageous source in comparison with animals.[2] Among the advantages, the availability of low-cost substrates, the extensive natural sources of microorganisms, and the simple procedures for isolation and purification of the final product may be cited.[3]

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Various bacteria,[2] actinomycetes,[4] yeasts,[5] and fungi[1] are known to secrete collagenases. Filamentous fungi, however, have distinct advantages as enzyme producers. They have high productivities and low production costs, the production processes are faster, and the resulting enzymes can be modified easily. Moreover, their hydrolytic enzymes are released extracellularly, which makes their recuperation from fermentation broths particularly easy.[6] Reports have recently appeared on collagenase biosynthesis by deuteromycetes belonging to the genera *Aspergillus*, *Cladosporium*, *Alternaria*, and *Penicillium*.[7]

In this decade, collagenase research has gained momentum because of additional therapeutic, industrial, and biotechnological applications other than those of conventional proteases.[8–13] Potential therapeutic applications include treatment of Peyronie's disease,[11] various types of destructive fibrosis such as arterial chronic occlusions[12] and postoperative abdominal adhesion.[13] Collagenases also find application in the leather industry, where they are used as biocatalysts to improve dye exhaustion[10] and in the food industry, for ripening and generating the flavor of dry-cured meat products.[8] In the pharmaceutical sector, various commercially available collagenase formulations are used for wound treatment.[14] Collagenase also hydrolyses bovine trachea cartilage, allowing the cultivation of intact mammalian cells and cleaning blood cells for improved screening in medical diagnostics.[9]

Collagens comprise the most prevalent family of proteins in the human body, particularly in the extracellular matrix.[15] Because of collagen's rigid structure (three helically polypeptide fibrils), its degradation is limited to certain proteases, being cleaved only by collagenases.[16] It has recently been shown that the enzymatic degradation of collagen, as well as that of gelatin (a denatured form of collagen), allows efficient utilization of these structural proteins. The degradation produces collagen peptides that have shown several biological activities of industrial and medical interest.[17] This, in turn, has led to several applications, e.g., immunotherapeutic agents, cosmetics moisturizers, preservative, seasoning and dietary materials, and agents for treating osteoporosis, gastric ulceration, and hypertension.[17,18]

Given the potential uses of collagenases and their high demand, there is an interest in finding new fungal strains able to produce collagenases with novel properties, and in developing low-cost industrial media formulations. Media composition is one of the most important parameters in the industrial production of enzymes, because 30–40% of the production costs are due to the growth media. Optimizing the cost/benefit ratio of such processes is therefore a main concern.[19] For biotechnological processes, in which even small improvements can be decisive for commercial success, process optimization is vital. In fermentation technology, productivity improvements of the microbial proteases are achieved, in general, via the manipulation of nutritional and physical parameters, such as carbon and nitrogen sources, pH, temperature, inoculum density, dissolved oxygen, and incubation time.[19,20]

Optimization by the traditional "one-variable-at-a-time" strategy, in which the level of a single independent variable is changed while the levels of the others are kept fixed, is a frequent choice in biotechnology. This procedure, however, is very time-consuming and expensive, especially if a large number of variables are being considered. Even worse, it may be unable to find a true optimum, mainly because of possible interactions between the factors under study.[21] Alternative approaches based on factorial designs and response surface methodologies (RSM) have increasingly attracted attention. These methods are also useful for understanding interactions among various physicochemical parameters, using a minimal number of experiments.[19]

RSM consists of mathematical and statistical techniques for fitting higher-order empirical models. When successfully applied, it evaluates the effects of the individual factors and also predicts optimal combinations of the variable levels yielding desirable response values. RSM is now customarily used in most media optimization studies.[6]

The cost of enzyme production is a major obstacle in successful industrial applications.[22] To be economically feasible, industrial collagenase production should have high yields and use an inexpensive medium. In this work, we report on the use of factorial designs and RSM to optimize collagenase production by *Penicillium aurantiogriseum* URM4622 in a cost-effective medium.

Materials and Methods

Microorganism and culture medium

The *Penicillium aurantiogriseum* dierckx (URM4622) strain was obtained from the Culture Collection of the Mycology Department of the Federal University of Pernambuco. The strain was isolated in the microscopy room air of the collection and qualitatively characterized at once, in a solid medium, for its ability to produce protease (data not published). Full characterization of this strain is currently under way.

The strain was maintained at 4°C in a malt extract agar medium, consisting of 0.5% (w/v) malt extract, 0.1% (w/v) peptone, 0.5% (w/v) glucose, and 1.5% (w/v) agar.

The soybean flour medium (SM) described by Porto et al.[23] and composed of filtered soybean flour at different concentrations, 0.1% (w/v) NH_4Cl, 0.06% (w/v) $MgSO_4.7H_2O$, 0.435% (w/v) K_2HPO_4, 0.01% (w/v) glucose, and 1.0% (v/v) mineral solution, was used for collagenase production. The mineral solution was obtained by adding, per 100 mL of distilled water, 100 mg $FeSO_4.7H_2O$, 100 mg $MnCl_2.4H_2O$, 100 mg $ZnSO_4.H_2O$, and 100 mg $CaCl_2.H_2O$. This fermentation medium was sterilized in an autoclave at 121°C for 20 min.

Inoculum preparation

Inoculum spores were produced in agar plates containing a culture of cells grown for 5 days at 30°C and suspended in a 3 mL of 0.9% (w/v) NaCl with 0.01% (v/v) Tween 80 solution previously sterilized at 121°C for 20 min. All the fermentations were performed in 250 mL Erlenmeyer flasks containing 50 mL of the culture medium inoculated with 10^6 spores mL^{-1}.

Kinetic of growth and collagenase production

Growth curve experiments were carried out over 108 h at 28°C in 250 mL Erlenmeyers flasks containing 50 mL of the culture medium (0.5% of soybean flour, pH 6.0) inoculated with 10^6 spores mL^{-1} and placed in orbital shaker (150 rpm). Samples were removed in triplicate every 12 h for

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Table 1. Real and Coded Factor Levels Used in the 2^3 Design to Investigate the Production of Collagenase by *P. aurantiogriseum* URM4622

Factors	Lower (−1)	Central (0)	Higher (+1)
X_1—Initial medium pH	7.0	8.0	9.0
X_2—Temperature (°C)	24	28	32
X_3—Substrate concentration (%, w/v)	0.50	0.75	1.00

Table 2. Real and Coded Levels of the Factors Used in the 2^2 Central Composite Design

Factors	Lower (−1)	Central (0)	Higher (+1)	Axial (−α)	Axial (+α)
X_1—Initial medium pH	6.0	7.0	8.0	5.0	9.0
X_2—Substrate concentration (%, w/v)	1.00	1.25	1.5	0.75	1.75

determinations of collagenolytic activity, biomass and protein concentrations.

According to Pirt[24] the growth rate of the microorganism (μ_m) was calculated by Eq. 1:

$$\mu_m = \frac{\ln X - \ln X_0}{t} \quad (1)$$

where, X is the final biomass concentration, X_0 the initial biomass concentration, and t the final time.

Screening of the fermentation parameters

A screening of the fermentation parameters for collagenase production by *Penicillium aurantiogriseum* URM4622 was carried out with a full two-level factorial design. Initial medium pH, soybean flour (substrate) concentration, and temperature were selected as the three independent variables (factors), whereas the responses were total protein, biomass concentration, volumetric collagenolytic activity, and specific collagenolytic activity. Eight runs, at all possible combinations of the factor levels, were performed. The real and coded values selected for the three factors are given in Table 1. These levels have been selected based on the results of a previous full two-level four-factor study (submitted for publication).

For model fitting, the actual factor levels were converted into coded, dimensionless values according to Eq 2:

$$x_i = \frac{X_i - X_0}{\Delta X_i/2}, \quad (2)$$

where x_i represents the coded value corresponding to the actual value X_i, X_0 is the average of the two extreme levels, and ΔX_i is the range of variation of the ith factor.

The full model for a 2^3 design includes a constant term, three main effects, three two-factor interactions, and a three-factor interaction. Since no replicates had been carried out at this point, the model was truncated after the two-factor interactions and the three-factor term was taken as an estimate of the error of an effect. With this assumption, the model was reduced to Eq. 3:

$$\hat{y} = b_0 + \sum b_i x_i + \sum b_{ij} x_i x_j, \quad (3)$$

where $\hat{y}$ is the predicted response, b_0 is the constant term, b_i are the linear coefficients, b_{ij} are the interaction coefficients and x_i and x_j are the coded values of the independent variables. The relative significance of the main and interaction coefficients was assessed from the error estimate based on the third-order term, at the 95% confidence level. All statistical and graphical analyses were carried out using the *Statistica 8.0* software (StatSoft, Tulsa, OK).

Optimization of collagenase production by Penicillium aurantiogriseum URM4622 using RSM

To optimize the production of collagenase, the incubation temperature was fixed at the best value found in the screening design (24°C), whereas the levels of the other two factors were varied according to a 2^2 central composite design (CCD). The levels of this second design were also chosen on the basis of the effect estimates obtained in the screening design. The CCD consists of a full 2^2 design augmented with four star (axial) points and a central point repeated six times (to provide an estimate, with five degrees of freedom, of the experimental error of a response). Since there are five levels for each factor, a full second-order model can be fitted to the new response values (Eq. 4):

$$\hat{y}_i = b_0 + \sum b_i x_i + \sum b_{ii} x_i^2 + \sum b_{ij} x_i x_j. \quad (4)$$

The CCD levels are given in Table 2. For simplicity, the star points were rounded off with respect to the theoretical values $\alpha = \pm\sqrt{2}$. This is a minor problem, which only makes the design slightly non-rotatable.

Biomass determination

The fungal mycelia were harvested by filtration using preweighted membranes with a 0.45-μm pore diameter. The dry weight was determined after heating at 80°C to constant weight.

Analytical methods

The Azo dye impregnated collagen-Azocoll (Sigma Chemical Co., St Louis, MO) assay was carried out with a modified version of the method developed by Chavira et al.[25] Azocoll was washed and suspended in a 0.05 M Tris-HCl buffer (pH 7.2) containing 1 mM $CaCl_2$ at a final concentration of 5 mg mL^{-1}. After this, 150 μL of the cell-free supernatant and 150 μL of the buffer were mixed with 270 μL of the azocoll suspension in a 2.0 mL-reaction tube. The reaction tubes were incubated at 37°C in a water bath under agitation. After 3 h of incubation the samples were chilled in ice for 5 min to stop the reaction, and then centrifuged at 10,000*g* and 4°C for 20 min (model KR-20000T, Kubota Seisakusho, Tokyo, Japan). The absorbance of the supernatant solution was measured at 520 nm (model B582 spectrophotometer, Micronal, São Paulo, Brazil). One unit of enzyme activity (U) was defined as the amount of enzyme, per mL of sample that led, after 3 h of incubation, to an absorbance increase of 0.1 at 520 nm, as a result of the formation of azo dye-linked soluble peptides.

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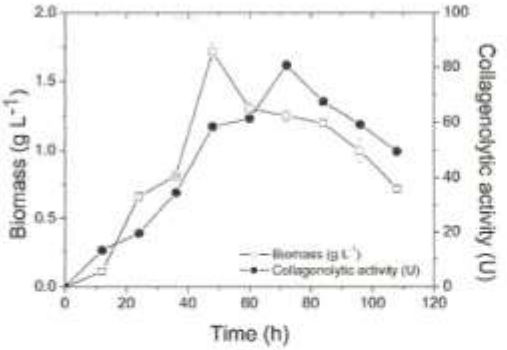

Figure 1. Kinetic of growth and collagenase production by *P. aurantiogriseum* in soybean flour medium.

The specific activity was calculated as the ratio of the enzymatic activity to the total protein in the sample, and expressed in U mg^{-1}.

Protein concentration was determined according to Smith et al.,[26] using bovine serum albumin as a standard.

Results

Kinetic of growth and collagenase production

To select the suitable time for collagenase production, fermentation was performed at 28°C, pH 6.0, and 0.5% (w/v) soybean flour at different time intervals. The behaviors of *Penicillium aurantiogriseum* URM4622 growth and collagenase production are shown in Figure 1. The results showed that the biosynthesis of the enzyme started during the microorganism growth, but the best collagenase production was observed in the stationary phase. It can be seen that *Penicillium aurantiogriseum* URM4622 grew very quickly, reaching a maximum biomass of 1.71 g L^{-1} after 48 h with μ_m of 0.034 h^{-1}. Collagenase was produced since the beginning of the fermentation and reached a maximum activity of 80.7 U after 72 h of incubation, i.e., in the middle of the stationary phase.

On the basis of these results, an incubation time of 72 h was always adopted in the subsequent optimization study.

Screening of the most significant fermentation parameters

The purpose of the first step was to identify the most significant fermentation parameters (experimental conditions) for collagenase production. The influence of the initial medium pH, soybean flour (substrate) concentration, and temperature on collagenase production by *Penicillium aurantiogriseum* URM4622 was evaluated according to the design defined by the levels given in Table 1. Experimental runs were carried out at all possible combinations of these levels. Equation 3 was then fitted to each of the four responses, namely biomass concentration, volumetric collagenolytic activity, total protein, and specific collagenolytic activity, but the second—volumetric collagenolytic activity—was considered the most interesting for industrial purposes and used to evaluate enzyme production effectiveness.

Table 3 contains the matrix of the design's experimental results. The fermentations were performed for an incubation time of 72 h and 200 rpm, following previous findings

Table 3. Matrix of the 2^3 Full Factorial Design Combinations (actual levels) and the Observed Response Values

Run*	pH	T (°C)	S_o (%)	X (g L^{-1})	A_c (U)
1	7	24	0.5	1.16	83.92
2	9	24	0.5	1.14	77.16
3	7	32	0.5	1.09	79.33
4	9	32	0.5	0.97	61.50
5	**7**	**24**	**1.0**	**2.43**	**118.00**
6	9	24	1.0	2.37	104.80
7	7	32	1.0	2.19	102.66
8	9	32	1.0	1.97	56.66

*Results refer to 72-h fermentation. pH = initial medium pH; S_o = soybean flour concentration; T = temperature; X = biomass concentration; A_c = volumetric collagenolytic activity. The best results are shown in boldface.

(results not shown). Of the eight runs in Table 3, the best results for volumetric collagenolytic activity and biomass (118.00 U and 2.43 g L^{-1}, respectively) were obtained in run no. 5, which was performed with 1% substrate concentration, at pH 7.0 and 24°C.

An analysis of variance for the volumetric collagenolytic activities shows that the model can be reduced to its linear form (Eq. 5):

$$\hat{A}_c = 88.64 - 10.47x_1 - 10.47x_2 + 10.02x_3. \quad (5)$$

All variables were significant at a confidence level of 95%, i.e., the main effects values of each one presented $P < 0.05$. The absolute values of the main effect coefficients [initial medium pH (−10.47), temperature (−10.47), and substrate concentration (10.02)] are similar, but the first two, corresponding to the initial medium pH and the temperature, are negative, whereas the third one, substrate concentration is positive (Eq. 5). According to this model, therefore, higher activities are expected at lower temperatures and more acidic pH values, together with increased substrate concentration. These results, then, suggest a displacement of the pH range to lower values and of substrate concentrations to higher values, as is in fact reflected by the CCD levels in Table 2. By the same argument, the temperature range should be displaced toward lower limits. However, the temperature was maintained at 24°C, because lowering it further might adversely affect the process kinetics and consequently the response values.

Optimization of collagenase production

The main responses obtained from the *Penicillium aurantiogriseum* URM4622 fermentations according to the 2^2 CCD used in this study are listed in Table 4. Overall, these values are much superior to the ones in Table 3, previously stated. The best responses of all were observed in the run n° 8 (pH = 7.0 and S_o = 1.75%): volumetric collagenolytic activity of 231.00 U and biomass concentration of 4.61 g L^{-1}.

When full quadratic models are fitted to the data in Table 4, the following equations are obtained:

$$\hat{X} = 3.28 + 0.87x_2, \quad (6)$$

$$\hat{A}_c = 214.43 + 8.34x_1 + 21.00x_2 - 19.28x_1^2 - 6.64x_2^2. \quad (7)$$

In these equations, only the statistically significant terms (at the 95% confidence level) were retained. For biomass response, the analysis of variance, based on the pure error

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Table 4. Level Combinations of the Two Factors (Initial Medium pH and Substrate Concentration) Used in the 2^2 Central Composite Design and Results of the Selected Responses (Biomass Concentrations and Volumetric Collagenolytic activity)

Run	pH	S_0 (%)	X (g L^{-1})	A_v (U)
1	6	1	2.176	156.33
2	6	1.5	3.866	208.42
3	8	1	2.248	160.08
4	8	1.5	3.808	207.17
5	5	1.25	3.082	158.67
6	9	1.25	3.314	204.11
7	7	0.75	1.986	182.33
8	**7**	**1.75**	**4.610**	**231.00**
9 (C)	7	1.25	3.292	214.52
10 (C)	7	1.25	3.316	215.33
11 (C)	7	1.25	3.380	215.56
12 (C)	7	1.25	3.342	213.17
13 (C)	7	1.25	3.066	214.67
14 (C)	7	1.25	3.302	213.32

All runs were performed at 24 °C. Notation as in Table 3. The best results are shown in boldface.

Table 5. Results obtained at the level combination for which a maximum is predicted by Eq. 5

Run	pH	S_0 (%)	X (g L^{-1})	A_v (U)
1	7.21	1.645	5.296	284.00
2	7.21	1.645	5.264	284.25
3	7.21	1.645	5.318	281.83

The temperature remained at 24°C. Notation as in previous tables.

estimate obtained from the replicates at the central point, showed no lack of fit. The form of Eq. 6 indicates that the response surface for biomass is essentially planar, with a positive slope on the substrate concentration. In other words, higher biomass values are expected for higher substrate concentrations, regardless of the pH, and no maximum can be calculated.

For the volumetric collagenolytic activity (Eq. 7), on the other hand, the function is fully quadratic, but no interaction between the factors appears to be significant, which is consistent with the result for biomass. The analysis of variance, however, exhibits a small lack of fit (3% of the regression mean sum of squares), leading to an adjusted R^2 value of 0.83. All the same, the form of the equation allows it to be differentiated with respect to the two factors, in search of a maximum. When this is done, a maximum activity is predicted at $x_1 = 0.216$ and $x_2 = 1.582$. In actual values, these conditions correspond to a pH of 7.21 and a substrate concentration of 1.645%. A confirmatory experiment at these conditions was run in triplicate, yielding the results reproduced in Table 5. The biomass and volumetric collagenolytic activity values are much larger than any of the values obtained so far. They are also highly reproducible, as shown by the low dispersion between the three lines of the table. A visual representation of the surface fitted to the volumetric collagenolytic activity values is shown in Figure 2.

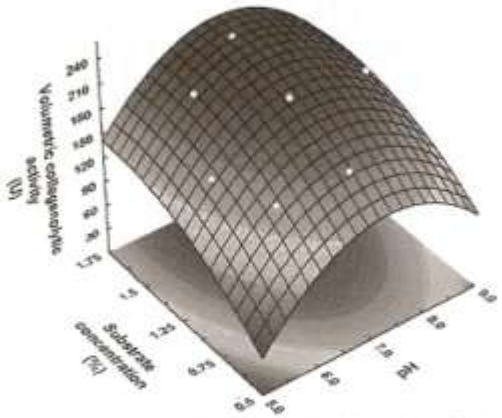

Figure 2. Response surface contour plot of the volumetric collagenolytic activity (U) as a function of coded values for the initial medium pH and substrate concentration (%).

Discussion

This study was conducted in an attempt to optimize the fermentation parameter levels, including medium composition and culture conditions, for maximal collagenase production by *Penicillium aurantiogriseum*. To the best of our knowledge, no previous attempt has been made to optimize collagenase production by the *Penicillium* genus. This is, therefore, the first report on this subject. A preliminary study on growth kinetics and collagenase production was performed at different time intervals to select the suitable time for collagenase production. The second step was a screening of the most significant fermentation parameters, with a 2^3 factorial design. This was followed by an attempt to find the optimal levels of the significant variables, based on a CCD on those two variables.

The results of the preliminary study on growth kinetics and collagenase production showed that the *Penicillium aurantiogriseum* URM4622 grew very quickly reaching a maximum biomass after 48 h, and the biosynthesis of the enzyme started during the microorganism growth, but the best collagenase production was observed in the middle of the stationary phase (72 h). The progressive reduction of the collagenase production after this time was probably due to depletion of nutrients available to the microorganism, as suggested in previous studies.[27,28] A literature survey reveals that the enzyme activity is highly dependent on the type of microorganism, medium composition and the incubation time.[29,30] To give only a few examples, maximum protease production by the thermophilic fungus *Thermoascus aurantiacus* was detected at the end of the stationary phase,[30] while protease synthesis and secretion by *Aspergillus terreus* started during the exponential growth phase and reached its maximum activity at the early stationary one.[31]

The improvement of microbial protease production has been the purpose of several investigations.[6,19–21,32,33] In general, no defined medium was found for the production of proteases by different microorganisms; each strain has specific conditions for maximum enzyme production. Moreover, the effect of environmental conditions on the production of extracellular proteolytic enzymes could play an important role in the induction or repression of the enzyme by specific compounds.[32]

At present, the overall cost of enzyme production is very high, because of the substrates and mediums used. Since the industrial use of proteases is expected to grow tremendously

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in the coming decade, developing novel processes to increase protease yields while lowering their production costs is of high commercial interest.[19] To this end, efforts have been directed to explore new and cheaper substrates and methods to increase protease production and decrease the market price of the desired enzyme.[19,34,35]

Many researchers have attempted to promote protease production by using glucose or starch, together with expensive nitrogen sources such as yeast extract, peptone or casamino acids. Much fewer attempts have been made to induce protease production using inexpensive carbon and nitrogen sources.[19,20] Using soybean flour as an alternative nitrogen source would be economically attractive, because it is an inexpensive and readily available substrate composed of approximately 40% of protein.[36]

The accumulation of extracellular enzymes in the culture media depends on its composition. One of the most important parameters influencing the fermentation systems is the level of substrates providing the nitrogen and carbon sources.[33,37] In this study, the collagenolytic enzyme production was improved when the soybean flour concentration was increased from 0.5% up to 1.645%. Laxman et al.[38] observed a similar increase of protease production by *Conidiobolus coronatus* when the soybean flour concentration was increased from 1% up to only 2%, and the maximum protease activities were obtained from *Aspergillus oryzae*,[6] *Bacillus subtilis* APP1,[39] and *Halobacterium sp.* SP1[33] using 4, 1.5, and 10% soybean flour, respectively.

Temperature is a very relevant factor for microbial protease production.[40] In fact, the incubation temperature has a profound effect on the length of the enzyme's synthesis phase.[41] Temperature not only regulates enzymatic synthesis but also possibly, by changing the properties of the cell wall, the secretion of the enzyme as well.[42] In this work, we found that temperature had a negative effect on collagenase production, the maximum yield being obtained at 24°C, the lower of the tested levels. Probably, protease secretion was improved at the lower temperature, to maintain adequate metabolic fluxes under these stress conditions, i.e., to sustain the assimilation of proteins as the substrate.[32,43]

The influence of temperature on protease production by bacteria is well studied with the genus *Bacillus*, but few studies with fungi have been reported. The *Penicillium aurantiogriseum* strain produced a maximum collagenase level at 24°C. In general, fungi show optimum growth and maximum protease production in the 10–30°C range; and only few of them appeared to require temperatures above 30°C.[38,41,44,45] To provide only a few examples, the maximum protease productions from *Aspergillus clavatus*[21] and *Aspergillus oryzae*[6] were found at 30°C and from *Botrytis cinerea*[45] and *Conidiobolus coronatus*[38] at 28°C.

It has been fairly well established that the initial pH of the culture medium influences many enzymatic processes, such as enzyme production, cell transport across membranes, and the expression of extracellular proteases.[20,42] In this report, we observed that the initial medium pH exerted a negative effect on collagenase production, the optimum production being achieved at pH 7.0. This is in agreement with the results of Hajji et al.,[21] who observed a decrease in protease production by *Aspergillus clavatus* after the initial medium pH was increased. On the other hand, Haddar et al.[19] observed a higher protease yield by *Bacillus mojavensis* A21 at pH 9.0. Taken together, these results confirm the importance of the initial medium pH on protease production, although the optimum value of this parameter is microorganism-dependent.

Statistical experimental designs are now widely used and have a growing acceptance in biotechnology, to optimize culture medium components and conditions.[33] In this study, RSM used for optimization of collagenase production by *Penicillium aurantiogriseum* indicated a significant effect of initial medium pH and substrate (soybean flour) concentration on enzyme production. Analysis of the CCD results for the collagenolytic activity, predicted maximum pH and substrate concentration of 7.21 and 1.645%, respectively. This prediction was confirmed by an experiment run in triplicate at these conditions. The result, 283.36 ± 1.33 U, represented an up to 5-fold increase with respect to the initial responses. Similar results were reported in a study by Beg et al.[46] for alkaline protease production by *Bacillus mojavensis*, who reported a 4.2-fold increase by optimizing culture conditions using RSM. Another study performed by Singh and Chatpar[47] showed that protease production by *Streptomyces* sp. A6 was improved up to 4.96-fold in a 5-L bioreactor using RSM. Our experiments also showed an increase in protease production higher than that reported by Reddy et al.[20] (2.3-fold), Thys et al.[40] (2.6-fold) and Puri et al.[48] (3.6-fold), in which optimization by RSM was used for the production of protease by *Bacillus* sp. RKY3, *Microbacterium* sp. and *Bacillus* sp., respectively.

Conclusion

Production of collagenase by *Penicillium aurantiogriseum* URM 4622 using the new inexpensive medium formulation, composed of soybean flour (1.645% w/v), proposed in this work resulted in a significant increase in collagenase production (5-fold) based on RSM that was quite simple, efficient and time- and materials saving. This study also introduces a new *P. aurantiogriseum* strain as a potential candidate for the production of a collagenase that could have potential applications in pharmaceutical, leather, and food industries.

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Abbreviations

CCD = central composite design
RSM = response surface methodologies
SM = soybean flour medium

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6. CAPÍTULO IV

Two-phase partitioning of a collagenase by *Penicillium aurantiogriseum* URM4622

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Two-phase partitioning of a collagenase by *Penicillium aurantiogriseum* URM4622

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Abstract

The potential application of aqueous two-phase PEG/phosphate systems to remove collagenase from a *Penicillium aurantiogriseum* fermentation broth was investigated. The experiments were carried out in the conditions specified by 2^4 full factorial design. PEG molar mass (M_{PEG}), PEG concentration (C_{PEG}), phosphate concentration (C_{PHOS}) and pH were chosen as the independent variables, while the purification factor (*PF*), the partition coefficient (*K*) and the activity yield (*Y*) were the responses. The analysis of the 2^4 factorial results indicated that *PF*, *K* and *Y* of collagenase in the top phase were increased with increasing C_{PEG} and C_{PHOS} and decreasing M_{PEG} and pH. The best *PF* value (5.23) was obtained with 1500 g/mol M_{PEG}, 17.5% (w/w) C_{PEG}, 15% (w/w) C_{PHOS} and pH 6.0. The highest *Y* value (167.01), in contrast, was obtained with the lower level of C_{PHOS} and the higher level pH 8.0. The electrophoretic study revealed that some protein contaminants were removed after the aqueous two-phase systems (ATPS). Aqueous two-phase systems are a promising alternative for primary collagenase purification from fermented broths.

Keywords: collagenase, aqueous two-phase system, *Penicillium aurantiogriseum*, Purification, factorial design.

1. Introduction

Microbial collagenases have received special attention in medicine and biotechnology, due to their ability to hydrolyze the native and denatured triple helix of collagen to small fragments [1]. They have been widely used in food, pharmaceutical-cosmetic, leather and other industries. In the food industry, for example, they have been used for meat tenderization [2]. In the pharmaceutical industry, various commercially available collagenase formulations are used for wound treatment [3]. In leather industries, collagenases have also been used for skin pre-tanning, softening and bating, for improving the dyeing properties of protein fibers, and decomposing or partially solubilizing protein fibers [4].

In recent years, the collagenolytic activity of these enzymes has been used for clinical and therapeutic applications, particularly for treating Dupuytrens' disease [5], various types of destructive fibrosis such as liver cirrhosis [6], postoperative abdominal adhesion [7], isolation and transplantation of cells such as skin cells [8], pancreatic islet cells [9] and cardiomyocytes [10]. Other uses of collagenases include their application as hydrolyzing agents for the release of collagen peptides. These peptides have several biological activities of industrial and medical interest, leading to various applications: antioxidant agents [11], production of functional food to relieve memory deficits associated with aging [12], and as ingredients for dietary materials and parentally-fed products [13].

For such a variety of uses, increasing quantities of highly pure collagenase are required. So far, collagenases have been purified by traditional methods combining operations like centrifugation, precipitation with ammonium sulfate, ultrafiltration, successive chromatographic steps (ion exchange, molecular exclusion and hydrophobic interaction), dialysis and final concentration of the product [14-15]. These operations are time-consuming, difficult to scale up and require some expensive reagents, contributing to raise the cost of downstream processing. They also increase macromolecular unfolding, with biological activity loss and a poor yield for the whole process. Development of new techniques for the separation and purification of these enzymes has therefore been an important motivation for many of the advances made in the biotechnology industry [16].

Partitioning in aqueous two-phase systems (ATPS) is a good alternative method to separate and purify protein mixtures. ATPS removals of contaminants are simple and economic, because they can be performed in a homogenate of a natural or genetically modified product [17].

An ATPS consists of two liquid phases that are immiscible beyond a critical concentration. The top phase is rich in one polymer and the bottom phase is rich in another

polymer or a salt. It is formed by mixing two incompatible polymers (polyethylene glycol, dextran, etc.) or a polymer and a salt (phosphate, citrate, sulfate, etc.) in water. The low cost of the phase-forming chemicals, especially polymer–salt systems (PEG/salt), makes such systems popular. Also, their viscosity is comparatively low [16; 18-19].

Aqueous two phase systems have a number of advantages over conventional methods for the isolation and purification of proteins: the partition equilibrium is reached very fast, they can easily be scaled up, they offer the possibility of continuous state operation, their cost is low and the materials are recyclable [16]. In general, an ATPS yields a specific environment suitable for maintaining enzymes in their native structure and for concentration/purification by means of selective partitioning of the enzyme to one of the phases [16, 18].

The basis of separation is the selective distribution of a given substance between the phases, depending on the characteristics of the phase system, the substance's properties and the interaction between them. Therefore, partitioning of a substance between the two phases can be manipulated by changing system conditions like type, concentration, and molecular mass of the phase-forming polymer, type and concentration of the phase-forming salt and added (co-solute) salts and also system pH. Environmental conditions, characteristic features (molecular weight, shape, charge and specific binding sites) and surface properties of the biomolecules are also very important factors affecting the separation process [19].

ATPSs have been used for the partitioning and recovery of various proteases such as papain [20], trypsin [21], chymosin and pepsin [22, 23] and proteases from fermentation broths [24, 25]. To the best of our knowledge, no previous attempt has been made to purify collagenases from fermentation broths by an ATPS; therefore, the main aim of this work was to make a first effort in this direction. For this purpose, collagenase produced by *Penicillium aurantiogriseum* URM4622 was extracted/concentrated directly from the fermented broth by a PEG/phosphate ATPS, and a 2^4 full factorial design was used to evaluate the most favorable levels of PEG molecular mass, pH, phosphate and PEG concentrations to perform this process.

2. Material and methods

2.1. Microorganism and culture medium

The *Penicillium aurantiogriseum* dierchx (URM4622) strain was obtained from the Culture Collection of the Mycology Department of the Federal University of Pernambuco.

The strain was maintained at 4ºC in a malt extract agar medium, consisting of 0.5% (w/v) malt extract, 0.1% (w/v) peptone, 0.5% (w/v) glucose and 1.5% (w/v) agar.

The soybean flour medium described by Porto et al. [26], composed of 1.65% (w/v) filtered soybean flour (SF), 0.1% (w/v) NH_4Cl, 0.06% (w/v) $MgSO_4.7H_2O$, 0.435% (w/v) K_2HPO_4, 0.01% (w/v) glucose and 1.0% (v/v) mineral solution, pH 7.21, was used for collagenase production. The mineral solution was prepared by adding, per 100 mL of distilled water, 100 mg $FeSO_4.7H_2O$, 100 mg $MnCl_2.4H_2O$, 100 mg $ZnSO_4.H_2O$ and 100 mg $CaCl_2.H_2O$. This medium was sterilized in an autoclave at 121ºC for 20 min.

2.2 Collagenase production

Inoculum spores were produced in agar plates containing a cell culture grown for 5 days at 28ºC and then suspended in 3 mL of a 0.9% (w/v) NaCl and 0.01% (v/v) Tween 80 solution previously sterilized at 121ºC for 20 min. After inoculation with 10^6 spores/mL, fermentations were carried out at 24 ºC and 200 rpm, in 250 mL Erlenmeyer flasks containing 50 mL of the culture medium. The broth obtained at the end of fermentation (72 h) was vacuum filtered through 0.45 µm pore diameter nitrocellulose membranes to remove the mycelia. Since the target collagenase was an extracellular one, the filtrate was analyzed to determine the final protein concentration and collagenase activity and used as an enzyme source (referred to as the crude extract). All the collagenase purification experiments were made on the crude extract.

2.3. Preparation of aqueous two-phase systems

A 40% (w/w) phosphate buffer solution was prepared 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 1000, 1500, 4000 and 8000 g/mol. These solutions were then added to 15 mL graduated tubes. Fermentation broth amounts 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 phases stored at -20ºC for later determinations of protein concentration and collagenase activity. To avoid any PEG or phosphate interference, all the samples were analyzed against protein-free standard solutions having the same phase composition.

2.4. Analytical techniques

2.4.1 Azocoll assay for collagenase 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. [27]. Azocoll was washed and suspended in a 0.05 M Tris-HCl buffer (pH 7.0) containing 1 mM $CaCl_2$ up to a final concentration of 0.5% (w/v). After this, 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 37ºC in a water bath under agitation. After 3 h of incubation, the samples were chilled in ice for 5 min to stop the reaction and centrifuged at 10,000 x g and 4ºC for 20 min (model KR-20000T, Kubota Seisakusho, Tokyo, Japan). The absorbance of the supernatant was measured at 520 nm by means of a UV-Vis spectrophotometer, model B582 (Micronal, São Paulo, Brazil). One unit of enzyme activity (U) was defined as the amount of enzyme per mL of crude extract that led, after 3 h of incubation, to an absorbance increase of 0.1 at 520 nm, as a result of the formation of azo dye-linked soluble peptides.

The specific activity was calculated as the ratio of the enzymatic activity to the total protein content of the sample, and expressed in U/mg.

2.4.2 Protein determination

Protein concentration was determined according to Smith et al. [28], using bovine serum albumin as the standard.

2.5. Experimental design and statistical analysis

A two-level full factorial designs (2^4) was carried out to study the effects and interactions of the factors PEG molar mass (M_{PEG}), PEG concentration (C_{PEG}), phosphate concentration (C_{PHOS}) and pH on collagenase partition and purification by the ATPS. For the 2^4 design, experiments were run at all combinations of the levels given in Table 1. The central point was run in quadruplicate, to provide an estimate of the pure error variance of the experimental responses. From that, experimental errors of the effects were estimated and used to assess the significance of the main effects and interactions of the four factors on collagenase partition and purification [29].

Three responses were monitored in all experimental runs: the collagenase partition coefficient (K), the activity yield (Y) and the purification factor (PF). The goodness-of-fit of

the full 2^4 model was evaluated by an analysis of variance, from which the statistical significance of each effect, at the 95% confidence level, was determined. All statistical and graphical analyses were carried out with the *Statistica 8.0* software (StatSoft Inc., Tulsa, OK, USA).

Table 1. Factor levels in the 2^4 full factorial design used for studying collagenase partition and purification by ATPS.

Factors	Level		
	Lower (-1)	Center (0)	Higher (+1)
PEG molar mass (M_{PEG})	1500	4000	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

2.6. Determination 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):

$$K = \frac{A_T}{A_B}. \qquad (1)$$

The activity yield was defined as the ratio of A_T in the top phase to the initial one in the fermentation broth (A_F), both multiplied by their respective volumes (V_T and V_F), and expressed as a percentage:

$$Y = \left(\frac{A_T \cdot V_T}{A_F \cdot V_F} \right) \cdot 100. \qquad (2)$$

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

$$PF = \frac{A_T / C_T}{A_F / C_F}, \quad (3)$$

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

The collagenase obtained from the ATPS fraction with the largest purification factor was set aside for further study.

2.7. Protein pattern of the crude extract from *P. aurantiogriseum* and its ATPS fraction

The crude extract and the extracted collagenase from the ATPS were submitted to SDS-PAGE in a 15% polyacrylamide gel, according to Laemmli [30]. The protein molecular markers used in this study 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). Samples containing PEG were precipitated by adding a TCA/acetone solution (1 g/mL) in a 9:1 ratio. Samples were kept overnight at 4 ºC and centrifuged for 10 min at 14,000 x g. Subsequently, the supernatant was discarded and the pellet was washed twice with 0.3 ml 90% (v/v) acetone and centrifuged for 10 min at 14,000 x g. The pellet was resuspended in 50 µL of distilled water. The gels were loaded with 20 µL of concentrated enzyme (equivalent to 50 µg of protein), and subjected to electrophoresis at a constant current of 100 V. The gels were stained overnight with 0.25% (w/v) Coomassie brilliant blue R-250 in methanol-acid acetic-water (45:10:45) and destained in methanol-acetic acid-water (45:10:45).

2.8. Zymogram

The proteolytic activity of enzyme band 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 into the gel. The gels were loaded with 20 µL of concentrated supernatant (equivalent to 50 µg of protein), 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. The gels were then incubated for 18 h at 37 °C in 50 mM Tris-HCl buffer, pH 7.5. The gels were stained overnight with 0.25% (w/v) Coomassie brilliant blue R-250 in methanol-acid acetic-water (45:10:45) and destained in methanol-acetic acid-water (45:10:45).

3. Results and discussion

3.1. Collagenase isolation by the ATPS

3.1.1. 2^4 full factorial design

With this design, our aim was to estimate the influence of PEG molar mass (M_{PEG}), PEG concentration (C_{PEG}), phosphate concentration (C_{PHOS}) and pH on ATPS collagenase partition and purification. The experimental responses are shown in Table 2. Run 5 did not form any biphasic system. Full factorial models were fitted to the three responses (*K*, *Y* and *PF*). The models contain four main effects, six two-factor interaction effects, four three-factor interactions and one four-factor interaction which, for hierarchical reasons, is usually assumed to be negligible [29]. The statistically significant effect estimates (at the 95% confidence level) are shown in Table 3. All main effects, on all three responses, are statistically significant. Most interactions are also significant, and should be taken into account in the analysis of a given factor's effect.

Table 2. 2^4 design factor level combinations and results for the extraction of collagenase from *P. aurantiogriseum* by a PEG/phosphate ATPS. No biphasic system was observed in run no. 5. (See text for details).

Run	M_{PEG}[a] (g/mol)	C_{PEG}[b] (% w/w)	pH	C_{PHOS}[c] (% w/w)	K^d	Y^e (%)	PF^f	PV^g (mL)
1	1500	12.5	6.0	10.0	0.91	115.02	3.12	4.8
2	8000	12.5	6.0	10.0	0.20	21.93	0.79	4.2
3	1500	17.5	6.0	10.0	0.73	106.30	2.34	5.8
4	8000	17.5	6.0	10.0	0.25	32.00	0.84	5.0
5	1500	12.5	8.0	10.0	-	-	-	-
6	8000	12.5	8.0	10.0	0.23	27.40	0.66	4.4
7	1500	17.5	8.0	10.0	1.04	167.01	3.35	6.0
8	8000	17.5	8.0	10.0	0.24	33.08	0.65	5.2
9	1500	12.5	6.0	15.0	0.84	63.48	3.50	3.6
10	8000	12.5	6.0	15.0	0.70	52.43	1.40	3.4
11	1500	17.5	6.0	15.0	1.20	110.70	5.23	4.4
12	8000	17.5	6.0	15.0	1.41	134.56	2.46	4.6
13	1500	12.5	8.0	15.0	1.13	96.44	2.15	3.8
14	8000	12.5	8.0	15.0	0.45	29.76	0.68	3.2
15	1500	17.5	8.0	15.0	1.52	135.71	2.65	4.4
16	8000	17.5	8.0	15.0	1.44	121.55	2.08	4.2
17(C)	4000	15.0	7.0	12.5	0.35	36.92	1.35	4.4
18(C)	4000	15.0	7.0	12.5	0.33	36.90	1.43	4.6
19(C)	4000	15.0	7.0	12.5	0.33	36.42	1.51	4.6
20(C)	4000	15.0	7.0	12.5	0.34	36.26	1.30	4.6

[a] PEG molar mass; [b] PEG concentration; [c] Phosphate concentration; [d] Partition coefficient; [e] Activity yield; [f] Purification factor; [g] Top phase volume.

Table 3. Statistically significant effect estimates ($p<0.05$) determined from the responses given in Table 2. Single digits indicate main effects; digit combinations stand for two- or three-factor interaction effects. Notation as in Table 2.

Effect	*PF*	*Y*	*K*
1 (M_{PEG})	-1.77	-39.23	-0.24
2 (C_{PEG})	0.65	52.35	0.45
3 (pH)	-0.67	-1.23	-0.06
4 (C_{PHOS})	0.79	28.29	0.66
12	------	-4.93	------
13	0.31	-6.05	------
14	------	27.69	0.11
23	------	20.89	0.21
24	0.52	11.55	0.16
34	-0.58	8.01	0.15
123	------	-19.56	-0.16
124	------	28.00	0.25
134	0.39	-18.56	-0.21
234	-0.36	-19.26	-0.14

3.1.1.1 Effects on the partition coefficient

The partition coefficient, *K*, is often used to measure the extent of the separation of biomolecules in aqueous two-phase polymeric systems. When *K* is significantly larger for the target biomolecule than for the other biomolecules present in the system, the extraction is more successful [31].

All four factors have significant main effects on the partition coefficient (*K*). There are also several significant interactions (**14**, **23**, **24**, **34**, **123**, **124**, **134** and **234**), which means that the effect of a given factor on *K* depends on which levels the other factors are set.

Phosphate concentration, C_{PHOS} (**4**), has the largest effect on *K*. Since this effect is positive, higher C_{PHOS} values are associated with larger *K* values. This can be ascribed to the "salting out" effect. As the salt concentration increases, the solubility of the biomolecules in the salt-rich (bottom) phase decreases, which results in increased partitioning of the biomolecules to the top phase [32]. Several researchers have reported similar results. For example, Babu et al. [32] observed that raising phosphate concentration from 14 to 20%

resulted in an increase in the partition coefficients of bromelain and polyphenol oxidase. Rawdkuen et al. [33] observed an increase, from 1.06 to 2.59, in the ATPS partition coefficient of the *Calotropis procera* latex protease when the phosphate concentration was raised from 14% to 20%.

K was also positively influenced by an increase in C_{PEG} (**2**). As C_{PEG} is raised, the number of polymer units involved in the biomolecular partitioning also increases and more protein molecules partition into the PEG phase due to hydrophobic interaction between the protein and PEG [34, 35]. Since the interaction effect between C_{PHOS} and C_{PEG}, **24**, is also positive, these two factors act synergistically. The increase, on the partition coefficient, of simultaneously raising the levels of both factors is larger than the sum of the results of individual level shifts.

In contrast, the main effects of M_{PEG} (**1**) and pH (**3**) are negative. M_{PEG} = 1500 g/mol resulted in more collagenase partitioning to the top phase than M_{PEG} = 4000 g/mol or 8000 g/mol. Larger M_{PEG} values result in less available space for collagenase in the top phase, which leads to a decrease in the partition coefficient. Several authors, such as Araújo et al. [36], who studied partition of lactate dehydrogenase from bovine heart crude extract in ATPS, Yan-Min et al. [37], who studied bovine serum albumin partitioning in ATPS and Rawdkuen et al. [33], who isolated a protease from *Calotropis procera* latex by an ATPS, agree that there seems to be a relation between the partition coefficient (*K*) and PEG molar mass, with higher molar masses leading to an increase in the exclusion effect and a decrease in *K*. A possible explanation is that an increase in polymeric molar mass decreases the available amount of solvent in the PEG-rich phase needed to solubilize the enzyme and the salt [38]. Moreover, in aqueous solution PEG acts as a highly mobile molecule with a large exclusion volume [38, 39].

The pH main effect on the collagenase partition was slight (-0.06), but its negative value indicates that in lower pH values the enzyme preferentially partitioned to the top phase. pH could affect partitioning either by changing the solute's charge or by altering the ratio of the charged species present [40].

3.1.1.2. Effects on the activity yield

Higher average collagenase activity yields are associated with higher C_{PEG} and lower M_{PEG} levels. Yields of over 100% were found with M_{PEG} of 1500 g/mol and C_{PEG} of 17.5%. Increases in *Calotropis procera* protease [41] and lactate dehydrogenase yields [36] were also found when low polymer molar masses were used in the ATPS. In the present study,

exceptions occurred in runs 12 and 16, for which PEG molar mass was 8000 g/mol. In these cases, since the phosphate concentration was 15% (w/w), salting out apparently prevailed over volume exclusion, leading to more collagenase partitioning to the top phase and resulting in high activity yields.

The main effect of C_{PHOS} was significantly positive while that of pH was significantly negative. Several large two- and three-factor interactions involving all factors were observed (Table 3), meaning that the effect of one of these factors is strongly dependent on the levels of the other three and making the interpretation of these effects very complex. The algebraic signs of the respective main effects suggest that higher *average* activity yield values are expected when M_{PEG} is at its lower level and C_{PEG} and C_{PHOS} are at their higher, but in fact the highest activity of all, 167.01 % (run no. 7 in Table 2), was obtained with M_{PEG} = 1500 g/mol, C_{PEG} = 17.5 % w/w, pH = 8.0 and C_{PHOS} = 10.0 % w/w.

Activity yield values higher than 100%, like many obtained in this study, have frequently been reported for enzyme extraction using liquid–liquid systems [42, 43, 44] as the result of a positive PEG interaction with the protein [39, 44, 45].

3.1.1.3. Effects on the purification factor

Though the results for the partition coefficient and activity yield are significant, the purification factor (*PF*) is considered the primordial response here, because the collagenase is being purified for clinical and therapeutic purposes. Considering first the PEG molar mass, lower levels are clearly associated with improved purification values (Table 2), as anticipated by its negative main effect. This result is in agreement with other reports based on biological extracts. For example, in investigations of ATPS's potential use for recovering bromelain from pineapple (*Ananas comosus* L. *Merr*.) [32] and lactate dehydrogenase from bovine heart crude extract [36], better purification factors were achieved with lower PEG molecular masses.

In the present study, M_{PEG} was responsible for the largest effects both on the purification factor in the top phase and on the activity yield (Table 3), a result that can be better visualized in Figure 1. A similar relationship was also described by Spelzini et al. [22], using a PEG-phosphate system, suggesting the non-specific nature of the interaction of PEG and a large amount of protein through hydrophobic regions. The high purification factors and yields observed in our study with PEG = 1500 g/mol could also be explained by the elimination of inhibitors during purification, which favors enzyme stability, as described for the partitioning of xylose reductase in a PEG-phosphate system [42].

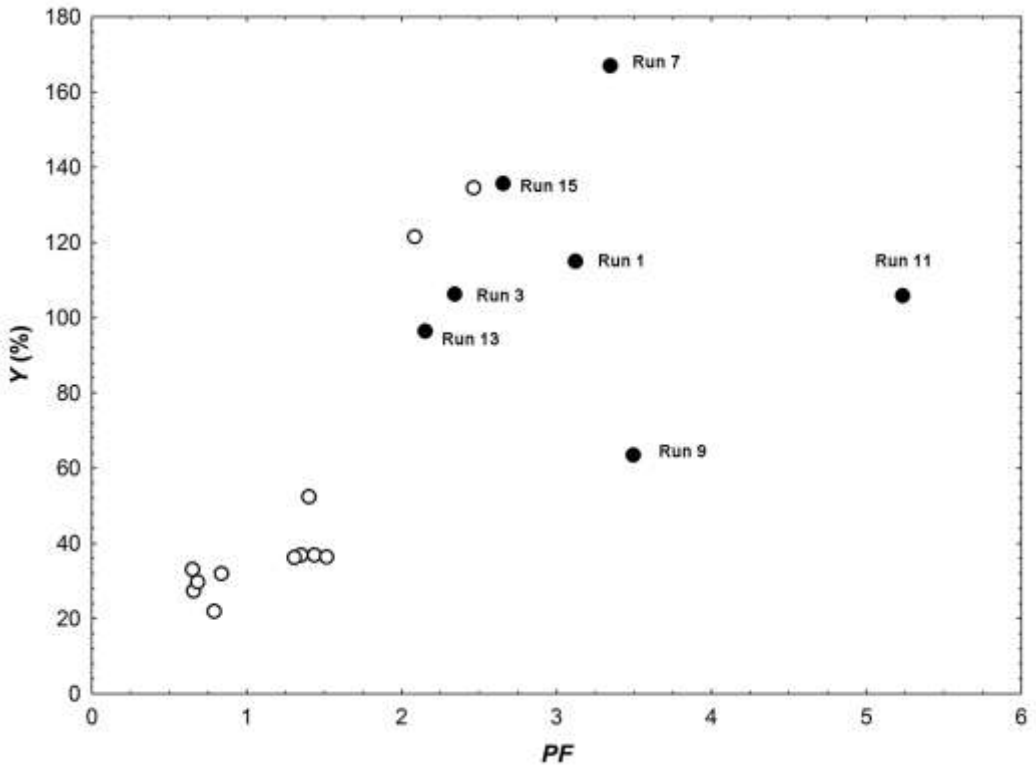

Figure 1. Activity yield as a function of the purification factor for collagenase purification by PEG/phosphate ATPS from a *Penicillium aurantiogriseum* fermentation broth. Black circles represent results obtained with M_{PEG} = 1500 g/mol.

C_{PHOS} also exerted an important influence on the collagenase purification. Since its effect, the second largest, is also positive, increasing C_{PHOS} should improve collagenase purification to the top phase. It has been reported that the purification factor of biomolecules in the top phase using ATPS changes with the concentration of salts added, an improvement being usually observed with higher salt concentrations. For example, the purification factor of a protease from the *Calotropis procera* latex isolated by an ATPS increased from 3.23 to 5.27 when C_{PHOS} was raised from 14 to 17% [34]. Ketnawa et al. [46], studying the effect of C_{PHOS} on bromelain partitioning, also observed a better purification factor with more concentrated phosphate solutions.

The absolute values of the other main effects are similar, that of C_{PEG} being positive, while the pH effect is negative (Table 3). Higher *PF* values, therefore, are expected at more acidic pH values and more concentrated PEG solutions. Considering all main effects together, the best level combination, with respect to the purification factor, is that of run no. 11 (1500 g/mol M_{PEG}, 17.5% (w/w) C_{PEG}, 15% (w/w) C_{PHOS} and pH 6.0), which indeed shows the best PF value of all, 5.23 (Table 2).

In this study we found that an increase in PEG concentration levels favours collagenase purification to the top phase. Babu et al. [32], Yang et al. [47] and Nalinanon et al. [23] observed similar increases in the purification of, respectively, polyphenol oxidase, xylanase and protease.

3.2. Protein pattern of crude extract from *P. aurantiogriseum* and its ATPS fraction

The purity of the collagenase from the crude *P. aurantiogriseum* extract after the ATPS was analyzed by SDS-PAGE (Fig. 2A). The crude extract contained a major band with a molecular mass (MM) of 39.16 KDa, but proteins with MMs of 83.55, 79.09, 67.82, 62.25, 43.61, 37.07, 32.49, 32.49, 28.17, 21.4, 18.15, 17.18, 13.79, 12.36 and 11.07 kDa were also found. After the ATPS, a major band with MM of 39.16 KDa, collagenase band confirmed by zymogram analysis (Fig. 2B), was observed in the top phase of the system with 1500 g/mol M_{PEG}, 17.5% (w/w) C_{PEG}, 15% (w/w) C_{PHOS} and pH 6.0. A large number of contaminating proteins were also removed after ATPS partitioning, mainly low molecular mass ones. Electrophoretic results clearly showed that this ATPS condition primarily purified the collagenase from the crude *P. aurantiogriseum* extract.

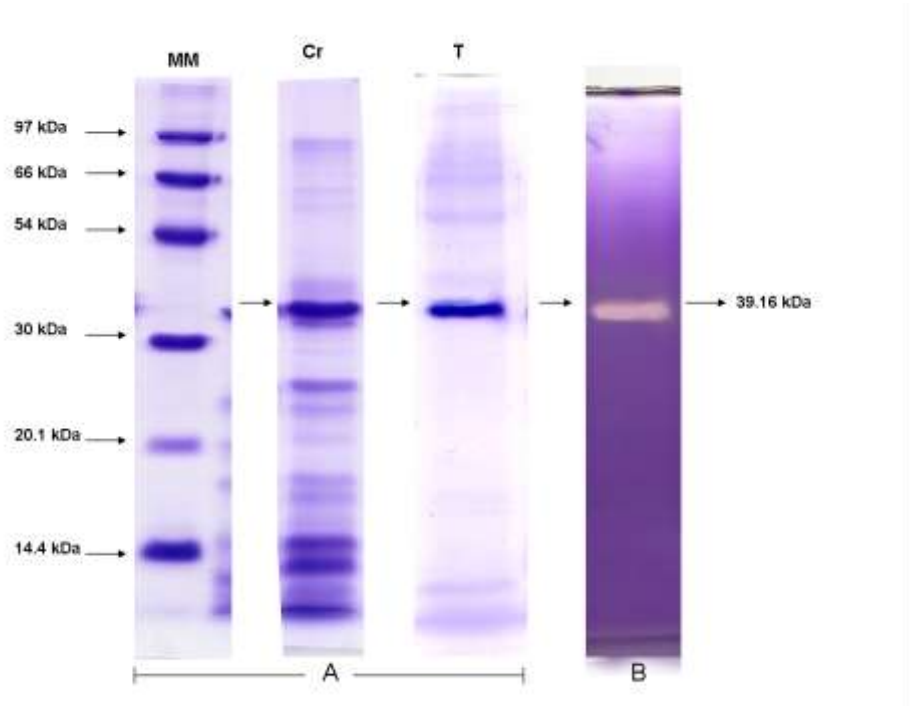

Figure 2. (A) SDS-PAGE patterns of *P. aurantiogriseum* extract and its top phase ATPS fraction obtained with 17.5% PEG1500, 15% phosphate concentration and pH 6.0. MM: molecular mass, Cr: crude extract; T: top phase of the ATPS. (B) Zymogram analysis of collagenase.

4. Conclusions

This paper describes a simplified strategy to use a PEG-phosphate aqueous two-phase system for the purification of a collagenase from *Penicillium aurantiogriseum* fermentation broths. The experimental models fitted to the experimental response values indicated that the collagenase can be readily extracted and purified. The most significant parameters influencing the purification factor, the activity yield and the partition coefficient were PEG molar mass and the PEG and phosphate concentrations ($M_{PEG,}$ C_{PEG} and C_{PHOS}, respectively), while the effect of pH was negligible, in the studied range. C_{PEG} and C_{PHOS} had positive main effects on all responses, and M_{PEG} had a negative effect. Different ATPS systems led to different partitioning of the target enzyme and contaminants. The highest purification factor (5.23) occurred with the factor level combination 1500 g/mol M_{PEG}, 17.5% (w/w) C_{PEG}, 15% (w/w) C_{PHOS} and pH 6.0. Based on these results, it is suggested that aqueous two-phase systems are a promising alternative for primary collagenase purification from fermented broths.

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7. CAPÍTULO V

Hydrolysis of bovine collagen type I by *Penicillium aurantiogriseum* URM 4622 collagenase and biological properties of the enzymatic hydrolysates

Artigo submetido para o periódico Food Chemistry

Hydrolysis of bovine collagen type I by *Penicillium aurantiogriseum* URM 4622 collagenase and biological properties of the enzymatic hydrolysates

RUNNING TITLE: Production of collagen peptides using *Penicillium aurantiogriseum* URM 4622 collagenase

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Abstract

A 2^3 full factorial design was used to identify the main effects and interactions of pH, collagen concentration, and temperature on the degree of collagen hydrolysis by collagenase from *Penicillium aurantiogriseum* URM 4622. The results indicated that pH and collagen concentration had significant positive effects, whereas temperature had a negative effect, on the degree of collagen hydrolysis. The most favourable hydrolysis conditions were 7.5 mg/mL collagen, pH 8.0, and 25 °C, which led to a degree of hydrolysis of 4.65 µg/mL. The peptide profile obtained under these conditions showed peptides with molecular weights less than 11 kDa, with several peptides having molecular weights of less than 2 kDa. These peptides exhibited antibacterial activity against *Escherichia coli* (MIC 0.625 mg/mL), *Bacillus subtilis* (MIC 5 mg/mL), *and Staphylococcus aureus* (MIC 0.55 mg/mL). The hydrolysates showed concentration-dependent ABTS radical-scavenging activity. An antioxidant activity of 84.7% ± 0.24% was obtained with 50 mg/mL of hydrolysates. This study demonstrated that collagen hydrolysed by *P. aurantiogriseum* URM 4622 collagenase has antibacterial and antioxidant activities. Such hydrolysates have potential applications in the food industry.

Keywords: collagen peptides, bioactive peptides, antioxidant activity, antibacterial activity, collagenase, *Penicillium aurantiogriseum*

1. Introduction

Collagen is the most abundant protein in vertebrates and constitutes about 30% of the total protein. Collagen, a right-handed triple superhelical rod, is unique in its ability to form insoluble fibres possessing high tensile strength. There are at least 27 different types of collagen, namely, type I–XXVII (Birk & Bruckner, 2005). Type I collagen is commonly found in the connective tissues, including tendons, bones, and skin. All members of the collagen family are characterized by domains with repetitions of the proline-rich tripeptide Gly-X-Y, which are involved in the formation of the triple helix (Muyonga, Cole, & Duodu, 2004).

Collagen is a natural material with good biological compatibility and biological degradability. Collagen has a wide range of applications in the leather and film industries, in cosmetic and biomedical materials, and as food (Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005). In pharmaceutical applications, collagen can be used to produce wound dressings, vitreous implants, and carriers for drug delivery. In addition, collagen is a source of biologically active peptides with promising applications (Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011).

Food proteins, like collagen, contain peptide sequences encrypted in their primary structures that are capable of modulating specific physiological functions. These protein fragments, termed bioactive peptides, are inactive within the sequence of the precursor protein, and can be released through enzymatic hydrolysis *in vivo* or *in vitro*. After release, they may exert several biological activities, and therefore, have potential applications in food science, technology, and nutrition (Sarmadi & Ismail, 2010).

In particular, collagen peptides are considered to be ideal ingredients in the field of functional foods because of their high nutritional value, high antihypertensive activity, and low antigenicity (Jian-xin & Zheng, 2009). Therefore, the interest of food, pharmaceutical, and cosmetic industries in the application of collagen hydrolysates has grown during the last two decades (Korhonen & Pihlanto, 2006). Collagen hydrolysates can be used as seasonings, non-allergic preservatives for drugs, ingredients for dietary materials and parentally fed products (Nakayama, Tsuruoka, Akai, &Nishino, 2000), and antioxidant and antimicrobial agents (Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011), and for treating diseases such as collagen-induced arthritis (Zhu et al., 2007).

The most common way to generate bioactive peptides is through enzymatic hydrolysis of whole protein molecules. The bioactivity of such hydrolysis-generated peptides appears to be inherent to size and specific amino acid sequences (Korhonen, 2009). Proteolytic enzymes

from various sources (animal, plant, and microbes) have been successfully employed in the production of these molecules (Korhonen & Pihlanto, 2006).

Since microorganisms can be cultured through well established methods to produce high amounts of enzyme, microbial proteases are attractive biocatalysts for commercial production of protein hydrolysates and bioactive peptides. In this sense, investigation of novel proteases is essential to obtain protein hydrolysates with the desired properties. The collagenolytic fungus *Penicillium aurantiogriseum* URM 4622 was shown to produce a biotechnologically relevant extracellular serine collagenase (Lima et al., 2011a; Lima et al., 2011b). In the current study, a collagenase preparation from this strain was employed in the hydrolysis of collagen type I. The molecular weight distribution of the collagen peptides obtained under optimal conditions of hydrolysis was identified, and the biological activities (antioxidant and antimicrobial) of the protein hydrolysates were assessed to evaluate the potential of the peptides for food applications.

2. Material and methods

2.1. Microorganism and culture medium

P. aurantiogriseum (URM 4622) strain was obtained from the Culture Collection of the Department of Mycology of the Federal University of Pernambuco (Micoteca). The strain was maintained at 4 °C in malt extract agar medium, consisting of 0.5% (w/v) malt extract, 0.1% (w/v) peptone, 0.5% (w/v) glucose, and 1.5% (w/v) agar.

The soybean flour medium described by Lima et al. (2011b), composed of 1.6% (w/v) filtered soybean flour (SF), 0.1% (w/v) NH_4Cl, 0.06% (w/v) $MgSO_4 \cdot 7H_2O$, 0.435% (w/v) K_2HPO_4, 0.01% (w/v) glucose, and 1.0% (v/v) mineral solution, was used for collagenase production. 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 $CaCl_2 \cdot H_2O$. This fermentation medium was sterilized in an autoclave at 121 °C for 20 min.

2.2. Collagenase production

Inoculum spores were produced in agar 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 10^6 spores/mL, fermentation was carried out at 24 °C and 200 rpm in 250-mL Erlenmeyer flasks containing 50 mL of the soybean flour medium (pH 7.21). The broth obtained at the end of fermentation

(72 h) was vacuum filtered through 0.45-µm nitrocellulose membranes to remove the mycelium. As the target collagenase is extracellular, the filtrate was analyzed to determine the final protein concentration and collagenase activity, and was used as a source of the enzyme (referred to as crude extract). Crude extract was used for collagenase purification.

2.3. Collagenase partial purification by aqueous two-phase system (ATPS)

A 40% (w/w) phosphate buffer solution was prepared at room temperature (25 ± 1 °C) by mixing the amounts of dibasic and monobasic sodium phosphates needed to reach pH 6.0. The required amounts of this solution were mixed with 60% (w/w) 1500 g/mol PEG solution. The resulting solutions were then added to 15-mL graduated tubes. Crude extract corresponding to 20% (w/w) of the total mass was subsequently added, along with enough water to give a 10-g system. After 1.0 min of vortex shaking, the two phases were left to separate by settling for 120 min. The extracted collagenase from the ATPS (PEG-rich phase) was used for collagen hydrolysis.

2.4. Azocoll assay for collagenase 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, Burnett, & Hageman (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 3 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 3 h of incubation, to an absorbance increase of 0.1 at 520 nm, because of the formation of azo dye-linked soluble peptides.

2.5. Protein determination

Protein concentration was determined according to Smith et al. (1985), using bovine serum albumin as a standard.

2.6. *Assay for collagen hydrolysis*

The experiments were performed according to Endo, Murakawa, & Shimizu (1987). The standard reaction mixture, containing different concentrations (2.5, 5.0, and 7.0 mg/mL) of collagen (type 1, bovine Achilles tendon; Sigma Chemical Co., St Louis, MO) in 5 mL of different buffers (0.05 M citrate (pH 6), 0.05 M Tris-HCl (pH 7 and 8)), was incubated with 1 mL of purified collagenase (60 U) at different temperatures (25, 37, and 49 °C). 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 degree of hydrolysis (DH) was quantified by assessing the hydroxyproline content (μg/mL) in the supernatant by the method of Creemers, Jansen, Veen-Reurings, Bos, & Everts (1996). The supernatant (peptide fraction) was further collected and concentrated. Its peptide profile was determined, and its antimicrobial and antioxidant activities were tested.

2.7. Effect of process parameters on the degree of hydrolysis (DH)

A 2^3 full factorial design was used to study the effects and interactions of independent variables, pH, substrate (collagen) concentration, and hydrolysis temperature on the degree of collagen hydrolysis. Eight runs, with all possible combinations of the factor levels, were performed. The real and coded values selected for the three factors are given in Table 1.

For model fitting, the actual factor levels were converted into coded, dimensionless values according to Eq. 1:

$$x_i = \frac{X_i - X_0}{\Delta X_i / 2} \quad (1)$$

where x_i represents the coded value corresponding to the actual value X_i, X_0 is the average of the two extreme levels, and ΔX_i is the range of variation of the i^{th} factor.

The full model for a 2^3 design includes a constant term, three main effects, three two-factor interactions, and a three-factor interaction. The following linear regression model was employed to predict the response (Eq. 2):

$$\hat{y} = b_0 + \sum b_i x_i + \sum b_y x_i x_j \quad (2)$$

where $\hat{y}$ is the predicted response, b_0 is the constant term, b_i are the linear coefficients, b_{ij} are the interaction coefficients, and x_i and x_j are the coded values of the independent variables. The relative significance of the main and interaction coefficients was assessed from the error estimate based on the third-order term, at the 95% confidence level. All statistical and graphical analyses were carried out using *Statistica 8.0* software (StatSoft Inc., Tulsa, OK).

Table 1. Real and coded factor levels used in the 2^3 design to investigate the degree of collagen hydrolysis by *P. aurantiogriseum* URM4622 collagenase.

Factors	Lower (-1)	Central (0)	Higher (+1)
X_1 – pH	6.0	7.0	8.0
X_2 - Hydrolysis temperature (ºC)	25	37	49
X_3 - Substrate concentration (mg/ml)	2.5	5.0	7.5

2.8. Peptide profile by mass spectrometry and amino acid sequence (MALDI TOF-MS/MS)

Peptide profiles were analyze by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) using an Autoflex III mass spectrometer (Bruker Daltonics, Bremen, Germany), equipped with a 355 nm Nd:YAG laser. The sample was mixed with the matrix solution (10 mg/mL α-cyano-4-hydroxycinnamic acid in 50% acetonitrile with 0.3% trifluoroacetic acid). Then, 0.5 µL of this mixture was spotted onto a MALDI target plate (MTP 384 ground steel, Bruker Daltonics, Bremen, Germany) and left to dry at room temperature. Mass spectra were acquired in positive reflectron mode with an acceleration voltage of 19 kV and a laser frequency of 100 Hz. The ion detection range was m/z 700–4480. After that, the selected parent ions were fragmented using LIFT mode. External calibration was performed using a standard mixture of peptides (Bruker Daltonics). Data were acquired using Flex Control software (Version 3.0, Bruker Daltonics), and spectra were processed using Flex Analysis software (Version 3.0, Bruker Daltonics). The obtained amino acid sequences were compared with sequences in the protein database using the NCBI BLAST program (www.ncbi.nlm.nih.gov).

2.9. Determination of the antimicrobial activity of hydrolysates

The antimicrobial activity of potential bioactive peptides contained in collagen type I hydrolysates generated by the collagenase from *P. aurantiogriseum* was tested by the

minimum inhibitory concentration (MIC) method as described by the Standard Clinical and Laboratory Standards Institute (CLSI) (2003). The microorganisms used were *Enterococcus faecalis* ATCC 6057, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 29665, and *Staphylococcus aureus* ATCC 6538. These strains were obtained from the Culture Collection of the Department of Antibiotics of the Federal University of Pernambuco. The amount of hydrolysate tested against these microorganisms ranged from 0.5 to 5 mg/mL.

2.10. Determination of the antioxidant activity of hydrolysates

Experiments were carried out using the ABTS assay (Re, Pellegrini, Proteggente, Pannala, & Rice-Evans, 1999), which involves generation of the 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical chromophore by oxidation of ABTS with potassium persulphate. The ABTS radical cation was produced by reacting 7 mmol ABTS stock solution with 140 mmol potassium persulphate (final concentration), and allowing the mixture to stand in the dark for at least 16 h at room temperature before use. For the assay, the ABTS•+ solution was diluted with ethanol to an absorbance of 0.7 (± 0.02) at 734 nm. A 30-μL sample (containing 5–50 mg/mL of hydrolysate) was mixed with 1 mL of diluted ABTS•+ solution. An absorbance (734 nm) reading was taken after 6 min. Trolox (6-hydroxy-2,5,7,8-tetrametichroman-2-carboxylic acid) was also used as a reference standard. The percentage inhibition of absorbance at 734 nm was calculated and plotted as a function of the concentration of the reference antioxidant (Trolox).

3.0. Results and discussion

3.1. Effect of process parameters on the degree of hydrolysis (DH)

The purpose of the first step was to identify the influence of pH, substrate (collagen) concentration, and hydrolysis temperature on the degree of collagen hydrolysis by *P. aurantiogriseum* collagenase, according to the 2^3 factorial design described in Section 2.7.

Table 2 lists the matrix and the observed response values of the experiments, which were performed for an incubation time of 18 h, according to previous findings (results not shown). The highest degree of hydrolysis (4.655 μg/mL) was obtained with run 6, carried out with 7.5 (mg/mL) collagen (substrate) at pH 8.0 and 25 °C.

Table 2. Matrix of the 2^3 full factorial design combinations (actual levels) and the observed response values. The best result is in boldface.

[a]Run	pH	T (ºC)	S_o (mg/ml)	DH (µg/ml)
1	6	25	2.5	0.562
2	8	25	2.5	2.096
3	6	49	2.5	0.23
4	8	49	2.5	1.963
5	6	25	7.5	0.938
6	**8**	**25**	**7.5**	**4.655**
7	6	49	7.5	0.857
8	8	49	7.5	3.785
9	7	37	5.0	2.749
10	7	37	5.0	2.896
11	7	37	5.0	3.057
12	7	37	5.0	2.915

[a]Results refer to 18-h of incubation. S_o = substrate (collagen) concentration; T = hydrolysis temperature; DH = degree of hydrolysis.

A full factorial model was fitted to the DH data. This model included three main effects, three two-factor interactions, and a three-factor interaction. The statistically significant estimates of the effects (at the 95% confidence level) are listed in Table 3. A simplified model based only on the effects of Table 3 showed a small lack of fit (2.88). The determination coefficient *(R^2)* was calculated as 0.86 for the DH, indicating that the statistical model can explain 86% of variability in response, and that the statistical model could significantly represent the actual relationships between the parameters chosen. The values of the significant effects indicate that, on an average, higher DH were obtained when factors 1 (pH) and 3 (collagen concentration) were at their highest levels and factor 2 (hydrolysis temperature) was at its lowest level (Table 3). As shown in Table 3, pH exerted the highest significant effect (2.47) within a 95% confidence interval ($p < 0.05$), followed by collagen concentration (1.34) within a 95% confidence interval ($p < 0.05$), while temperature exerted the lowest effect (-0.35) within a 95% confidence interval ($p < 0.05$). In addition, a significant interaction was also observed between pH (1) and collagen concentration (3) (Table 3). The

positive interaction effect means that an increase in pH with a simultaneous increase in collagen concentration led to an increase in DH.

Table 3. Statistically main effects and interactions estimated from the degree of hydrolysis (DH) values listed in Table 2

Factors	Effects on DH
(1) pH	2.47
(2) Hydrolysis temperature	-0.35
(3) Substrate concentration	1.34
1*2	-0.14*
1*3	0.84
2*3	0.12*

* Not significant.

These results were similar to the study by Jian-xin & Zheng (2009), which reported that the major-minor order of factors affecting the degree of collagen hydrolysis was pH, collagen concentration, and temperature. Similarly, Zhuang, Sun, & Li (2010) observed that pH exerted the highest significant effect on the enzymatic hydrolysis of collagen. Kong et al. (2011) also observed that the degree of collagen hydrolysis is dependent of the collagen concentration and temperature of hydrolysis. In the study done by these authors, the highest degree of collagen hydrolysis was obtained when the highest collagen concentration and the lowest temperature were used; however, the effect of pH did not have significant influence.

Since the DH is reported to affect the antioxidant and antimicrobial activity of protein hydrolysates, the most favourable condition for collagen hydrolysis was set aside for further study.

3.2. Peptide profile by mass spectrometry and amino acid sequence (MALDI-TOF MS/MS)

The molecular weight ranges of digested collagen type I were analyzed with MALDI-TOF MS (Fig. 1A and 1B). Peptides from collagen I have molecular weights less than 11 kDa, with several peptides less than 2 kDa. This result indicates that the collagenase produced by *P. aurantiogriseum* effectively hydrolysed collagen and produced several small collagen peptides. Thus, its biological activity should be further investigated.

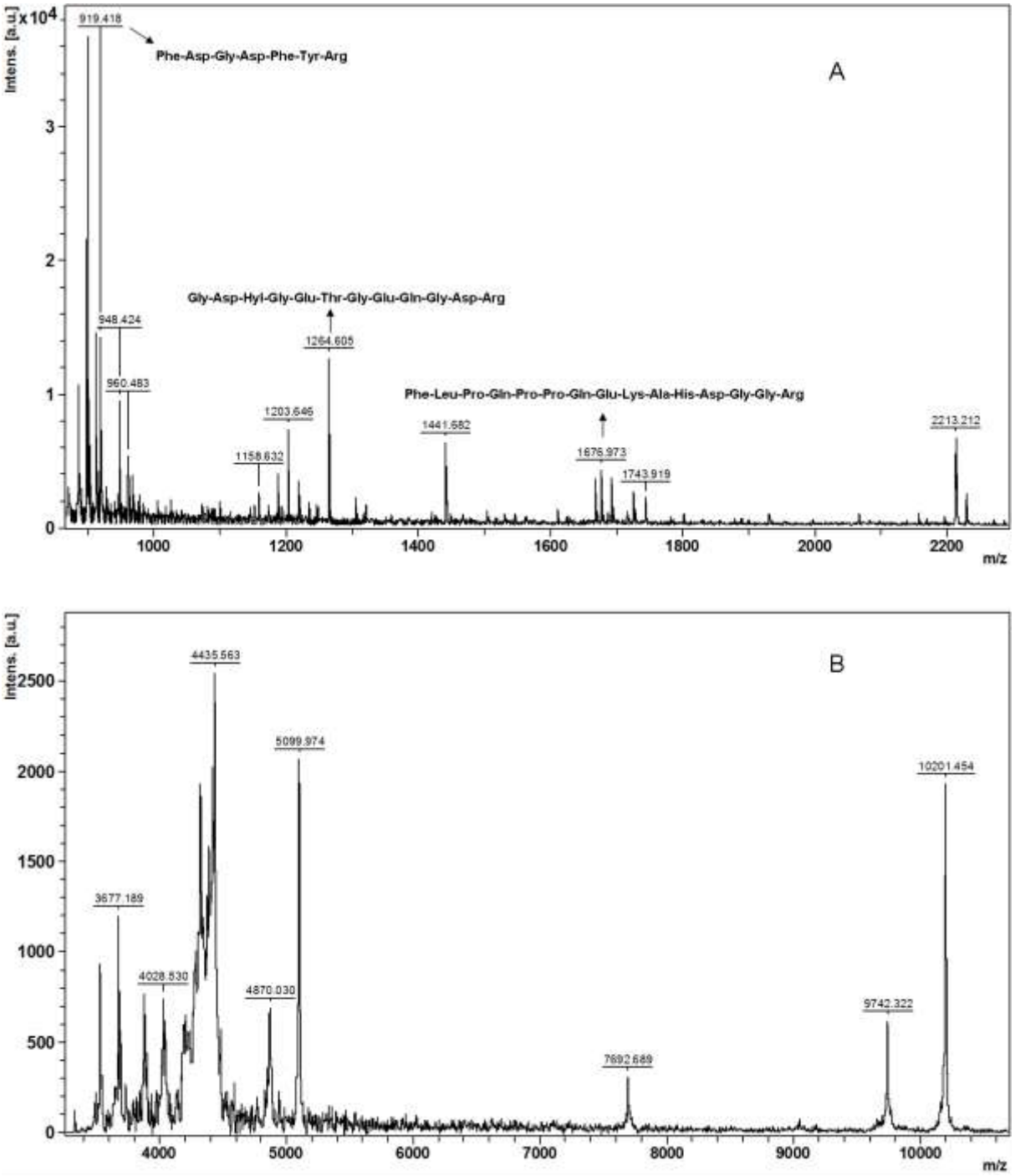

Figure 1. Peptide profile by mass spectrometry and amino acid sequence (MALDI TOF-MS/MS). (A) Mass spectrum in the range of 0–3 kDa. (B) Mass spectrum in the range of 3–20 kDa.

To identify the cleavage sites within the collagen triple helix, the amino acid sequences of three peptides (919.41 kDa, 1264.60 kDa, and 1676.97 kDa) were determined by MALDI-TOF MS/MS. The experimentally obtained sequences (Fig 1A) were compared with the sequences for the α_1 and α_2 chains of bovine type I collagen. The sequences Gly-Asp-Hyl-Gly-Glu-Thr-Gly-Glu-Gln-Gly-Asp-Arg and Phe-Leu-Pro-Gln-Pro-Pro-Gln-Glu-Lys-Ala-His-Asp-Gly-Gly-Arg were identical to sequences in the α_1 chain commencing at Gly1093 and Phe1198, respectively (Fig 2). The sequence Phe-Asp-Gly-Asp-Phe-Tyr-Arg was identical to a sequence in the α_2 chain commencing at Phe1109 (Fig 2). The cleavage pattern of the collagenase produced by *P. aurantiogriseum* differs from that of other known collagenases. For example, the cleavage sites of the *Clostridium histolyticum* collagenase were identified at amino acid residues 397 and 406 in both the α_1 and α_2 chains (French, Bhown, & Van Wart, 1992), while the cleavage sites of the collagenase from ginger were identified at amino acid residues 286, 499, and 712 in both the α_1 and α_2 chains (Kim et al. 2007).

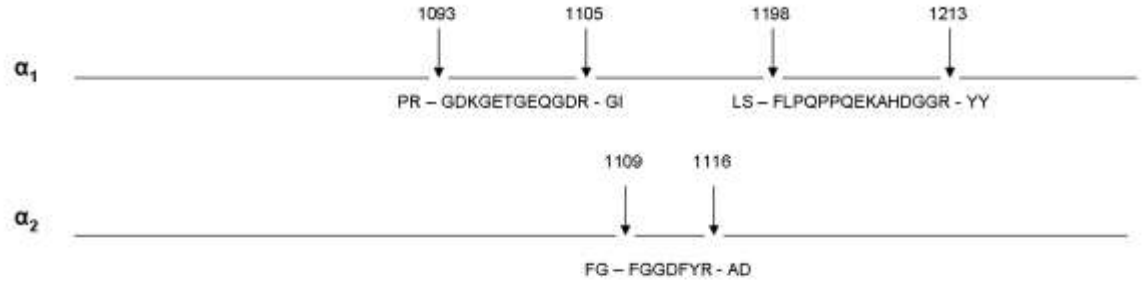

Figure 2. *Penicillium aurantiogriseum* collagenase cleavage sites in bovine collagen type I. Cleavage sites are indicated by commencing residue numbers in the triplets of collagen type I.

3.3. Antimicrobial activity of collagen hydrolysates

The ability of collagen hydrolysates to inhibit the growth of many bacteria was investigated. The results are shown in Table 4. Collagen hydrolysed for 18 h with the collagenase preparation from *P. aurantiogriseum* showed antibacterial activity against *E. coli* (MIC 0.625 mg/mL), *B. subtilis* (MIC 5 mg/mL), *and S. aureus* (MIC 0.55 mg/mL). In our results, both gram-positive and gram-negative bacteria were inhibited. The cell envelope of gram-negative bacteria is both structurally and functionally more complex than that of gram-positive bacteria. These differences in bacterial membrane composition suggest that the mode of action of the collagen peptides produced in the present work probably involved the cytoplasmic membrane or specific targets within the cell. According to Floris, Recio,

Berkhout, & Visser (2003), differences in membrane composition have implications for the mode of action and the specificity of antibacterial compounds.

Published information on the antimicrobial properties of hydrolysates or peptides from collagen or gelatin is very scarce. As previously described in Section 3.2, the molecular weights of the peptides obtained from the hydrolysis of collagen type I by *P. aurantiogriseum* collagenase were <11 kDa, and several peptides were <2 kDa. Gómez-Guillén, Giménez, López-Caballero, & Montero (2011) reported antimicrobial activity in peptide fractions from tuna and squid skin gelatins within a range of 1–10 kDa and <1 kDa. The hydrolysates were tested using the agar diffusion assay against 18 strains of bacteria (both gram-positive and gram-negative). *Lactobacillus acidophilus*, *Bifidobacterium animalis* subsp. lactis, *Shewanella putrefaciens*, and *Photobacterium phosphoreum* were the most susceptible species.

According to Gómez-Guillén, Giménez, López-Caballero, & Montero (2011), the reduced molecular weight of the peptide fractions (which was related to the elimination of aggregates), better exposure of the amino acid residues and their charges, and structure acquisition may facilitate the interaction with bacterial membranes.

The three peptides sequenced (GDKGETGEQGDR, FLPQPPQEKAHDGGR, and FGGDFYR) in the present study had hydrophobic amino acids in their sequences. The hydrophobic amino acids would allow peptides to enter the bacterial membrane (Wieprecht et al., 1997).

The relationship between peptide characteristics and antimicrobial activity has not yet been clearly stated. Several factors such as amino acid composition, sequence, molecular weight, and type of bacteria need to be considered. Thus, both the sequence and concentration of the peptide and the composition of the bacterial membrane would influence the mode of interaction (Di Bernardini et al., 2011).

Antimicrobial peptides generated from food proteins (particularly collagen) have the great advantage of being produced from harmless and inexpensive sources. Hence, there is a growing interest in the utilization of these peptides, for instance, as food-grade biopreservatives or as health-promoting food supplements in the food industry (Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011).

Table 4. Determination of the minimal inhibitory concentration (MIC) of collagen hydrolysates against *Enterococcus faecalis*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus*.

Peptides concentration (mg/ml) / Microorganisms	5.0	2.5	1.6	1.25	1.0	0.83	0.71	0.625	0.55	0.5
Enterococcus faecalis (ATCC 6057)	-	-	-	-	-	-	-	-	-	-
Escherichia coli (ATCC 25922)	+	+	+	+	+	+	+	+	-	-
Pseudomonas aeruginosa (ATCC 27853)	-	-	-	-	-	-	-	-	-	-
Bacillus subtilis (ATCC 6633)	+	-	-	-	-	-	-	-	-	-
Klebsiella pneumonae (ATCC 29665)	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus (ATCC 6538)	+	+	+	+	+	+	+	+	+	-

(+) presence of growth inhibition; (-) absence of growth inhibition

3.4. Antioxidant activity of collagen hydrolysates

The antioxidant activity of the collagen hydrolysates at seven different concentrations is shown in Table 5. The antioxidant activity of the collagen hydrolysates was determined using radical ABTS. The radical ABTS is reduced with concomitant conversion to a colourless product in the presence of antioxidants with hydrogen-donating or chain-breaking properties. The antioxidant activity was dependent on the concentration. A significant

increase was observed in this parameter with increasing concentrations of collagen hydrolysates (Table 5).

Table 5. Antioxidant activity of collagen type I hydrolysates.

Peptides concentration (mg/ml)	ABTS radical scavenging activity (%)
50.0	84.7 ± 0.24
40.0	82.7 ± 0.91
20.0	73.0 ± 0.49
10.0	45.4 ± 1.49
8.0	43.0 ± 0.18
6.7	40.5 ± 0.25
5.0	37.7 ± 0.78

Peptides and protein hydrolysates obtained from the proteolysis of various food proteins are reported to possess antioxidant activities. Antioxidant mechanisms include radical-scavenging (both hydrogen-donating capability and free radical quenching) activity, inhibition of lipid peroxidation, metal ion chelation, or a combination of these properties (Sarmadi & Ismail, 2010; Rival, Boeriu, & Wichers, 2001). Antioxidant activities might protect biological systems against damage related to oxidative stress in human diseases. These antioxidant peptides and hydrolysates might also be employed to prevent oxidation reactions (such as lipid peroxidation) that lead to the deterioration of foods and foodstuffs (Hogan, Zhang, Li, Wang, & Zhou, 2009; Zhang, Li, & Zhou, 2010).

The use of microbial enzymes obtained by fermentation is a good alternative method for producing collagen protein hydrolysates and collagen-derived peptides with antioxidant activity. Functional foods with such natural antioxidants can potentially be employed without the toxic side effects associated with the use of synthetic equivalents. Moreover, apart from their functional/physiological properties, antioxidants from protein hydrolysates might confer nutritional value, an additional advantage over their synthetic counterparts (Gómez-Ruiz, López-Expósito, Pihlanto, Ramos, & Recio, 2008).

4. Conclusion

This study describes a simplified strategy to hydrolyse collagen type I by using collagenase produced by *P. aurantiogriseum.* The most significant variables for collagen hydrolysis were singled out, along with their interactions. Collagen hydrolysis was improved

when the collagen concentration and pH were raised, and the temperature was lowered. The molecular weight distribution of the collagen peptides obtained under the most favourable hydrolysis conditions demonstrated the presence of a large proportion of low-molecular-weight peptides. The peptides with antioxidant and antimicrobial activities were produced through hydrolysis with a novel microbial collagenase preparation. The bioactivities of the protein hydrolysates could have resulted from the synergistic effect of different peptides within the mixture. Such collagen hydrolysates may be useful for applications in the food industry that aim to increase the nutritional value and shelf life of food products, as well as in the development of functional foods. The physicochemical characterization and properties of collagen hydrolysates are under investigation.

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8. CONCLUSÕES

Os estudos desenvolvidos no presente trabalho mostraram que:

- A análise do planejamento estatístico 2^4 utilizado para identificar as variáveis que influenciam o processo de produção da colagenase demonstrou que as variáveis mais significativas foram a concentração do substrato e o pH inicial do meio de cultura que apresentaram efeito positivo e a temperatura que apresentou efeito negativo;
- A maior produção da colagenase em cultura líquida submersa (283,36 ± 1,33 U) predita pela superfície de resposta foi obtida quando se utilizou 1,645% de farinha de soja, a pH 7,21 e 24 ºC;
- A colagenase produzida pelo *P. aurantiogriseum* URM4622 apresentou atividade máxima em pH 9,0 e temperatura de 37 ºC;
- A colagenase mostrou-se estável em uma ampla faixa de pH (6,0 a 10,0) e temperatura (25 a 45 ºC);
- A colagenase foi fortemente inibida por 10 mM de fenilmetilsulfonil fluoride, um potente iniidor de serino proteases;
- Os efeitos termodinâmicos principais encontrados foram E^*_d = 107.4 kJ/mol e ΔH^*_d = 104.7 kJ/mol;
- O sistema de duas fases aquosas (SDFA) foi seletivo para a colagenase e a enzima particionou preferencialmente para a fase rica em PEG;
- A análise dos resultados do planejamento fatorial 2^4 utilizado para estudar a influência da massa molar do PEG (M_{PEG}), da concentração do PEG (C_{PEG}), da concentração do fosfato (C_{FOSF}) e do pH na extração da colagenase no SDFA indicou que o fator de purificação (FP), o coeficiente de partição (K) e o rendimento em atividade (Y) da colagenase na fase superior aumentou com o aumento da C_{PEG} e da C_{FOSF} e com a diminuição da M_{PEG} e do pH;
- A análise dos planejamentos estatísticos identificou que a melhor condição de extração da colagenase no SDFA foi com: massa molar do PEG de 1500 (g/mol), as concentrações do PEG e do fosfato, respectivamente 17,5% (m/m) e 15,0% (m/m) e pH 6,0;

- É possível extrair colagenase do meio de fermentação do *P. aurantiogriseum* (URM 4622) através de SDFA PEG/fosfato com grau de purificação de 5,23 e recuperação em atividade de 110,70%;
- O estudo eletroforético revelou que muitas proteínas contaminantes foram removidas após o processo do SDFA, demonstrando que o SDFA pode ser uma potente ferramenta como um primeiro passo no processo de purificação da colagenase do *P. aurantiogriseum*;
- A análise do planejamento estatístico 2^3 utilizado para identificar as variáveis que influenciam no grau de hidrólise do colágeno bovino tipo demonstrou que as variáveis pH e concentração do substrato apresentaram efeito positivo, enquanto que a temperatura apresentou efeito negativo;
- O grau de hidrólise máximo (4.65 µg/mL) foi obitido utilizando-se 7.5 mg/mL de colágeno, pH 8,0 e 25 °C.
- O perfil de peptídeos, obtido nas condições mais favoráveis de hidrólise, demonstrou a presença de peptídeos com peso molecular inferior a 2 kDa.
- Os peptídeos apresentaram atividade antibacteriana contra *Escherichia coli* (CIM 0,625 mg/mL), *Bacillus subtilis* (CIM 5 mg/mL) e *Staphylococcus aureus* (CIM 0,55 mg/mL).
- Uma atividade antioxidante de 84,7 ± 0,24% foi obtida quando se utilizou uma concentração de peptídeos de 50 mg/mL.

9.0. ANEXOS

9.1. Normas para a redação de artigos para a revista "Journal of Chromatography B"

Preparation

Use of wordprocessing software

It is important that the file be saved in the native format of the wordprocessor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the wordprocessor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: http://www.elsevier.com/guidepublication). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.

To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your wordprocessor.

Article structure

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Theory/calculation

A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• Title. Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• Author names and affiliations. Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• Corresponding author. Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. Ensure that telephone and fax numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.
• Present/permanent address. If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Highlights

Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). See http://www.elsevier.com/highlights for examples.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at

their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Nomenclature

Widely accepted symbols, abbreviations and units (SI) should be used. If there is any doubt about a particular symbol or abbreviation, the full expression followed by the abbreviation should be given the first time it appears in the text. Abbreviations used in tables and figures should be explained in the captions. In general, the recommendations of the International Union of Pure and Applied Chemistry (IUPAC) should be followed and attention should be given to the recommendations of the Analytical Chemistry Division in the journal Pure and Applied Chemistry: Nomenclature for Chromatography, Pure Appl. Chem., 65 (1993) 819-872. Decimal points should be indicated by full stops. All decimal numbers smaller than unity should include a leading zero (e.g. 0.11).

Math formulae

Present simple formulae in the line of normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article, using superscript Arabic numbers. Many wordprocessors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Table footnotes

Indicate each footnote in a table with a superscript lowercase letter.

Artwork

Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.

• Save text in illustrations as 'graphics' or enclose the font.

• Only use the following fonts in your illustrations: Arial, Courier, Times, Symbol.

• Number the illustrations according to their sequence in the text.

• Use a logical naming convention for your artwork files.

• Provide captions to illustrations separately.

• Produce images near to the desired size of the printed version.

• Submit each figure as a separate file.

A detailed guide on electronic artwork is available on our website:

http://www.elsevier.com/artworkinstructions

You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

Regardless of the application used, when your electronic artwork is finalised, please 'save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS: Vector drawings. Embed the font or save the text as 'graphics'.

TIFF: Color or grayscale photographs (halftones): always use a minimum of 300 dpi.

TIFF: Bitmapped line drawings: use a minimum of 1000 dpi.

TIFF: Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required.

If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is'.

Please do not:

• Supply files that are optimised for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low;

• Supply files that are too low in resolution;

• Submit graphics that are disproportionately large for the content.

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Please make sure that artwork files are in an acceptable format (TIFF, EPS or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article. Please indicate your preference for color: in print or on the Web only. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.

Please note: Because of technical complications which can arise by converting color figures to 'gray scale' (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (not on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with

either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference management software

This journal has standard templates available in key reference management packages EndNote (http://www.endnote.com/support/enstyles.asp) and Reference Manager (http://refman.com/support/rmstyles.asp). Using plug-ins to wordprocessing packages, authors only need to select the appropriate journal template when preparing their article and the list of references and citations to these will be formatted according to the journal style which is described below.

Reference Style

Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.

Example: "..... as demonstrated [3,6]. Barnaby and Jones [8] obtained a different result ...."

List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.

Examples:

Reference to a journal publication:

[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, J. Sci. Commun. 163 (2000) 51.

Reference to a book:

[2] W. Strunk Jr., E.B. White, The Elements of Style, Macmillan, New York, 3rd ed., 1979.

Reference to a chapter in an edited book:

[3] G.R. Mettam, L.B. Adams, in: B.S. Jones, R.Z. Smith (Eds.),

Introduction to the Electronic Age, E-Publishing, New York, 1994, p. 281.

Journal abbreviations source

Journal names should be abbreviated according to

Index Medicus journal abbreviations: http://www.nlm.nih.gov/tsd/serials/lji.html;
List of title word abbreviations: http://www.issn.org/2-22661-LTWA-online.php;
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Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:

• E-mail address

• Full postal address

• Telephone and fax numbers

All necessary files have been uploaded, and contain:

• Keywords

• All figure captions

• All tables (including title, description, footnotes)

Further considerations

• Manuscript has been 'spell-checked' and 'grammar-checked'

• References are in the correct format for this journal

• All references mentioned in the Reference list are cited in the text, and vice versa

• Permission has been obtained for use of copyrighted material from other sources (including the Web)

• Color figures are clearly marked as being intended for color reproduction on the Web (free of charge) and in print, or to be reproduced in color on the Web (free of charge) and in black-and-white in print

• If only color on the Web is required, black-and-white versions of the figures are also supplied for printing purposes

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9.2. Normas para a redação de artigos para a revista "Food Chemistry"

Preparation

Use of wordprocessing software

General: Manuscripts must be typewritten, double-spaced with wide margins on one side of white paper. Each page must be numbered, and lines must be consecutively numbered from the start to the end of the manuscript. Good quality printouts with a font size of 12 or 10 pt are required. The corresponding author should be identified (include a Fax number and E-mail

address). Full postal addresses must be given for all co-authors. Authors should consult a recent issue of the journal for style if possible. An electronic copy of the paper should accompany the final version. The Editors reserve the right to adjust style to certain standards of uniformity. Authors should retain a copy of their manuscript since we cannot accept responsibility for damage or loss of papers. Original manuscripts are discarded one month after publication unless the Publisher is asked to return original material after use.

Article structure

Follow this order when typing manuscripts: Title, Authors, Affiliations, Abstract, Keywords, Main text, Acknowledgements, Appendix, References, Vitae, Figure Captions and then Tables. Do not import the Figures or Tables into your text. The corresponding author should be identified with an asterisk and footnote. All other footnotes (except for table footnotes) should be identified with superscript Arabic numbers. The title of the paper should unambiguously reflect its contents. Where the title exceeds 70 characters a suggestion for an abbreviated running title should be given.

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Essential title page information

• Title. Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• Author names and affiliations. Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• Corresponding author. Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. Ensure that telephone and fax numbers (with

country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.

• Present/permanent address. If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

The abstract should not exceed 150 words.

Highlights

Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). See http://www.elsevier.com/highlights for examples.

Units

Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

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Elsevier aims at connecting online articles with external databases which are useful in their respective research communities. If your article contains relevant unique identifiers or accession numbers (bioinformatics) linking to information on entities (genes, proteins, diseases, etc.) or structures deposited in public databases, then please indicate those entities according to the standard explained below.

Authors should explicitly mention the database abbreviation (as mentioned below) together with the actual database number, bearing in mind that an error in a letter or number can result in a dead link in the online version of the article.

Please use the following format: Database ID: xxxx

Links can be provided in your online article to the following databases (examples of citations are given in parentheses):

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- PDB: Worldwide Protein Data Bank (PDB ID: 1TUP)
- CCDC: Cambridge Crystallographic Data Centre (CCDC ID: AI631510)
- TAIR: The Arabidopsis Information Resource database (TAIR ID: AT1G01020)
- NCT: ClinicalTrials.gov (NCT ID: NCT00222573)
- OMIM: Online Mendelian Inheritance in Man (OMIM ID: 601240)
- MINT: Molecular INTeractions database (MINT ID: 6166710)
- MI: EMBL-EBI OLS Molecular Interaction Ontology (MI ID: 0218)
- UniProt: Universal Protein Resource Knowledgebase (UniProt ID: Q9H0H5)

Artwork

Electronic artwork

General points

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TIFF: Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required.

If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is'.

Please do not:

• Supply files that are optimised for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low;

• Supply files that are too low in resolution;

• Submit graphics that are disproportionately large for the content.

Please insert the following text before the standard text - Photographs, charts and diagrams are all to be referred to as "Figure(s)" and should be numbered consecutively in the order to which they are referred. They should accompany the manuscript, but should not be included within the text. All illustrations should be clearly marked with the figure number and the author's name. All figures are to have a caption. Captions should be supplied on a separate sheet.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF, EPS or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article. Please indicate your preference for color: in print or on the Web only. For further

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Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (not on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References

Citation in text

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Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

All publications cited in the text should be presented in a list of references following the text of the manuscript. No more than 30 references should be cited in your manuscript. In the text refer to the author's name (without initials) and year of publication (e.g. "Steventon, Donald and Gladden (1994) studied the effects..." or "...similar to values reported by others (Anderson, Douglas, Morrison & Weiping, 1990)..."). For 2-6 authors all authors are to be listed at first citation. At subsequent citations use first author et al.. When there are more than 6 authors, first author et al. should be used throughout the text. The list of references should be arranged alphabetically by authors' names and should be as full as possible, listing all authors, the full title of articles and journals, publisher and year. The manuscript should be carefully checked to ensure that the spelling of authors' names and dates are exactly the same in the text as in the reference list.

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List: references should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Examples:

Reference to a journal publication:

Van der Geer, J., Hanraads, J. A. J., & Lupton, R. A. (2010). The art of writing a scientific article. Journal of Scientific Communications, 163, 51–59.

Reference to a book:

Strunk, W., Jr., & White, E. B. (1979). The elements of style. (4th ed.). New York: Longman, (Chapter 4).

Reference to a chapter in an edited book:

Mettam, G. R., & Adams, L. B. (2009). How to prepare an electronic version of your article. In B. S. Jones, & R. Z. Smith (Eds.), Introduction to the electronic age (pp. 281–304). New York: E-Publishing Inc.

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All necessary files have been uploaded, and contain:

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• All figure captions

• All tables (including title, description, footnotes)

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• References are in the correct format for this journal

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Abbreviations for units should follow the suggestions of the British Standards publication BS 1991. The full stop should not be included in abbreviations, e.g. m (not m.), ppm (not p.p.m.), % and '/' should be used in preference to 'per cent' and 'per'. Where abbreviations are likely to cause ambiguity or may not be readily understood by an international readership, units should be put in full.

Current recognised (IUPAC) chemical nomenclature should be used, although commonly accepted trivial names may be used where there is no risk of ambiguity.

The use of proprietary names should be avoided. Papers essentially of an advertising nature will not be accepted.