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Purificação parcial de colagenase produzida por *Penicillium aurantiogriseum* (URM-4622) via sistema de duas fases aquosas PEG-fosfato

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"Purificação parcial de colagenase produzida por *Penicillium aurantiogriseum* URM4622 utilizando sistema de duas fases aquosas PEG-fosfato"

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

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RESUMO

As colagenases são enzimas, obtidas a partir de procariotos e eucariotos, que clivam a cadeia protéica do colágeno em pH e temperatura fisiológicos. Estas enzimas são empregadas em diversas aplicações industriais com destaque para a indústria farmacêutica, onde são aplicadas no tratamento médico de feridas, cicatrizes e queimaduras. A proposta para a utilização do sistema de duas fases aquosas (SDFA) é devido à alta relação custo-benefício, baixa tensão interfacial, fácil escalonamento e a sua capacidade de purificar uma proteína em um ambiente rico em água (80-90%), o que favorece a estrutura protéica e retém assim a sua atividade biológica após purificação. Este trabalho visa à purificação de colagenase produzida por *Penicillium aurantiogriseum* (URM-4622) utilizando SDFA PEG/fosfato. O planejamento experimental (2^3) foi usado para selecionar as variáveis significativas no processo de purificação, e a massa molar do PEG (MM_{PEG}), concentração do PEG (C_{PEG}) e concentração do fosfato (C_{FOS}) foram as variáveis estudadas. O sistema de duas fases aquosas foi composto de PEG 550, 1500 e 4000 g/mol nas concentrações de 15, 17,5 e 20% (m/m) e concentrações de fosfato de 12,5, 15 e 17,5% (m/m). As variáveis de resposta escolhidas foram: coeficiente de partição (K), rendimento de atividade (Y) e fator de purificação (PF). Os dados e gráficos obtidos passaram por análise estatística. Observou-se que PEG de massa molar 550 (g/mol) em concentração de 20% (p/p), concentração de fosfato 17,5% (p/p) e pH 6.0 foram as melhores condições para purificação de colagenase. Estas condições geraram um coeficiente de partição de 1,01, um rendimento de atividade de 242% e fator de purificação de 23,5. Os resultados mostraram que SDFA foi seletivo para colagenase e esta enzima particionou para a fase rica em polímero.

Palavras-chave: Colagenases; Penicillium; SDFA; PEG/Fosfato; Desenho Experimental

ABSTRACT

Collagenases are enzymes obtained from prokaryotes and eukaryotes that cleave the main body chain of the structural protein collagen at physiological pH and temperature. These enzymes are used in several industrial applications pointing out in pharmaceutical industry, where they are applied in medical treatment of wounds, scar and burns. The techniques for the purification of collagenases are generally based on chromatography and ultrafiltration techniques. However, these techniques are usually expensive and difficult to scale up. The proposal of aqueous two phase system (ATPS) usage is due to its high cost-benefit relation, low interfacial tension, easy scale up, and its allowance to purify a protein in a high water content environment (80-90%) favoring protein structure and thus its biological activity after purification. This work deals with the purification of collagenase produced by *Penicillium aurantiogriseum* URM4622 using an aqueous two phase system (ATPS) PEG/phosphate. Experimental design (2^3) were used to choose the significant variables for purification process, and PEG molar mass (M_{PEG}), PEG concentration (C_{PEG}) and phosphate concentration (C_{PHOS}) were the variables studied. The aqueous two phase system was composed by PEG 550, 1500 and 4000 g/mol under concentrations of 15, 17.5 and 20% (w/w) and phosphate concentrations of 12.5, 15 and 17.5 (w/w). Selected responses were: partition coefficient (K), activity yield (Y) and purification factor (PF). Statistical analysis of data and graphics was carried. It was observed that PEG molar mass 550 (g/mol) in a 20% (w/w) concentration, phosphate concentration of 17.5% (w/w) and a 6.0 pH were the best conditions to collagenase purification. This condition generated a partition coefficient of 1.01, an activity yield of 242% and Purification factor of 23.5. The results showed that ATPS has been selective to the collagenase and this enzyme partitioned to the polymer rich-phase.

Keywords: Collagenases; Penicillium; ATPS; PEG/phosphate; Experimental Design

1. INTRODUÇÃO

As colagenases são metaloproteases, que compõem o grupo mais diverso das proteases (BARRET, 1995 e RAO, 1998). São caracterizadas pela necessidade de um íon metálico divalente para que possam expressar atividade e são bastante específicas agindo apenas em colágeno e gelatina e não em outros tipos de substratos protéicos. Foram inicialmente descobertas no caldo fermentado pela bactéria anaeróbia *Clostridium hystolyticum* como componente de seus produtos tóxicos. São também produzidas pela bactéria anaeróbia *Achromobacter iophagus* e por outros microrganismos incluindo os fungos (RAO, 1998).

Os fungos, como produtores de enzimas, possuem muitas vantagens, dentre elas, a capacidade de produzir enzimas que são normalmente extracelulares (BIDOCHKA e KHACHATOURIANS, 1988 ; SANDHYA et al., 2005), o que torna a sua recuperação a partir do caldo fermentado mais fácil, sendo considerados mais seguros, no que se refere às questões sanitárias do que o uso de bactérias com esta finalidade (PANDEY, 1992 ; MITRA et al., 1996).

O fungo *Penicillium aurantiogriseum* é comumente encontrado em cereais estocados e como contaminante de produtos derivados dos mesmos (WEIDENBORNER et al., 2000). Trabalhos como de Agrawal e colaboradores (2003) e Rodrigues e colaboradores (2008) relatam *P. aurantiogriseum* como bom produtor de proteases, dentre elas a colagenase, sendo ele o microrganismo proposto a ser estudado por este trabalho.

Sabe-se que as metaloproteases produzidas por fungos são ativas em pH próximo de 7,0 e são inibidas por agentes quelantes (RAO, 1998). A colagenase é normalmente ativa em pH próximo ao fisiológico (MANDL, 1961), suas características próprias de especificidade ao colágeno fez dessas enzimas úteis para aplicação pela medicina em cicatrizes pós-operatórias, limpeza de feridas necrosadas, queimaduras, escaras e tratamento de psoríase e pediculoses (BATURIN, 2007; MARKOVICK, 2008) provando ser de interesse biotecnológico.

Nos processos biotecnológicos industriais a recuperação de produtos do meio fermentado é essencial e depende da natureza do produto e viabilidade econômica

do processo escolhido. Principalmente devido a este, a otimização dos processos de recuperação e purificação de proteínas passaram a ser de vital importância na produção e obtenção industrial de biomoléculas (SEADER et al., 1998).

Beijerinck (1896) foi o primeiro a descrever os sistemas de duas fases aquosas (SDFAs), ao misturar gelatina, ágar e água, em certas concentrações observou a formação de duas fases, sendo a fase superior rica em gelatina e a fase inferior rica em ágar. Per-Aka Albertsson, na década de 50, descobriu que o polietileno glicol (PEG), fosfato de potássio e água também formavam sistemas bifásicos aquosos, assim como o PEG, a dextrana e água. Desde então os sistemas de PEG/dextrana/água e PEG/sal/água têm sido os mais freqüentemente empregados na purificação de um grande número de biomoléculas, principalmente os sistemas PEG/sal/água que devido a seus materiais de baixo custo tornam o uso de SDFAs em purificações industriais atrativo.

Foi, no entanto, Albertsson (1958) que reconheceu a possível utilização destes sistemas supracitados como método de separação aplicado a biomoléculas, partículas celulares e células intactas sob condições que preservem a sua atividade biológica, estabelecendo inclusive um grande número de diagramas de fase para vários SDFAs.

Os SDFAs são geralmente formados por uma solução aquosa de um ou dois polímeros hidrófilos, ou de polímero com determinados sais. Acima de uma dada concentração crítica destes componentes ocorre espontaneamente a separação de fases, predominando um ou outro componente em cada uma das duas fases resultantes (TUBIO et al., 2004).

Este trabalho teve como objetivo purificar colagenase produzida por *Penicillium aurantiogriseum* utilizando o sistema de duas fases aquosas PEG/Sais de fosfato.

2.0. REVISÃO DE LITERATURA

2.1. Proteases Microbianas

As proteases, de acordo com o Comitê Internacional de Nomenclatura da União de Bioquímica e Biologia Molecular, por definição, são enzimas capazes de hidrolisar a ligação peptídica de uma molécula de proteína, sendo então também chamadas de hidrolases (subgrupo 4 do grupo 3 – E. C. 3. 4). Podem ser encontradas em plantas, animais e microrganismos. Contudo, os microrganismos são a fonte preferencial para a produção devido a sua ampla diversidade bioquímica e susceptibilidade a manipulação genética. As proteases microbianas correspondem a 40% da venda de enzimas mundiais (SANDHYA et al., 2005).

Dentre os microrganismos (vírus, bactérias e fungos), alguns fungos, principalmente as leveduras oferecem vantagens para produção de enzimas uma vez que estas são GRAS (“generally regarded as safe” – consideradas seguras no geral – menos infecciosas que outros microorganismos) e, suas enzimas são extracelulares o que facilita a recuperação do meio fermentado (SANDHYA et al., 2005). Existem diversos trabalhos que apontam a biossíntese de proteases por fungos pertencentes aos gêneros Aspergillus (FAN-CHING et al., 1998), Penicillium (CHRZANOWSKA et al., 1993) e Rhizopus (FARLEY e IKASAR, 1992).

Atualmente as proteases são classificadas com base em três critérios: (i) relacionamento evolucionário com estrutura de referência (ii) tipo de reação catalizada e (iii) natureza química do sítio catalítico.

Baseando-se em suas seqüências de aminoácidos as proteases são classificadas de acordo com seu tipo de sítio catalítico e depois acomodadas em “clãs” para acomodar os conjuntos de peptidases que divergiram de ancestrais em comum (ARGOS, 1987; RAWLINGS et. al, 1993).

Quanto ao tipo de reação catalisada podemos ter: Endoproteases ou exoproteases, as primeiras clivam a ligação peptídica distante da região amino ou carboxiterminal do substrato e as últimas clivam próximo a estas. As exoproteases são divididas com base no seu mecanismo de ação em aminopeptidases e carboxipeptidases (MONOD et al., 2002). As aminopeptidases atuam na região

aminoterminal livre da cadeia polipeptídica podendo liberar um único resíduo de aminoácido, um dipeptídeo ou um tripeptídeo. As carboxipeptidases por sua vez atuam na região carboxiterminal da cadeia peptídica e liberam um único aminoácido ou um dipeptídeo.

Pelo grupamento funcional do sítio catalítico as proteases podem ser classificadas em: Serino Proteases (S), Aspártico Proteases (A), Cisteíno Proteases (C) e Metaloproteases (M), ou de tipo desconhecido (U).

As serino proteases caracterizam-se pela presença do grupo serina no sítio ativo. São comumente ativas em pH neutro e alcalino, com região de maior atividade ótima entre os valores de pH 7 e 11, no entanto o maior grupo delas são as fortemente alcalinas (RAO et al., 1998).

As aspártico proteases, também conhecidas como proteases ácidas por exibirem atividade máxima entre pH 3 e 4, são endopeptidases que dependem do resíduo ácido aspártico para a realização da atividade catalítica (RAO et al., 1998).

As cisteíno proteases são produzidas tanto por eucariotas como por procariotas e sua atividade depende da presença da dupla cisteína-histidina no centro catalítico, independente da ordem em que aparecem cis-his ou his-cis. A maioria das cisteíno proteases tem pH ótimo na faixa neutra com algumas exceções que apresentam atividade na faixa ácida de pH, como por exemplo, proteases lisossomais. Geralmente, as cisteíno proteases são ativas apenas na presença de agentes redutores como HCN (RAO et al., 1998).

As metaloproteases são o grupo mais diverso das proteases, são caracterizadas por requerimento de íons bivalentes em pH neutro e alcalino para que ocorra sua atividade e engloba enzimas de variadas origens como toxinas hemorrágicas de cobras venenosas, termolisina de bactérias e mesmo as colagenases de organismos superiores (RAO et al., 1998).

2.2. Colagenases

As colagenases são enzimas proteolíticas capazes de degradar tanto moléculas de colágeno nativo como desnaturado (TRAN e NAGANO, 2002). Em geral, outras proteases não digerem a tripla hélice do colágeno, essa degradação só

é possível através da ação de enzimas específicas, as chamadas metaloproteases (GOSHEV et al., 2005).

Existe um grande interesse na busca de colagenases de outras fontes que não a animal, pois, esta possui a capacidade de clivar somente o colágeno nativo. As de microrganismos, no entanto, possuem a capacidade de hidrolisar tanto o colágeno nativo quanto o colágeno desnaturado e apresentam afinidade por vários sítios ao longo da cadeia de aminoácidos, representando assim, uma fonte promissora para pesquisas e aplicações biotecnológicas (JUNG e WINTER, 1998).

De acordo com o grupo de microrganismos produtores e os meios utilizados às colagenases microbianas apresentam diferenças físico-químicas. A colagenase produzida pelo *Streptomyces* sp., em meio contendo amido, apresentou pH ótimo de 7,5 e peso molecular de 116 kDa (PETROVA et al., 2006), a colagenase de *Bacillus* sp. produzida em meio contendo gelatina, apresentou pH ótimo de 3,9 (NAKAYAMA et al., 2000), enquanto que uma enzima colagenolítica obtida em meio contendo colágeno insolúvel, por *Bacillus subtilis,* apresentou sua atividade máxima a pH 9,0, temperatura de 50ºC e peso molecular de 125 kDa (NAGANO E TO, 1999).

2.2.1. Penicilium aurantiogriseum

O *Penicillium aurantiogriseum* é um fungo filamentoso encontrado como típico contaminante de cereais e seus produtos derivados. Apresenta temperatura mínima de crescimento perto de 21ºC e máxima em 30ºC, sendo a temperatura ótima de 23ºC (ZARDETTO et. al, 2005). Seu crescimento é estimulado com concentração de 10% de CO_2 (MAGAN e LACEY, 1984) e segue crescendo com até 30% de dióxido de carbono, embora assim, nestas condições, ocorra um aumento na fase lag pela diminuição do ritmo de crescimento (ZARDETTO et. al, 2004).

Germano e colaboradores (2003) e Agarwal e colaboradores (2003), relataram o uso de *Penicillium sp.* em fermentação sólida utilizando a soja como fonte de carbono e nitrogênio para produção de proteases enquanto Rodrigrues e colaboradores (2008) relataram a produção de protease com alta atividade em substrato de colágeno em cultura submersa com meio de farinha se soja.

2.2.2. Aplicações das Colagenases

As colagenases têm sido largamente utilizadas na medicina com o propósito de limpar feridas necrosadas, escaras, cicatrizes pós-operatórias, e no tratamento de psoríase e pediculoses (MARKOVICH, 2008).

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

2.3. Sistema de Duas Fases Aquosas (SDFA)

O uso de enzimas industriais aumentou nos últimos anos; assim se fez necessário desenvolver novos métodos para isolar e purificar proteínas com considerável pureza, baixo custo e aplicabilidade industrial. Os métodos tradicionais de purificação e isolamento de proteínas envolvem alguns passos que requerem longo período para execução e um alto custo, tais quais precipitação com sulfato de amônio, cromatografias iônicas e de afinidade, diálise e concentração final do produto (REH et al., 2007). Estas operações costumam corresponder entre 50-80% do custo total de produção de enzimas industriais e ainda induzem um desdobramento da macromolécula protéica com perda de atividade biológica e baixo rendimento no final do processo (SARAVANAN et al, 2008).

Em 1896, Beijerinck observou que quando soluções aquosas de amido e gelatina ou Agar-agar e gelatina eram misturadas, um sistema composto por duas fases aquosas era formado, com a parte inferior rica em amido (ou Agar-agar) e a superior rica em gelatina. Segundo Carvalho e colaboradores (2008), Ostwald e Hertel mais tarde, demonstraram que diferentes concentrações de amido misturadas

eram necessárias para a separação das fases. Em 1947, Dobry e Boyer-Kawenoki testaram inúmeros pares de polímeros e observaram separação de fases na maioria deles. Foi Albertsson, no entanto, em 1958 quem propôs a aplicação do sistema de duas fases aquosas (SDFA) como ferramenta de biosseparação. Ele usou sistemas constituídos por polietileno glicol (PEG) e dextrana para separar com sucesso várias biomoléculas. Desde então, diversos estudos vêm sendo realizados expandindo o uso de SDFAs na partição de células, organelas, enzimas, metais, esporos, ácidos nucléicos e proteínas.

É conhecido que ao se misturar dois polímeros ou um polímero e um sal, acima de certa concentração crítica, um sistema bifásico se forma espontaneamente (ALBERTSSON, 1986). As concentrações críticas dos componentes de um sistema bifásico são estabelecidas pelas curvas binodais (Figura 1).

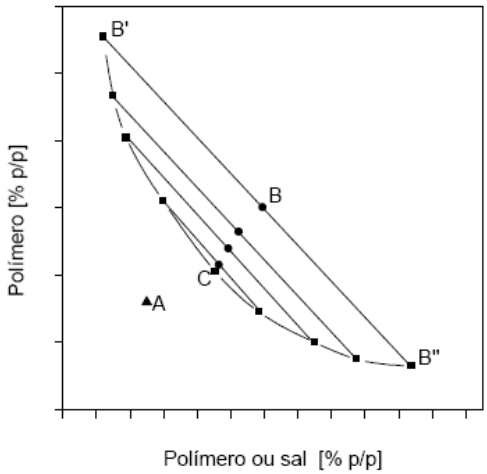

Figura 1. Diagrama de fases para um sistema de duas fases aquosas. Binodal (−■−), linha de amarração ou “tie line” (−●−), região monofásica (A), região de duas fases (B) onde B’ e B’’ são as composições das fases superior e inferior do sistema e (C) o ponto crítico. Fonte: PEREIRA, 2005.

O sistema de duas fases aquosas apresenta as seguintes vantagens sobre os métodos tradicionais: a) permite o uso de polietileno glicol (PEG), polímero biodegradável, de baixo custo e disponível para uso comercial; b) não há adição de solventes orgânicos na extração; c) diversos anions inorgânicos podem ser utilizados como agentes de extração solúveis em água; d) a partição ocorre entre duas fases aquosas imiscíveis, assim sendo, o efeito de desidratação de soluto na

extração é diminuído, mantendo estabilidade da biomolécula no final do processo (BULGARIU et al., 2008).

Para propósitos industriais, os sistemas constituídos por polímero/sal, mais especificamente, PEG/(fosfato/sulfato) são os mais comumente empregados, contudo as altas concentrações de sal requeridas representam um problema no seu descarte, levando a preocupações ambientais. Estudos demonstraram que substituindo os sais inorgânicos por outros biodegradáveis e não tóxicos, tal qual o citrato poderia ser considerado uma boa alternativa, uma vez que o mesmo pode ser lançado diretamente ao sistema de esgoto e degradado normalmente pelos processos de tratamento de água (MALPIEDI et al., 2008). Contudo, em qualquer sistema PEG/sal a dificuldade de remover o PEG dos produtos de interesse quando as biomoléculas de interesse são seletivamente particionadas para a fase superior, rica em polímero, uma nova etapa de recuperação se faz necessária na fase superior, como o processo de ultrafiltração, aumentando assim o custo do produto e o número de etapas de purificação empregadas (TUBIO et al., 2008).

O comportamento da molécula alvo de interesse pode ser avaliado pelo coeficiente de partição (K), e este é resultado de interações: de van der Waals, hidrofóbicas, ligações de hidrogênio e interações iônicas das moléculas da fase circundante (MALPIEDI et al., 2008). Deve-se levar em consideração que a partição de uma molécula alvo em sistema de duas fases aquosas depende de muitos fatores, tanto intrínsecos quanto extrínsecos. Propriedades intrínsecas incluem tamanho da molécula, sua natureza eletroquímica e hidrofobicidade superficial, enquanto que as propriedades extrínsecas incluem peso molecular e concentração dos componentes das fases, força iônica, pH, tipo de tampão, temperatura, entre outros (AZEVEDO et al, 2008). Pouco se pode prever do comportamento de uma molécula sob diversos fatores, sabe-se, no entanto que, 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 seus contaminantes (SALGADO et al, 2008).

2.3.1. Efeito da Massa Molar e Concentração do Polímero

Quanto maior a massa molar do polímero, menor é o volume de solvente disponível, reduzindo assim a solubilidade das proteínas na fase rica em polímero e conseqüentemente diminuindo o coeficiente de partição, efeito esse conhecido como teoria do volume excluído (ALBERTSSON, 1986).

O efeito, acima mencionado, depende da própria massa molar da biomolécula a ser separada. Proteínas de massas molares maiores são mais influenciadas por variações na massa molar dos polímeros que as de menor massa (ASENJO, 1990).

No que se refere à concentração do polímero, seu aumento desloca o sistema de fases em direção à região bifásica e aumenta a viscosidade das fases, podendo influenciar a partição da proteína alvo (ASENJO, 1990; ALBERTSSON, 1986).

Estudos feitos por Porto e colaboradores (2008) mostraram que elevando a concentração de PEG em um sistema PEG/citrato em apenas 4% ocasionou aumento de 2,94 vezes no coeficiente de partição de protease produzida por *Clostridium perfringens*.

Os efeitos da massa molar e concentração do polímero estão intimamente relacionados, Oliveira e colaboradores (2001), descreveram que um aumento na massa molar do polímero polietileno glicol de 4000 para 8000 levou a concentrações menores necessárias do mesmo para que ocorresse a separação de fases em um sistema PEG-goma de cajueiro (policajú).

2.3.2. Efeito da Temperatura

A temperatura afeta a composição das fases em equilíbrio, assim como alteração da estrutura da biomolécula e sua possível desnaturação (SARUBBO, 2000). Geralmente, para temperaturas abaixo de 20°C a curva binodal desloca-se em direção às baixas concentrações dos componentes que formam as fases. Os sistemas de fases próximos do ponto crítico podem ser mais influenciados pela mudança de temperatura devido à sua instabilidade, podendo assim passar facilmente para a região monofásica (BAMBERGER et al., 1985; TJERNELD et al., 1990).

De acordo com o tipo de sistema empregado, polímero/sal ou polímero/polímero o efeito da temperatura varia. Para o sistema PEG/sal constatou-se que temperaturas maiores ou próximas à ambiente (25ºC ±2) favorecem a separação das fases do sistema e também a um aumento da concentração de PEG na fase superior, resultando em redução da concentração do polímero na fase inferior (FORCINITI & HALL, 1991; ZASLAVSKY, 1995). No entanto, sistema PEG/dextrana requer temperaturas inferiores à ambiente (25ºC ±2) para que a separação das fases seja favorecida.

A relação do coeficiente de partição com a temperatura ainda não é totalmente esclarecida. Existem trabalhos que relatam uma tendência no aumento do coeficiente de partição com o aumento da temperatura (JOHANSSON et al., 1984) enquanto outros, afirmam que não há relação entre os dois (TJERNELD et al., 1985), demonstrando a necessidade de mais estudos que possam esclarecer o efeito deste parâmetro sobre o fenômeno da partição.

Zafarani-Moattar e colaboradores (2005), em estudo variando a temperatura (25,15ºC; 35,15ºC e 45,15ºC) nos sistemas Polivinilpilorridona (PVP)-fosfato de tripotássio e PVP-fosfato de hidrogênio dipotassio afirmam que as curvas binodais são muito próximas mesmo a diferentes temperaturas. No entanto, com concentrações maiores de sal o efeito se tornava mais notável. Também observou que a inclinação e comprimento das “tie-lines” de equilíbrio aumentavam com a temperatura. Resultados semelhantes foram encontrados por Carvalho e colaboradores (2008) ao estudar a influência da temperatura e tipo de sal no equilíbrio das fases de sistemas de duas fases aquosas PEG/fosfato e PEG/citrato.

2.3.3. Força iônica e pH

O efeito de diferentes sais inorgânicos no comportamento das fases em um sistema de duas fases aquosas pode ser atribuído a uma alteração da estrutura da água nos sistemas. Os sais podem ser classificados como “desestruturadores”, de efeito similar à elevação da temperatura em um dado sistema, ou, “estruturadores”, com efeito similar à queda da temperatura (ZASLAVSKY, 1995).

De acordo com o estudo de Gupta e colaboradores (2002) os íons de sais estruturadores incluem os cátions Li^{+}, Na^{+} $NH^{4+,}$ Ca^{2+}, $Mg^{2+,}$ etc., e anions, F^{-}, SO_4^{2-}, CO_3^{2-}, PO_4^{3-}, etc, enquanto que os íons desestruturadores seriam K^{+}, Rb^{2+}, Cs^{+}, Cl^{-}, Br^{-}, I^{-}, NO^{3-}, etc. Afirmam também que os cátions sódio (estruturador) e potássio (desestruturador) afetaram a formação das fases, onde sódio necessita menor concentração de PEG (m/m), independente do ânion em relação ao potássio.

Outro fator a ser levado em consideração é a partição dos íons entre as fases do sistema gerar um potencial elétrico entre as fases, que por sua vez direciona a partição de materiais biológicos carregados (SARUBBO, 2000).

O pH do sistema influencia a dissociação dos grupos ionizáveis das proteínas alterando as cargas da sua superfície e conseqüentemente, o seu coeficiente de partição (LEHNINGER, 1976). Em linhas gerais, proteínas carregadas negativamente (pH>pI) tendem a particionar na fase superior rica em PEG. A influência do pH depende muito do sal empregado uma vez que o potencial elétrico nas fases causado pelos sais interfere na movimentação da proteína. Condições extremas de pH podem desnaturar proteínas modificando seu comportamento de separação. Enzimas desnaturadas exibem a região hidrofóbica e apresentam uma maior área de superfície (ALBERTSSON, 1986).

O grande número de variáveis que interferem na partição confere considerável versatilidade aos sistemas de duas fases aquosas na separação de misturas de componentes. Entretanto, a existência de tantas variáveis, a sua grande maioria interdependentes, torna extremamente difícil a previsão teórica do coeficiente de partição de um dado soluto, obrigando por vezes a um trabalho experimental exaustivo (KULA et al., 1982).

2.3.4. Efeito da Adição de Suspensões Biológicas em SDFA

A presença de fragmentos celulares (*debris*) e polímeros intracelulares das suspensões biológicas, das quais se recuperam as proteínas alvo, em SDFA tem uma forte influência na modificação da posição da curva binodal do diagrama de fases por reduzirem a quantidade crítica de reagentes para a separação das fases.

Rito-Palomares e Cueto (2000) estudaram as influências na posição da curva binodal de três diferentes suspensões biológicas, leveduras lisadas, homogeneizado de *Escherichia coli* e caldo fermentado de *Trichoderma harzianum*. A variável de resposta empregada foi a mudança na razão dos volumes das fases superior e inferior ($V_r=V_{sup}./V_{inf}.$) e a própria posição da curva binodal em SDFA acrescido da biomassa. Os ensaios realizados envolveram a montagem de sistemas de duas fases aquosas tanto com a adição de suspensão biológica quanto com água deionizada como substituinte da anterior. Seus resultados indicaram que acúmulo de biomassa em dada fase causava um aumento de seu volume. A razão de volumes aumentava pelo acúmulo de biomassa na fase superior ou diminuía com o acúmulo de biomassa na fase inferior. Notaram também que sistemas cuja suspensão de origem era caldo fermentado ocorria um grande deslocamento da curva binodal no ponto crítico do diagrama de fases. Tal comportamento da curva pode ser explicado pela presença de biopolímeros produzidos durante o processo fermentativo gerando alta viscosidade e reduzindo a quantidade de reagentes necessários para a separação das fases.

2.3.5. Comportamento do Coeficiente de Partição de Biomoléculas em Sistema 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 2. Em tais sistemas a partição depende do efeito do volume de exclusão na fase rica em polímero (fase superior) e do efeito de *salting out* na fase rica em sal (inferior) (Figura 2a). O volume ocupado pelo polímero aumenta com o aumento da concentração (Figura 2b) e da massa molar do próprio polímero (Figura 2c), que resultam em um menor espaço para a biomolécula na fase superior, assim as biomoléculas tendem a particionar na fase inferior, efeito chamado de volume de exclusão (BABU et al, 2008).

A solubilidade de biomoléculas na fase inferior, rica em sal, diminui com o aumento da concentração de sal (Figura 2d), o que resulta em aumento de partição de biomoléculas na fase superior e a este efeito dá-se o nome de "*salting out*".

Os sistemas que compreendem alta concentração de polímero ou alto peso molar do mesmo, justamente com altas concentrações de sal (Figura 2e) resultam em partição das biomoléculas para a interfase, devido à influência concomitante dos efeitos citados, volume de exclusão e "*salting out*".

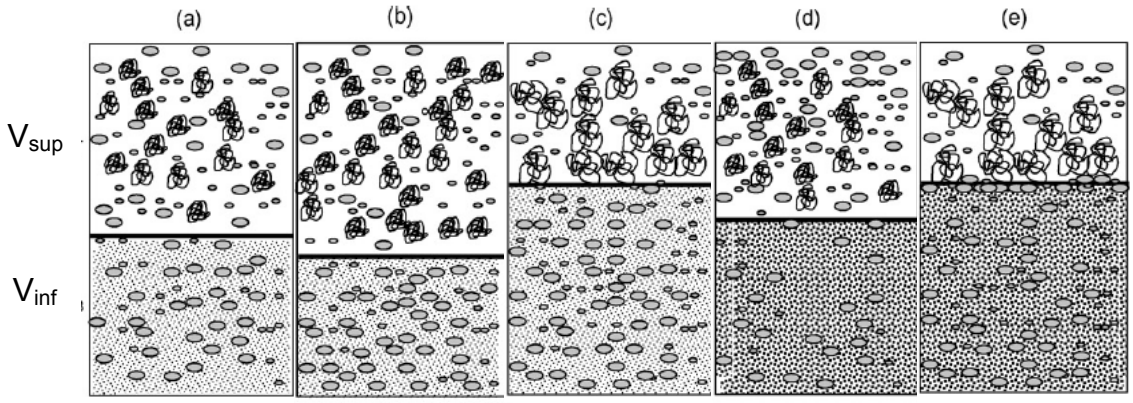

Figura 2. Representação esquemática do comportamento do coeficiente de partição de biomoléculas em SDFA: (a) sistema PEG/sal típico; (b) efeito do aumento da concentração do polímero; (c) efeito do aumento da massa molar do polímero; (d) efeito do aumento da concentração de sal; (e) efeito combinado 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.

2.4. Aplicação de Planejamentos Experimentais em SDFA

A utilização de planejamentos experimentais é uma boa ferramenta para se conhecer as relações entre os principais fatores que influenciam um SDFA com cada tipo de extração e de proteína que se deseja separar. Alguns dos fatores, conhecidos como de primeira ordem, que se analisa são: massa molar do polímero, concentração do polímero e do sal, pH e temperatura. As relações dos fatores de primeira ordem entre si são conhecidas como interações de segunda ordem.

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 seu nome (AHMAD et. al, 2008).

Se for possível assumir que certas interações de primeira ordem no sistema de duas fases aquosas possuem efeitos negligenciáveis então as informações dos principais efeitos juntamente com as interações de segunda ordem podem ser obtidas executando apenas uma fração do planejamento completo (AHMAD et. al, 2008).

Mayerhoff e colaboradores (2004) utilizaram um planejamento experimental 2^4 para avaliar a influência das variáveis massa molar do PEG, concentração do PEG, concentração de fosfato e concentração de NaCl na extração de xilose redutase utilizando sistemas de duas fases aquosas.

Porto e colaboradores (2008) otimizaram a extração de proteases de *Clostridium perfrigens* utilizando três planejamentos experimentais sucessivos (um 2^4 e dois 2^3) em SDFA PEG/citrato. A massa molar do PEG (fixa no 2^3), concentração do PEG, concentração do citrato e pH foram as variáveis independentes enquanto que, coeficiente de partição, rendimento de atividade, fator de purificação e seletividade foram as variáveis de resposta.

Moktharani e colaboradores (2008) estudaram o coeficiente de partição do antibiótico Ciprofloxacin em sistema de duas fases aquosas PEG/Na_2SO_4 utilizando um planejamento experimental completo 2^3, avaliando as influências da temperatura, concentração de sal, concentração do polímero e sua massa molar.

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 indica os efeitos de interação entre as mesmas.

3.0. OBJETIVOS

3.1. OBJETIVO GERAL

Purificar colagenase a partir de caldo fermentado de *Penicillium aurantiogriseum* URM4622 utilizando o processo de extração líquido-líquido em sistema de duas fases aquosas PEG/Sais de fosfato.

3.2. OBJETIVOS ESPECÍFICOS

- Produzir colagenase a partir de *Penicillium aurantiogriseum* URM4622;
- Estudar a influência das variáveis, massa molar do PEG (MM_{PEG}), concentração do PEG (C_{PEG}) e concentração de fosfato (C_{FOSF}), sobre a extração de colagenase utilizando o SDFA com auxílio de planejamento fatorial, tendo o coeficiente de partição (K), a recuperação de atividade (Y) e o fator de purificação (FP) como as variáveis de resposta;
- Estabelecer as melhores condições de purificação da colagenase utilizando sistema de duas fases aquosas após análise dos resultados do planejamento fatorial.

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5. ARTIGO – Collagenase partitioning and purification in poly (ethylene glycol)/phosphate aqueous two-phase systems

ESTE ARTIGO FOI SUBMETIDO À REVISTA SEPARATION AND PURIFICATION TECHNOLOGY (SEPPUR-D-09-00342)

Partitioning and extraction collagenase from *Penicillium aurantiogriseum* in poly (ethylene glycol)/phosphate aqueous two-phase system

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Abstract

Purification of collagenase produced by *Penicillium aurantiogriseum* was carried using an aqueous two-phase system (ATPS) PEG/phosphate. A 2^3-full experimental design was used to select the significant variables for the extraction process, being PEG molar mass, PEG and phosphate concentrations the independent variables investigated. ATPS was composed by PEG with molar mass of 550, 1 500 and 4 000 g/mol at concentrations of 15.0, 17.5 and 20% (w/w) and phosphate concentrations of 12.5, 15.0 and 17.5 (w/w). On the other hand, the selected responses were the partition coefficient, activity yield and purification factor. The best results of one-step extraction of collagenase contained in the fermentation broth (partition coefficient of 1.01, activity yield of 242% and purification factor of 23.5) were obtained pH 6.0 using 20% (w/w) PEG 550, 17.5% (w/w) phosphate. The preliminary results of this study are very promising and demonstrate that ATPS is selective to the collagenase.

Keywords: Collagenase, *Penicillium aurantiogriseum,* aqueous two-phase system, liquid-liquid extraction.

1. Introduction

Proteases are ubiquitously found in plants, animals and microorganisms. However, microorganisms are the preferred source of proteases owing to their broad biochemical diversity and their susceptibility to genetic manipulation. The microbial proteases account for approximately 40% of total worldwide enzyme sales [1]. Fungal proteases have many advantages, among which their extracellular localization, which makes its recuperation from the fermentation broth easier [2].

Collagenases are proteases that can hydrolyze both native and denatured collagens [3]. Collagenolytic proteases have been directly employed in clinical therapy and, as experimental reagents, in laboratory-scale studies. Their direct therapeutic use includes wound healing, treatment of sciatica in herniated intervertebral discs, treatment of retained placenta, and pretreatment for enhancing adenovirus-mediated cancer gene therapy. Collagenolytic proteases have also been used in the lab-scale preparation of isolated rat liver cells and the scission of collagen-like peptides in fusion proteins [4]. Up to now, however, the most common source of collagenases is *Clostridium hystolyticum* [5], which is a pathogenic microorganism; therefore, alternative safer sources of these enzymes are hoped for, like species of *Candida* [6], *Penicillium*, and so on.

According to Diamond and Hsu [7], 50–90% of production costs of biological products are due to the purification strategy; therefore, the downstream processing of biological materials requires effective and low-cost separation, recovery and purification techniques [8].

The development of techniques for the separation and purification of proteins has been an important prerequisite for many of the advances made in the

biotechnology industry. Liquid–liquid extraction using aqueous two-phase systems (ATPSs) is one of the most promising bioseparation processes, which can be used in the early steps of a purification process (e.g., separation of proteins from cell debris), replacing difficult solid-liquid separations, and also for further purification. Several advantages of ATPSs can be summarized as follows: (a) the high water content of both phases (70-80%, w/w), which means high biocompatibility and low interfacial tension, minimizing degradation of biomolecules; (b) the easiness to be scaled-up; (c) the low material costs; (d) the possibility of polymer recycling [9, 10].

Liquid-liquid extraction in ATPS is a powerful, non-chromatographic, unit operation for the separation of biomolecules, which has been successfully applied in the purification of different biological materials, such as cells, virus, organelles, nucleic acids, proteins and enzymes [9, 11, 12]. Moreover, this technique is relatively simple and inexpensive, has sometimes high resolution capacity and allows clarification, concentration and purification to simultaneously occur in just one step [13].

To the best of our knowledge, no previous attempt has been made to purify collagenases by ATPS; therefore, the main aim of this work was to make a first attempt in this direction. To this purpose, collagenase produced by *Penicillium aurantiogriseum* URM4622 fermentation was extracted/concentrated directly from the fermented broth by ATPS composed of PEG/phosphate, and a 2^3-factorial design was used to identify the best conditions of PEG molar mass, phosphate and PEG concentrations to perform this process.

2. Materials and methods

2.1. Chemicals

Polyethylene glycol with different molar masses was obtained from Fluka Biochemika (Buschs, Switzerland). The substrate Azocoll (<50 mesh) was acquired from Calbiochem/Merck (Darmstadt, Germany) and used as a collagen source. All the other reagents were of analytical grade.

2.2. Microorganism and culture medium

Penicillium aurantiogriseum URM4622 was supplied by the Micoteca of Mycology Department of the Federal University of Pernambuco (UFPE). The strain was maintained at 28ºC in malt extract agar, consisting 2 % (w/v) malt extract, 0.1 % (w/v) peptone, 2 % (w/v) glucose and 1.5 % (w/v) agar.

Soy flour medium (SM), as described by Porto et al. [14], was used for collagenase production. SM was composed by 2 % (w/v) filtered soy flour, 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 0.8 mL mineral solution. The composition of the mineral solution, per 100 mL of distilled water, was 100 mg of $FeSO_4.7H_2O$, 100 mg of $MnCl_2.4H_2O$, 100 mg of $ZnSO_4.H_2O$ and 100 mg of $CaCl_2.H_2O$. The fermentation medium was sterilized in autoclave at 121 ºC, for 20 min.

2.3. Collagenase production

To prepare the inoculum, spores were removed from the agar plates using 3 mL of a solution containing 0.9 % (w/v) NaCl and 0.01 % (v/v) Tween 80, previously sterilized at 121ºC for 20 min. Fermentations were done in 250 mL-Erlenmeyer flasks

containing 50 mL of the SM and 10^6 spores/mL. Flasks were incubated at 28ºC, with constant shaking at 150 rpm for 72 h.

2.4. Preparation of aqueous two-phase systems

A concentrated 40% (w/w) phosphate buffer solution was prepared by mixing appropriate amounts of dibasic sodium phosphate and monobasic sodium phosphate at room temperature (25 ± 1°C), pH 6.0. The required amount of this solution was mixed with 50% (w/w) PEG solutions with different molar masses, specifically 550, 1500 and 4000 g/mol. Such solutions were then added to 15 mL-graduated tubes. Fermentation broth representing 20% (w/w) of total mass was later added along with water to complete a 10 g system. After 1.0 min-vortex shaking, the two phases were separated by settling for 60 min. Then phase volumes were measured, separated and stored at -20ºC for later protein concentration and protease activity determinations. To avoid interference of PEG and phosphate, all samples were analyzed against blanks containing the same phase composition but without proteins.

2.5. Analytical techniques

Protein concentration was determined by the Bradford method [15] using Comassie brilliant blue G-250 as dye and bovine serum albumin as a standard.

Azocoll assay was carried out by the method described by Chavira et al. [16] modified as follows. Azocoll was washed and suspended in 0.05 M Tris-HCl buffer (pH 7.2) containing 1.0 mM $CaCl_2$ up to a final concentration of 5 mg/mL. Briefly, 150 µL of samples (cell-free supernatants) and 150 µL of buffer were mixed with 270 µL of azocoll suspension in a 2.0 mL-reaction tube. The reaction tubes were incubated at 37°C in a water bath under agitation at 330 rpm. After 3 h-incubation, the reaction

was stopped by centrifugation of samples at 10 000 × g (Kubota KR-20000T, Tokyo, Japan) at 4°C for 8 min. The absorbance of the supernatant solution was measured at 520 nm using a spectrophotometer, DU640 model (Beckman, Fullerton, CA). One activity unit (U) was defined as the amount of enzyme per mL that produces an increase in the optical density of 0.1 after 3 h at 520 nm. The specific activity was calculated as the ratio between the enzymatic activity (U) and the total protein concentration in the sample (mg/mL) and expressed as U/mg.

2.6. Experimental design and statistical analysis

PEG molar mass (M_{PEG}), PEG concentration (C_{PEG}) and concentration of phosphate (C_{PHOS}) were selected as the independent variables influencing the collagenase partition and purification by ATPS. The responses investigated were the collagenase partition coefficient (K), activity yield (Y) and purification factor (PF). Table 1 shows the experimental conditions for each system studied. The central point was run in quadruplicate to allow for estimation of pure error. The results were statistically analyzed by analysis of variance (ANOVA) at a significance level (p) ≤ 0.05. All statistical and graphical analyses were performed using the “Statistica 8.0” software (StatSoft, Inc., 2008, Tulsa, OK).

2.7. Determinations of the partition coefficient, activity yield and purification factor

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

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

Activity yield (Y) was defined as the ratio of activity in the top phase (A_T) to the initial one in the fermentation broth (A_I) and expressed as percentage:

$$Y = \left(\frac{A_T \cdot V_T}{A_I \cdot V_I} \right) \cdot 100 \quad (2)$$

where V_T and V_I represent the volumes of the top phase and the initial fermentation broth, respectively.

The purification factor was calculated as the ratio of the specific activity in the top phase to the initial specific activity in the fermentation broth before partition:

$$PF = \frac{A_T / C_T}{A_I / C_I} \quad (3)$$

where C_T and C_I represent the protein concentrations, expressed as mg/mL, in the top phase and fermentation broth, respectively.

3. Results and discussion

Table 2 lists the main results of collagenase extraction tests using directly the broth fermented by *P. aurantiogriseum*. The variables levels were selected based on previous literature reports [17, 18]. However, run A1 did not form any biphasic system, because the component complexity of the fermentation medium led to

deviation of the curve towards the monophasic region of the binodal curve, like those already reported by other authors for similar protein ATPS [19].

The statistical analysis of the partition coefficient (K) showed that the three independent variables were significant. The PEG molar mass exerted the greatest effect (Table 3), in that the lowest molar mass of the polymer (550 g/mol) ensured the highest value of this response (Table 2). Similar decreases in K were observed for many other enzyme systems with increasing PEG molar mass in ATPS [10,20].

On the contrary, K was positively influenced by both PEG and phosphate concentrations, although there was a significant interaction between the three variables (Table 3). Figure 1 shows the simultaneous effects of the independent variables on the partition coefficient and points out as the best conditions for this response M_{PEG} = 550 g/mol, C_{PEG} = 20% (w/w) and C_{PHOS} = 17.5% (w/w). The partition coefficient of collagenase was < 1 in almost all runs, which indicates that collagenase preferentially partitioned to the bottom phase.

As is well known, in PEG-salt systems partitioning of biomolecules depends on volume exclusion effect of the polymer in the polymer rich (top) phase and salting out in the salt rich (bottom) phase. Briefly, the volume occupied by the polymer increases with an increase in polymer concentration and polymer chain length or molar mass, which results in reduced space for biomolecules in the top phase. As a result, the biomolecules tend to partition to the bottom phase, which is inferred as “volume exclusion effect” [18, 21, 22]. However, the solubility of biomolecules in the salt rich (bottom) phase decreases with an increase in salt concentration, which results in increased partitioning of biomolecules to the top phase, and is inferred as “salting out effect” [18,20].

On the basis of these considerations, the increase in concentration of collagenase in the bottom phase observed in almost all the runs of the present study was likely due to a volume exclusion effect prevailing over the salting out. This hypothesis appears to be confirmed by the fact that the values of the partition coefficient in the presence of PEG 550 were always higher than with PEG 4 000 (Table 2).

However, in only one condition (run A7) was K >1 (1.01), and the enzyme partitioned mostly to the other phase. This behavior is indeed difficult to explain on the basis of the present knowledge on the characteristics of this enzyme from *P. aurantiogriseum*. Nevertheless, it suggests that the salting out effect could have become predominant over the volume exclusion under these conditions of high polarity and charge levels in the bottom phase (linked to the high concentrations of PEG and phosphate, respectively), thus driving to protein towards the other phase. Similar increase in K was already observed in PEG/phosphate system for thaumatin partition with decreasing PEG molar mass [23].

It was previously shown that the protein partition coefficient was strongly influenced when the tie line length was increased as a result of the increased difference between the polymer and salt concentrations in the top and bottom phases [24]. For those systems in which proteins exhibited a great affinity for the top phase, as it occurred for run 7 of this work (K > 1), the partition equilibrium was displaced to the top phase as the tie line increased, whereas for proteins which preferred the salt rich-phase (K < 1), such as trypsinogen, the opposite behavior was observed [8].

The decrease in molar mass and the increase in PEG concentration exalted the activity yield of collagenase (Y). Although the interaction among the three independent variables was statistically significant –and similar to that obtained for the

partition coefficient–, the independent effect of C_{PHOS} was not (Table 3); therefore, the best conditions for the recovery of collagenase (Y = 376%) were obtained with M_{PEG} = 550 g/mol and C_{PEG} = 20% (w/w), but at the lowest C_{PHOS} value (12.5% w/w) (Table 2).

Values of the yield higher than 100%, like most of those obtained in this study, have frequently been reported for enzyme extraction using liquid–liquid systems [18, 25, 26, 27], as the likely result of positive PEG interaction with the protein [18, 28, 29].

The same analysis of the results was performed for the purification factor (PF), which presented several statistically significant effects. Although the interaction among the three variables was not statistically significant, likewise for K, all the main variables (M_{PEG}, C_{PEG} and C_{PHOS}) showed statistically significant independent effects, and only the interactions M_{PEG} x C_{PHOS} and M_{PEG} x C_{PEG} were so (Table 3). As a result of these factor combinations, the best conditions for the purification factor (PF = 23.5) were the same as for K (run A7) (Table 2).

Fig. 2 illustrates the simultaneous influence of M_{PEG} and C_{PHOS} on PF, while Fig. 3 that of M_{PEG} and C_{PEG} on the same response. In particular, at phosphate concentration of 17.5% (w/w), PF in the top phase increased from 4.9 (run A8) to 23.5 (run A7), when M_{PEG} was reduced from 4 000 to 550 g/mol at a given level of PEG (20% w/w) (Fig. 2), and from 14.7 (run A5) to 23.5 (run A7), when PEG concentration increased from 15 to 20% (w/w) at a given value of M_{PEG} (550 g/mol) (Fig. 3). Both behaviors can be explained by an effect of salting out (highest phosphate concentration) stronger than that of volume exclusion (lowest PEG molar mass and highest PEG concentration), which likely promoted the purification in the top phase.

The highest purification factor (PF = 23.5) was close to that (PF = 21.2) found by Mayerhoff et al. [27], who utilized a PEG/phosphate system to remove xylose reductase from a broth fermented by *Candida mogii* and observed M_{PEG}, C_{PEG} and C_{PHOS} effects on PF qualitatively coincident to those of the present study. Similarly, Cascone et al. [23] obtained a 20-fold purification of thaumatin in a single purification step with the same system.

4.0. Conclusions

There are many factors influencing the partition of collagenase in aqueous two-phase systems (ATPS), especially when the target protein is contained in a complex mixture like a fermented broth. Among the several factors influencing the collagenase partitioning in PEG/phosphate ATPS, three of them (PEG molar mass, PEG concentration and phosphate concentration) were selected as the independent variables and investigated according to a 2^3-experimental design. The partition coefficient (K), the activity yield (Y) and the purification factor (PF) of collagenase from *P. aurantiogriseum* URM4622 were selected as the responses. The highest values of both responses (PF = 23.5 and Y = 242%), obtained using 20% (w/w) PEG 550, 17.5% (w/w) phosphate at pH 6.0, were better than those reported in the literature for similar ATPS. Taking into account additional advantages such as the easiness to scale-up and low material costs, it is possible to conclude that ATPS constitutes a viable and potentially powerful strategy for the purification of collagenase from *P. aurantiogriseum*. The preliminary results of this study allowed

defining the operating conditions for collagenase purification from a broth fermented by this microorganism to be optimized in future attempts.

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Table 1

Factor levels of the 2^3-experimental design used for the study of collagenase purification by ATPS

Variables	Levels		
	Low (-1)	Central (0)	High (+1)
PEG molar mass (g/mol)	550	1 500	4 000
PEG concentration (%)	15.0	17.5	20.0
Phosphate concentration (%)	12.5	15.0	17.5

Table 2

Conditions and results of collagenase extraction by PEG/ phosphate ATPS according to the 2^3–experimental design

Run	M_{PEG}[a] (g/mol)	C_{PEG}[b] (% w/w)	C_{PHOS}[c] (% w/w)	K[d] (—)	Y[e] (%)	PF[f] (—)
A1	550	15.0	12.5	-	-	-
A2	4 000	15.0	12.5	0.27	85.6	4.8
A3	550	20.0	12.5	0.91	376.7	14.7
A4	4 000	20.0	12.5	0.25	86.0	7.7
A5	550	15.0	17.5	0.90	222.9	14.7
A6	4 000	15.0	17.5	0.20	41.8	5.9
A7	550	20.0	17.5	1.01	242.0	23.5
A8	4 000	20.0	17.5	0.50	96.3	4.9
A9(C)	1 500	17.5	15.0	0.74	190.4	9.8
A10(C)	1 500	17.5	15.0	0.90	236.5	14.4
A11(C)	1 500	17.5	15.0	0.85	194.9	11.1
A12(C)	1 500	17.5	15.0	0.80	186.7	11.4

[a] PEG molar mass; [b] PEG concentration; [c] Phosphate concentration; [d] Partition coefficient; [e] Activity yield; [f] Purification factor in the top phase; - no biphasic system.

Table 3

Statistical effects calculated for the responses according to the experimental plan of Table 2.

Variables	K[a]	Y[b]	PF[c]
1(M_{PEG})[d]	-8.18*	-8.11*	-5.37*
2(C_{PEG})[e]	6.54*	6.87*	4.60*
3(C_{PHOS})[f]	5.98*	0.83	3.96*
1-2[g]	-3.92*	-5.20*	-3.90*
1-3[g]	-4.18*	-1.85	-4.56*
2-3[g]	-2.51	-4.63*	-1.80
1-2-3[g]	5.60*	6.28*	0.36

[a] Partition coefficient; [b] Activity yield; [c] Purification factor; [d] PEG molar mass; [e] PEG concentration; [f] Phosphate concentration; [g] Terms of variable interactions; *Statistically significant values (at the 95% confidence level).

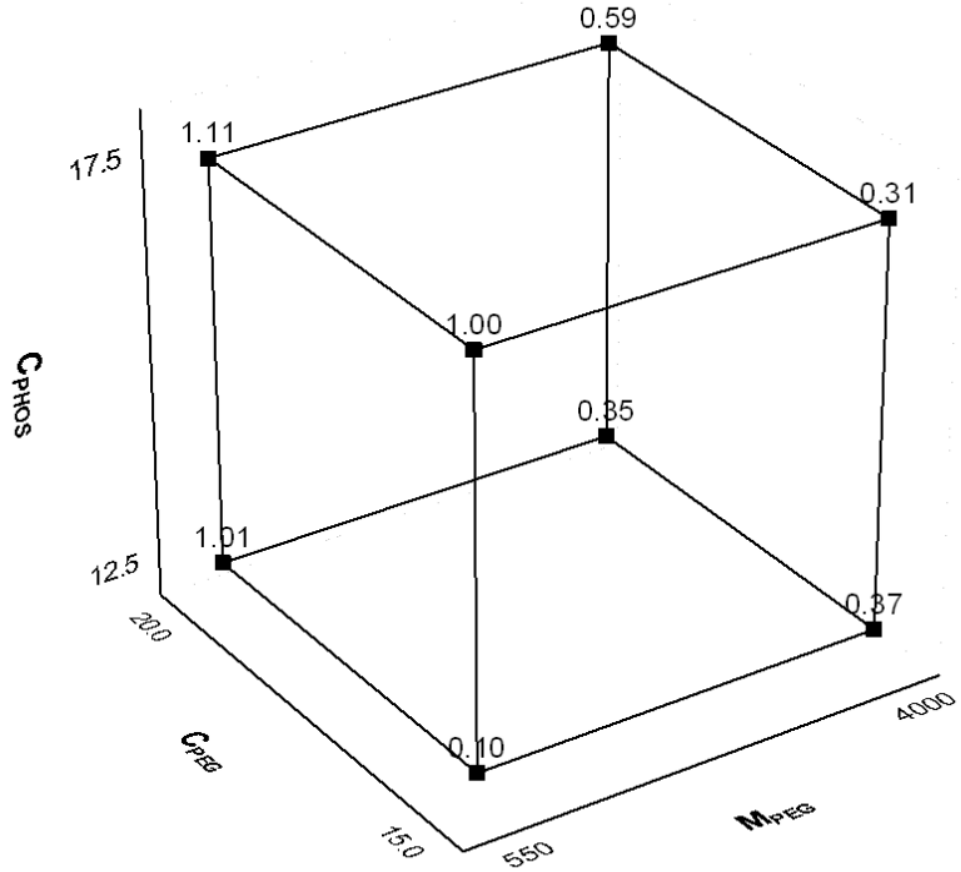

Fig. 1. Cubic plot of the partition coefficient as a function of PEG molar mass (M_{PEG}) and concentrations of PEG (C_{PEG}) and phosphate (C_{PHOS}) according to the experimental plan of Table 2.

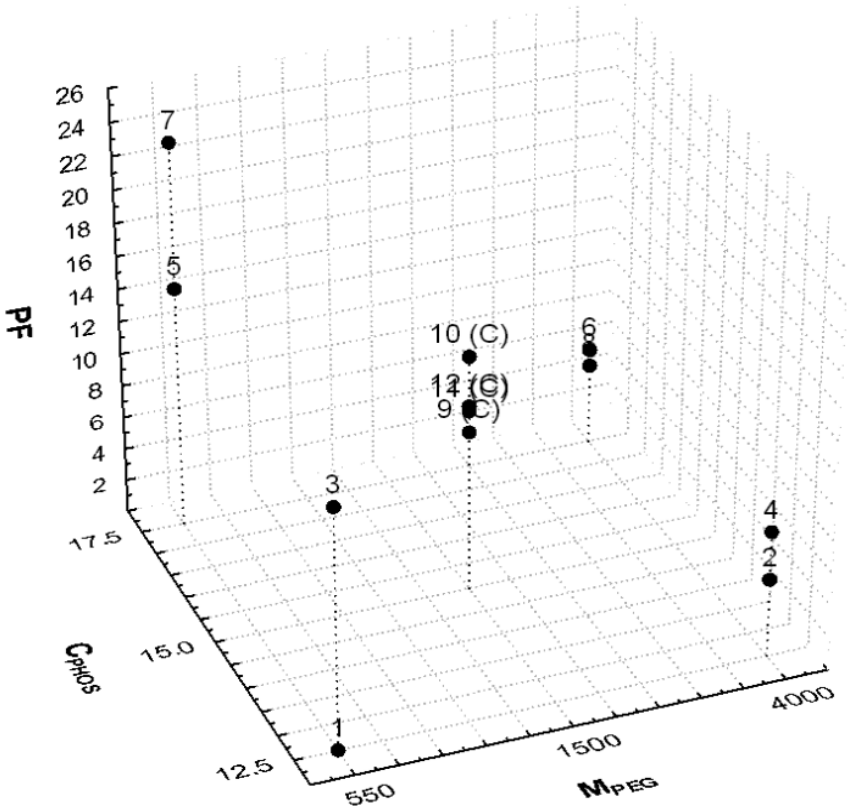

Fig.2. Simultaneous effects of PEG molar mass (M_{PEG}, g/mol) and phosphate concentration (C_{PHOS}, % w/w) on the purification factor of collagenase from *P. aurantiogriseum* by ATPS. Experiments were performed according to the 2^3-experimental design.

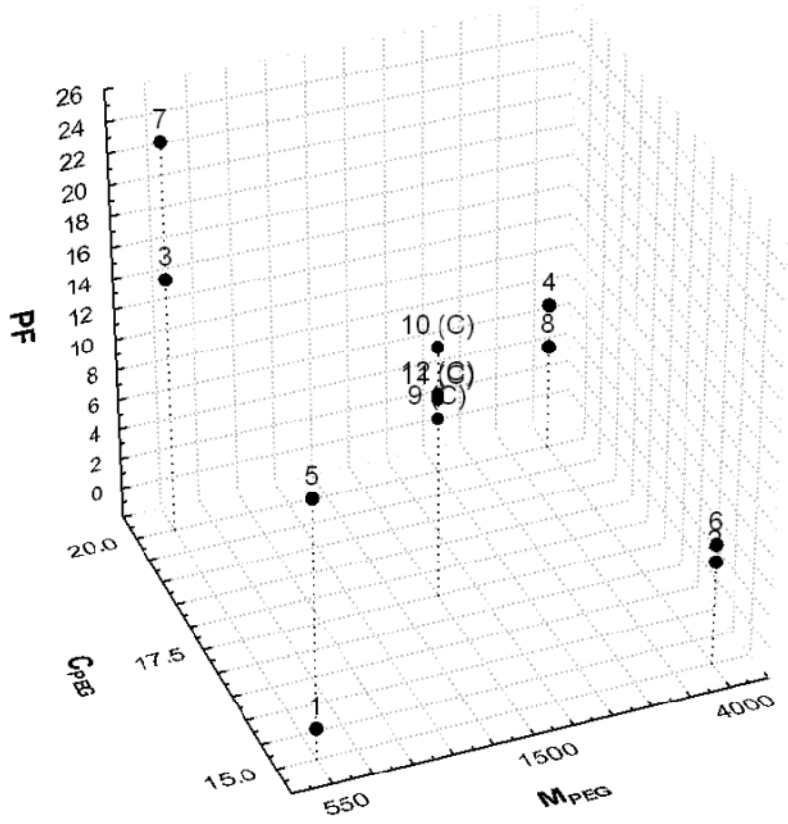

Fig.3. Simultaneous effects of PEG molar mass (M_{PEG}, g/mol) and PEG concentration (C_{PEG}, % w/w) on the purification factor of collagenase from *P. aurantiogriseum* by ATPS. Experiments were performed according to the 2^3-experimental design.

6.0. CONCLUSÕES

Após a realização dos experimentos foi possível concluir que:

- 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 do planejamento estatístico 2^3 identificou que a melhor condição de extração foi com: massa molar do PEG mais baixa 550 (g/mol) e as concentrações do PEG e do fosfato mais altas, respectivamente 20% (m/m) e 17,5% (m/m);

- É possível extrair colagenase do fermentado de *P. aurantiogriseum* (URM 4622) através de SDFA PEG/fosfato com grau de purificação 23,5 e recuperação 242%, demonstrando ser uma potente ferramenta como um primeiro passo no processo de purificação.

7. ANEXOS

7.1. Normas da Revista Separation and Purification Technology

Guide for Authors

INTRODUCTION

Types of Paper - Contact Details for Submission

BEFORE YOU BEGIN

Ethics in Publishing - Conflict of interest - Submission declaration - Copyright - Retained author rights - Role of the funding source - Funding body agreements and policies - Language Services - Submission - Referees

PREPARATION

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AFTER ACCEPTANCE

Use of the Digital Object Identifier - Proofs - Offprints

AUTHOR INQUIRIES

INTRODUCTION

Separation and Purification Technology is an international journal providing a comprehensive coverage of all aspects of separation and purification of homogeneous gaseous and liquid solutions and heterogeneous mixtures, such as emulsions or gas/solid, liquid/solid, and solid mixtures. *Separation and Purification Technology* welcomes in particular contributions focused on applications in the environmental protection industry, the recycling of valuable materials from industrial wastes, and the treatment of separation and purification problems in emerging technologies including energy storage and conversion systems, raw material and renewable energy exploitation, biotechnology and chemical or petrochemical processing.

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Contact Details for Submission

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Andre de Haan: extraction, leaching, distillation, absorption, flotation, crystallization, precipitation; membranes (liquid, reverse osmosis, nanofiltration, gas permeation, pervaporation).
Chi Tien: adsorption, ion exchange and chromatographic separations, absorption with chemical reactions, filtration (depth, cake and crossflow membrane filtrations) and other mechanical separations.

BEFORE YOU BEGIN

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PREPARATION

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Please write your text in good English (American or British usage is accepted, but not a mixture of these). Use decimal points (not decimal commas); use a space for thousands (10 000 and above).

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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.

Experimental

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.

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Results should be clear and concise.

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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.

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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.

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Examples:

Reference to a journal publication:

[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, J. Sci. Commun. 163 (2000) 51-59.

Reference to a book:

[2] W. Strunk Jr., E.B. White, The Elements of Style, third ed., Macmillan, New York, 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, Inc. New York, 1994, pp. 281-304.

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7.2. Indicativos de produção 2008-2009

7.2.1. Resumos em congressos

Resumo no ENZITEC 2008

ROSSO, B. U., LIMA, C. A., PORTO, T.S., NASCIMENTO, C. O., PORTO, A. L. F. E CARNEIRO-DA-CUNHA, M. G. **Recuperação de colagenase produzida por *Penicilium aurantiogriseum* em sistemas de duas fases aquosas PEG-fosfato.** In: Enzitec – VIII Seminário Brasileiro de Tecnologia Enzimática, 2008, Rio de Janeiro, 13 a 15 de agosto.
Palavras-chave: SDFAs; *Penicillium aurantiogriseum*; Extração; PEG/fosfato; Colagenase

7.2.2. Trabalhos completos em congressos

Paper submetido ao SINAFERM 2009

Bruno Ubertino Rosso, Carolina de Albuquerque Lima, Tatiana Souza Porto, Cynthia de Oliveira Nascimento, Adalberto Pessoa Junior, Maria das Graças Carneiro-da-Cunha, Ana Lúcia Figueiredo Porto. **Collagenase Partitioning and Extraction in Poly (Ethylene Glycol)/Phosphate Aqueous Two-phase System.** In: XVII Simpósio Nacional de Bioprocessos – SINAFERM, 2009, Natal, 02 a 05 de agosto. Keywords: Collagenases, *Penicillium aurantiogriseum,* ATPS, liquid-liquid extraction.