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PRODUÇÃO DE PROTEASES COM ATIVIDADE FIBRINOLÍTICA POR FUNGOS FILAMENTOSOS DE SOLOS DA CAATINGA UTILIZANDO FERMENTAÇÃO EM ESTADO SÓLIDO

Thiago Pajeú Nascimento

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Co – Orientadora: Prof.ª Dr.ª Tatiana Souza Porto

Recife, 2014

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BANCA EXAMINADORA

Presidente: Prof ª. Dr.ª Ana Lúcia Figueiredo Porto
(Departamento de Morfologia e Fisiologia Animal/Área de Bioquímica-UFRPE)
Orientadora

Membro Interno-Titular: Prof ª. Dr.ª Galba Maria de Campos Takaki
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Membros Suplentes:

Maria Tereza dos Santos Correia (Departamento de Bioquímica – UFPE)

Maria Taciana Cavalcanti Vieira Soares (Departamento de Morfologia e Fisiologia Animal – UFRPE)

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"Paciência e perseverança tem o efeito mágico de fazer as dificuldades desaparecerem e os obstáculos sumirem".

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RESUMO

Enzimas fibrinolíticas têm recebido atenção, devido ao seu potencial medicinal para doenças trombolíticas, estas estão se tornando uma das principais causas de morbidade e mortalidade em todo o mundo. Este trabalho teve como finalidade obter condições de produção e caracterização de proteases fibrinolítica, produzida por fungos filamentosos isolados de solo da Caatinga-PE-Brasil, utilizando fermentação em estado sólido, e purificar essa enzima utilizando Sistema de Duas Fases Aquosas. Dentre os fungos estudados, foi selecionado o *Mucor subtillissimus* SIS42 como melhor produtor da protease e entre os resíduos agroindustriais utilizados, o melhor substrato foi o farelo de trigo. Através de um planejamento fatorial 2^3 as condições otimizadas de produção foram: 3g de farelo de trigo, 50% de umidade, submetidos a 25ºC após 72 horas de fermentação, com uma atividade fibrinolítica e proteásica de 144,58 U/mL e 48,33 U/mL, respectivamente. A protease fibrinolítica contida no extrato bruto apresentou uma temperatura ótima de 45ºC e foi estável nesta temperatura por 150 minutos, a mesma teve sua atividade proteásica aumentada na presença de K^+, Ca^+ e Mn^+ e diminuída na adição de Cu^+. De acordo com a especificidade a substratos cromogênicos e a presença de PMSF, esta enzima foi classificada como uma serino protease do tipo quimiotrispsina. A partir dos resultados obtidos, foi realizado um planejamento experimental (2^3) para purificação da protease fibrinolítica produzido pelo *M. subtillissimus* SIS42 utilizando o sistema de duas fases aquosas, onde foi possível avaliar a influência das variáveis: concentração e massa molar do PEG e concentração de sulfato de sódio na purificação da enzima. Três variáveis-respostas foram avaliadas (coeficiente de partição, recuperação da enzima e fator de purificação). Utilizando um planejamento central composto para ampliar os estudos e efeitos na purificação da enzima, foram estudadas as variáveis: concentração de PEG (6000 g/mol) e sulfato de sódio. Os resultados atingidos foram: coeficiente de partição para a fase rica em sal (0,049 a 0,795), um aumento de pureza de 10 com uma recuperação de 102% da atividade enzimática na fase inferior do sistema. O sistema que proporcionou as melhores condições de purificação foi constituído por: PEG 6000 (g/mol) e concentração de 30,00% (m/m) com uma concentração de sulfato de sódio de 13,20% (m/m). A protease fibrinolítica apresentou através de uma SDS-PAGE uma massa massa molar após a purificação de 94kDa e atividade no zimograma de fibrina. Após a purificação, por sistema de duas fases aquosas a protease fibrinolítica foi novamente avaliada a presença de PMSF e a substratos cromogênicos, indicando a enzima como uma quimiotripsina.

Palavras chaves: protease fibrinolítica, planejamento fatorial, fermentação em estado sólido, farelo de trigo, *Mucor subtillissimus*, sistema de duas fases aquosas.

ABSTRACT

Fibrinolytic enzymes have received attention owing to their medicinal potential for thrombolytic diseases these are becoming a leading cause of morbidity and mortality worldwide. This work aimed to obtain conditions for the production and characterization of fibrinolytic proteases, produced by filamentous fungi isolated from soil of Caatinga-PE-Brazil, using solid-state fermentation and purification of this enzyme by aqueous two-phase system. Among the studied species, *Mucor subtillissimus* SIS42 was selected as the best protease producer and wheat bran was the best of all agroindustrial residues.. Using a 2^3 factorial design, the best conditions of production were: 3g wheat bran, 50% moisture, subjected to 25°C after 72 h, showing a protease activity and fibrinolytic activity of 144.58 U/mL and 48.33 U/mL, respectively. The fibrinolytic protease contained in the crude extract showed an optimal temperature of 45°C and was stable at the same temperature for 150 minutes, increasing in the presence of a K^+, Ca^+, Mn^+ and decreasing the protease activity in the addition of Cu^+. According to a specific chromogenic substrate and the presence of PMSF, the enzyme was classified as a serine protease chymotrypsin-like. According of the obtained results, was applied an experimental design (2^3) for purification of fibrinolytic protease produced by *M. subtillissimus* SIS42 using aqueous two-phase system how to evaluate the influence of variables: PEG molar mass concentration and sodium sulfate concentration in the enzyme purification. Three response variables were analysed (partition coefficient, enzyme yield and purification factor). Using a central composite design, to expand the studies and effects on enzyme purification, the variables were studied: PEG concentration (6000 g/mol) and sodium sulphate concentration. The results were: the partition coefficient for the salt-rich phase (0.049 to 0.795) increased purity of 10, with a recovery of 102% of the enzyme activity in the bottom phase of the system. The best purification conditions were constituted by: PEG 6000 (g/mol) and concentration 30.00% (w/w) with a sodium sulfate concentration of 13.20% (w/w). The fibrinolytic protease presented through a SDS-PAGE molecular mass of 94kDa after purification and activity on fibrin zymography. After purification by aqueous two-phase system the fibrinolytic protease was again evaluated in the presence of PMSF and chromogenic substrates, confirming the enzyme as chymotrypsin-like.

Key words: fibrinolytic protease, factorial design, solid-state fermentation, wheat bran, *Mucor subtillissimus*, aqueous two-phase system.

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LISTA DE ABREVIAÇÕES

AVC – Acidente vascular cerebral

AVCI – Acidente vascular cerebral isquêmico

DCP – Doença vascular periférica

DCV – Doença cardíaca valvular

DCVs – Doenças cardiovasculares

EC – Classificação Enzimática

FES – Fermentação em estado sólido

FS – Fermentação submersa

IAM – Infarto agudo do miocárdio

IM – Infarto do miocárdio

LIP – Lignina peroxidase

MnP – Manganês peroxidase

MRS – Metodologia de superfície de resposta

OMS – Organização mundial de saúde

PAI – Inibidor do ativador do plasminogênio

PAI 1 - Inibidor do ativador do plasminogênio 1

PAI 2 - Inibidor do ativador do plasminogênio 2

SDFA – Sistema de duas fases aquosas

t-PA – Ativador do plasminogênio tecidual

U/mL – Unidades por

u-PA – Uroquinase

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1. INTRODUÇÃO

Com o aumento da produção agroindustrial, a geração de resíduos têm se tornado um problema para a sociedade contemporânea, principalmente para os setores de alimentos. Contudo, é de fundamental importância utilizar tecnologias que aproveitem este crescente volume de resíduos gerados, entre essas tecnologias, destaca–se a fermentação em estado sólido (FES) (ALEXANDRINO et al., 2007).

A FES, também chamada por alguns autores de fermentação semi-sólida (FSS) caracteriza-se pelo crescimento microbiano sobre um material orgânico sólido que funciona como substrato e/ou suporte, é um processo bastante eficiente e muito promissor no desenvolvimento de vários bioprocessos, como na obtenção de produtos bioativos provenientes do metabolismo primário e secundário de micro-organismos (alcaloides, ácidos orgânicos, biopesticidas, antibióticos e enzimas) (BOGAR et al., 2003).

Vários micro-organismos podem crescer nos substratos sólidos, como bactérias, leveduras e fungos, entretanto os fungos filamentosos se destacam, por crescerem com pouca água e muitos sólidos presentes, além da sua forma de crescimento favorecer a colonização no meio em virtude da formação de hifas (DURAND A., 2003).

Em 2003, entre as diferentes enzimas produzidas pelos micro-organismos, as proteases constituíram um dos mais relevantes grupos de enzimas, devido a sua aplicabilidade serem bastante ampla no campo médico e industrial, representando aproximadamente 60% do total de enzimas comercializadas no mundo (SINGH et al., 2003; BARRET et al., 2001). Algumas destas proteases são enzimas fibrinolíticas capazes de lisar a fibrina, proteína fundamental no processo de coagulação sanguínea (PENG et al., 2005). Essas enzimas são capazes de prevenir ou curar doenças trombóticas pela degradação de coágulos sanguíneos, mantendo o fluxo sanguíneo em sítios de lesão vascular (UEDA et al., 2007; CHOI et al., 2011; WANG et al., 2011).

Entretanto, após o processo de produção das proteases, é necessário que se tenha uma segunda fase relativa à recuperação e a pré-purificação dessas biomoléculas (YEGIN et al., 2011). O sistema de duas fases aquosas (SDFA) vem sendo utilizado como um bom método alternativo para ser utilizado como uma pré–purificação, principalmente de biomoléculas, biomateriais e engenharia de tecidos, uma de suas características é que o SDFA pode ser formado pela adição de dois ou mais polímeros ou um polímero e um sal específico, sendo amplamente utilizado para biosseparação de enzimas e proteínas por conta do seu baixo custo em relação a outros processos de separação (JARA & PILOSOF, 2011; ELBERT, 2011; MARINI et al., 2011), sendo o sistema PEG/SAL um dos mais adequados para a separação e purificação de proteases (PERICIN et al., 2009).

2. OBJETIVOS

2.1 Objetivo Geral

Produzir e caracterizar proteases com atividade fibrinolítica por espécies de fungos filamentosos isolados de solo da Caatinga–PE, Brasil por fermentação em estado sólido e pré-purificar esta enzima utilizando sistema de duas fases aquosas.

2.2 Objetivos específicos

- Produzir proteases com atividade fibrinolítica por FES utilizando resíduos agroindustriais;
- Selecionar qualitativamente as culturas produtoras de proteases com atividade fibrinolítica;
- Selecionar o melhor substrato para a produção de proteases com atividade fibrinolítica;
- Estudar a influência das variáveis: quantidade de substrato, umidade e temperatura sobre a produção das proteases com atividade fibrinolítica por FES, utilizando um planejamento fatorial (2^3);
- Caracterizar bioquimicamente a protease com atividade fibrinolítica no extrato bruto e pré-purificado, em relação à temperatura ótima e estabilidade à temperatura; efeito de inibidores, íons metálicos e substratos cromogênicos sobre a atividade da enzima;
- Purificar a protease utilizando o sistema de duas fases aquosas, utilizando PEG/Sulfato de sódio e estudar a influência das variáveis: Massa molar e concentração do PEG e concentração do sulfato de sódio sobre a protease, com atividade fibrinolítica utilizando um planejamento fatorial (2^3) e metodologia de superfície de resposta;
- Determinar a massa molar da enzima através de SDS-PAGE e realizar um zimograma de fibrina.

3. REVISÃO BIBLIOGRÁFICA

3.1 Proteases: Classificação, aplicação terapêutica e micro-oganismos produtores

As proteases também conhecidas como peptídeo–hidrolases ou peptidases são enzimas responsáveis pela clivagem hidrolítica das ligações peptídicas das proteínas e formam a subclasse EC 3.4. das hidrolases. As proteases podem ser divididas em dois grandes grupos: As exopeptidases (EC 3.4.11. até EC 3.4.19.) que clivam sequencialmente as extremidades dos aminoácidos a partir do grupamento amino ou carboxílico, sendo este grupo subdividido em aminopeptidases (ácidos aminados com cisão no N-terminal da cadeia polipeptídica), carboxipeptidases (ácidos aminados com cisão no C-terminal da cadeia polipeptídica), dipeptidases (substratos dipeptídicos) e dipeptidil e tripeptidil-peptidases (responsáveis por catalisarem a clivagem N-terminal de dois ou três peptídeos de uma cadeia polipeptídica). O segundo grupo, são constituídos pelas endopeptidases (EC 3.4.21. até EC 3.4.99.) responsáveis pela catálise iniciada dentro de uma cadeia de polipeptídeos (ESPOSITO; AZEVEDO, 2010; CASTRO et al., 2011; YIKE, 2011).

As proteases agem dentro de uma rede complexa que são compostas de pequenas moléculas que podem ser ativadores e/ou inibidores, peptídeos, receptores, substratos e domínios de ligação, influenciando na localização espacial e temporal da sua atividade (LI et al., 2013). Com relação ao seu pH ótimo, as proteases podem ser classificadas em ácidas, alcalinas ou neutras e de acordo com seu mecanismo de ação, elas podem ser classificadas em cinco principais classes: as metalo proteases (~200), as serino proteases (~180), as cisteíno proteases (~160), treonina proteases (~30) e as proteases do ácido aspártico ou aspártico proteases (~25), sendo as demais proteases, pertencentes a grupos com mecanismos catalíticos desconhecidos (DRAG; SALVESEN, 2010; YIKE, 2011).

Essas enzimas proteásicas realizam uma infinidade de funções, atuando desde a reciclagem de proteínas intracelulares para a digestão de nutrientes até a amplificação em cascata do sistema imunológico, atuando ainda na ativação do sistema complemento, na patogênese, na apoptose, na resposta imune e no metabolismo secundário, dentre outras funções (BI et al., 2011; LI et al., 2013).

As proteases constituem o grupo de enzimas mais explorado e mais importante, por possuírem atuações e aplicações tanto na pesquisa básica, quanto nos setores industriais, chegam a representar cerca de 60% do mercado industrial de enzimas, sendo empregada no amaciamento de carnes, estabilização de vinhos e cervejas, clareamento de sucos, fabricação e amaciamento do couro, como agentes hidrolíticos na coagulação do leite e na maturação do queijo, em indústrias de panificação, de detergentes, papeís, celulose, seda, têxteis, indústrias farmacêuticas, indústrias de revestimento anti-incrustantes, processos de biorremediação, e podem ser utilizadas também na recuperação da prata de películas fotográficas, além de poder atuar na síntese de peptídeos em meios orgânicos e no processamento de proteínas (SILVA et al., 2009; RAI; MUKHERJEE, 2009; LIMA et al., 2011; ANBU, 2013; GOHEL; SINGH, 2013).

Embora tenha um vasto uso, a produção de enzimas proteolíticas ainda não é o suficiente para atender a demanda crescente do mercado mundial (SUNDARARAJAN et al., 2011). No ano 2005, o

mercado externo brasileiro de enzimas foi avaliado em 147,2 milhões de dólares, correspondendo a apenas 3,7% do mercado internacional (ZIMMER et al., 2009). Dentre essas enzimas, as proteases são especialmente importantes porque são enzimas mais estáveis e ativas a pH alcalino, a temperaturas elevadas e a presença de tensioativos, íons metálicos, agentes oxidantes e solventes orgânicos (ANBU, 2013).

As proteases podem ser encontradas em uma ampla diversidade de fontes, sendo constituintes essenciais de todas as formas de vida na terra e fisiologicamente necessárias para os organismos vivos, podendo ser produzidas por: animais, vírus e plantas, entretanto as proteases produzidas por micro-organismos são as mais desejáveis uma vez que elas possuem todas as características essenciais para aplicações biotecnológicas, onde através dos micro-organismos pode-se aumentar a reprodutibilidade das proteases, permitindo um menor tempo de geração da enzima através da manipulação genética (RAO; NARASU, 2007; RAVIKUMAR et al., 2012; ABO-ELMAGD; HOUSSEINY, 2012).

A pesquisa por novas drogas é um processo multidisciplinar e envolve duas fases distintas: a sua descoberta e o seu desenvolvimento (LUCO; MARCHEVSKY, 2006). Nos últimos anos a relação entre a engenharia genética, biotecnologia e a bioquímica têm sido cada vez mais frequentes em busca de especificidades de reações individuais e adaptadas conforme a necessidade específica e terapêutica do seu uso (LI et al., 2013).

A utilização de proteases para aplicações terapêuticas tem sido um dos objetivos das indústrias farmacêuticas nos últimos anos, pois a atividade catalítica dessas enzimas permite o uso de menores doses para os tratamentos, com um potencial alvo e uma maior eficácia, além de diminuir efeitos colaterais dos medicamentos já existentes, mantendo ao mesmo tempo os benefícios terapêuticos desejados e reduzindo custos, tornando-se interessantes para o ramo farmacêutico industrial, estima-se que cerca de 5 a 10% de todos os alvos farmacêuticos para o desenvolvimento de drogas sejam proteases (DRAG; SALVESEN, 2010; LI et al., 2013).

Essas enzimas terapêuticas tem se representado como alvo de drogas potenciais no combate a doenças, estudos sugerem que cerca de 60 proteases humanas são alvos de drogas, por exemplo, no retardado da AIDS demonstrando um sucesso terapêutico substancial nos últimos anos e também no combate a doenças cardiovasculares (CASTRO et al., 2011; RAVIKUMAR et al., 2012), câncer, e na ação contra parasitas e outros vírus (ZIMMER et al., 2009) como pode ser observado na Tabela 1.

Tabela 1. Uso de proteases na aplicação terapêutica

Enzima	Fonte	Uso terapêutico	Referência
Estreptoquinase	*Streptococcus β – hemolítico*	Coágulo sanguíneo	ZIMMER et al. (2009)
Serino protease com atividade colagenolítica	*Penicillium aurantiogriseum*	Úlceras de pele; Úlceras intestinais	LIMA et al. (2011)
Papaína	*Carica papaya*	Digestão; Verminose	ZIMMER et al. (2009)
Metalo protease	*Bacillus subtilis K42*	Anticoagulante	HASSANEIN et al. (2011)
Protease fibrinolítica	*Aspergillus oryzae* KSK-3	Degradação de coágulos sanguíneos	SHIRASAKA et al. (2012)
Colagenase	*Clostridium histolyticum*	Úlceras de pele	ZIMMER et al. (2009)
Serino protease com atividade fibrinolítica	*Cirriformia tentaculata*	Trombose	PARK et al. (2013)
Protease fibrinolítica	*Bacillus* sp. UFPEDA 485	Degradação de coágulos sanguíneos	SALES et al. (2013)

3.2 Fibrinólise

Um sistema hemostático saudável é constituído de plaquetas, vasos, proteínas envolvidas no processo de coagulação sanguínea, anticoagulantes naturais e pelo sistema de fibrinólise. A hemostasia consegue suprimir o desenvolvimento de coágulos de sangue em circulação normal, através da remoção rápida por um sistema de fibrinólise eficaz, mas reage extensivamente no caso de uma lesão vascular contrapondo–se a perda excessiva de sangue e evitando a formação de trombos intravasculares decorrentes da formação excessiva de fibrina (FRANCO, 2001; BANERJEE et al., 2004; LU; CHEN, 2010).

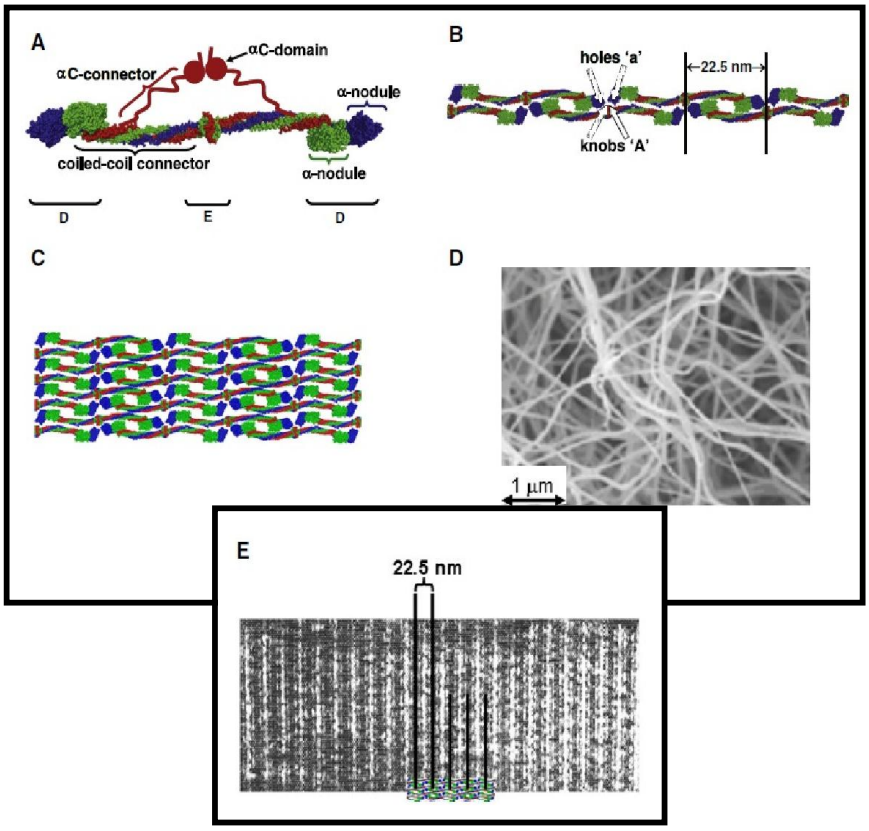
A formação dos coágulos é um fator de proteção pelo corpo humano para se evitar o excesso de sangramento de feridas e lesões, entretanto elas podem atuar negativamente promovendo o bloqueio do fluxo sanguíneo (DEEPAK et al., 2010; RAAFAT et al., 2012). A presença do trombo (do grego *thrómbos* que significa coágulo sanguíneo) no sistema circulatório vascular representa um processo crítico para a manutenção da integridade vascular podendo ocasionar um bloqueio da passagem sanguínea levando a consequências graves como: infarto do miocárdio (IM), acidente vascular cerebral (AVC), embolia pulmonar, doença cardíaca valvular (DCV), doença vascular periférica (DCP), arritmias cardíacas e outras doenças cardiovasculares sendo que a trombose vascular, ou o tromboembolismo venoso pode inclusive levar o paciente a óbito, esse problema clínico tem sido cada vez mais frequente, sobretudo nos países

ocidentais desenvolvidos (BARBOSA et al., 2009; CASTRO et al., 2011; VAIDYA et al., 2011; SHIRASAKA et al., 2012).

A prevalência de doenças cardiovasculares impõe um impacto cada vez maior em nossa sociedade tanto emocionalmente, quanto socialmente e financeiramente (KIM et al., 2011). Segundo a Organização Mundial de Saúde (OMS), as doenças cardiovasculares (DCVs) assim como a pressão alta, se apresentaram como a causa número um de mortes globais nos últimos anos, onde cerca de 17 milhões de pessoas morrem a cada ano, vitimas de doenças cardiovasculares (BHAVANI et al., 2012; BAJAJ et al., 2013). Dentre estas doenças, a trombose é uma das mais prevalentes e que mais acometem a sociedade moderna (MAHAJAN et al., 2012).

A dissolução dos coágulos quando mediado por uma enzima é conhecida como trombólise ou fibrinólise, estudos nessa área tem sido feitos para o melhor entendimento a cerca da compreensão da formação dos coágulos e da estrutura da fibrina (Figura 1) e sua dissolução (BANERJEE et al., 2004; WHITTAKER; PRZYKLENK, 2009).

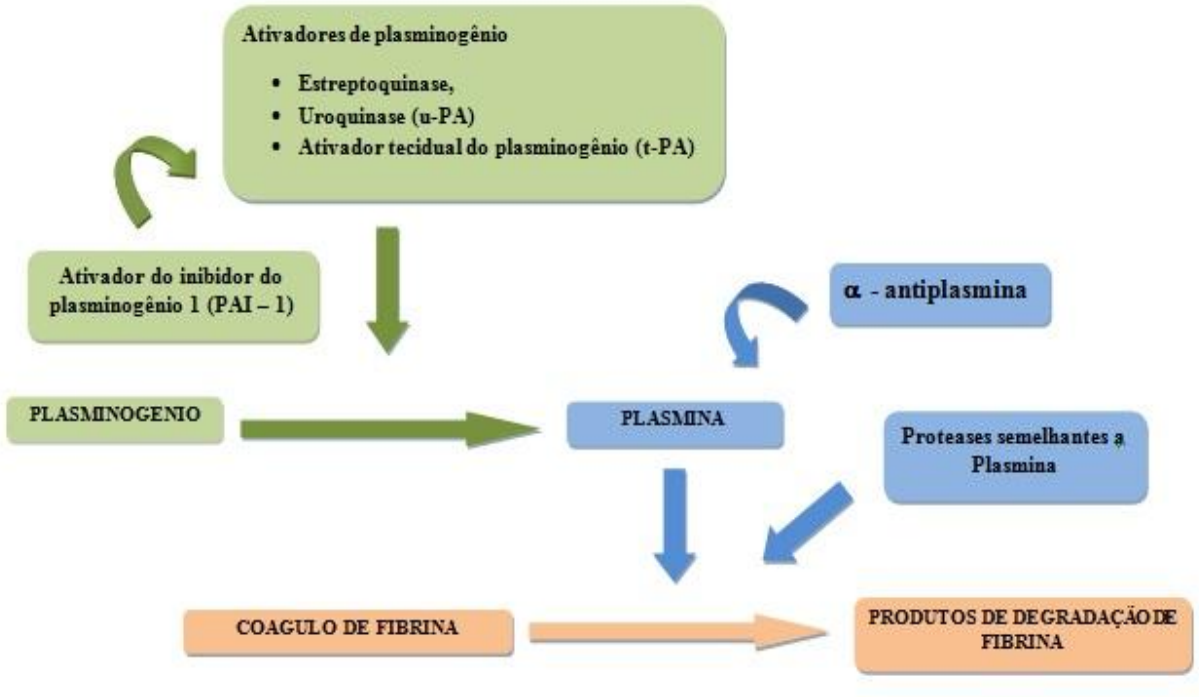
Figura 1. Estrutura do fibrinogênio e da fibrina. (A) Modelo da estrutura do fibrinogênio humano, (B) Modelo da estrutura da fibrina mostrando a cadeia αA. (C) Modelo da fibra de fibrina. (D) Micrografia eletrônica do coágulo de fibrina. (E) Segmentos de fibra de fibrina através de micrografia eletrônica de sobreposição de imagem. (Fonte: FALVO et al., 2010).



A fibrinólise também promove a cicatrização de feridas, restabelecendo o fluxo sanguíneo, sendo efetuada pelo sistema de ativação do plasminogênio, que na circulação de mamíferos convertem o plasminogênio inativo em zimogênio ativo através de uma serino protease denominada de plasmina (EC 3.4.21.7), uma enzima (semelhante à tripsina) fibrinolíticamente ativa, que é produzida a partir do plasminogênio da proteína inativa presente na circulação (BANERJEE et al., 2004; DRAG; SALVESEN, 2010; STIE; FOX, 2012). Devido ao seu efeito direto na degradação dos coágulos de fibrina, a plasmina tem grande importância para o tratamento do acidente vascular cerebral isquêmico (AVCI) e também como ativadora de microglia, e da ativação dos fatores de crescimento e da matriz de metalo proteases (RADNAABAZAR et al., 2011).

A conversão do plasminogênio em plasmina inativa envolve uma clivagem proteolítica e uma complexa cascata de interações que medeia a fibrinólise necessária para a homeostase vascular, ou a degradação e remodelação do tecido necessária para a função imune, bem como os processos de crescimento do tecido e seu desenvolvimento e reparação. Essa conversão, é mediada por dois tipos de ativadores de plasminogênio: o do tipo uroquinase ativador do plasminogênio (u–PA) e do tipo ativador do plasminogênio tecidual (t–PA) (BANERJEE et al., 2004; DRAG; SALVESEN, 2010; STIE; FOX, 2012). A atividade proteolítica destes dois ativadores de plasminogênio é modulada pelo inibidor do ativador do plasminogênio (PAI) que pode ser do tipo PAI-1 ou PAI-2, enquanto que a atividade da plasmina é controlada pela antiplasmina $\alpha 2$ (MEDCALF, 2007) como pode ser observado na Figura 2.

Figura 2. Modelo de ação dos agentes fibrinolíticos e ativadores da ativação proteolítica do plasminogênio em plasmina e a representação esquemática da fibrinólise. (adaptado de ARBIND; JAGDEEP, 2011; BANERJEE et al., 2004).



3. 3 Características dos diversos agentes fibrinolíticos

Um dos tratamentos terapêuticos oferecidos com o intuito de melhorar a sobrevida dos pacientes submetidos a doenças cardiovasculares e diminuir a sua morbidade, é a perfusão intravenosa imediata no início do tratamento com agentes trombolíticos, que estão atualmente em uso clínico para a dissolução de trombos arteriais em particular nos vasos sanguíneos cardíacos. Esses agentes são utilizados para permitir o fluxo coronariano após a ocorrência de uma oclusão coronariana aguda, sobretudo em pacientes com IAM, podendo salvar este indivíduo da mortalidade e mobilidade (LU; CHEN, 2010; VAIDYA et al., 2011; RAAFAT et al., 2012; NAVEENA; GOPINATH; et al., 2012a).

Como foi esclarecido por OLIVEIRA (2001) o nome “trombolítico” é utilizado indevidamente para estes agentes, na verdade estes agentes deveriam ser chamados de agentes fibrinolíticos, pois os mesmos não degradam a fibrina presente no trombo diretamente, mas sim catalisa a ativação proteolítica do plasminogênio em plasmina, a principal enzima responsável pela quebra dos coágulos sanguíneos (MURCIANO et al., 2003; PARK et al., 2013). Entretanto o termo “agentes trombolíticos” continua sendo bastante difundido. Uma variedade desses agentes, tais como a estreptoquinase, a uroquinase (u–PA) e o ativador tecidual do plasminogênio (t-PA) são utilizados no uso clínico, sendo as formas farmacológicas

mais ativas e disponíveis no mercado (VAIDYA et al., 2011; NAVEENA; GOPINATH; et al., 2012a) como pode ser observado na Figura 2.

Os principais agentes podem ser classificados em dois tipos. Os ativadores de plasminogênio: uroquinase, ativador do plasminogênio tipo tecido (t-PA), e estreptoquinase, responsáveis por ativarem o plasminogênio em plasmina, e os tipos de proteínas de plasmina, tais como: a nattoquinase e a lumbroquinase, que agem degradando diretamente à fibrina (CHUNG et al., 2010).

A estreptoquinase (EC 3.2.1.35) é um dos agentes trombolíticos mais importantes, sua produção nas indústrias farmacêuticas é produzida através de micro–organismos, e a sua utilização continua sendo a terapia vital mais acessível especialmente nos sistemas de saúde mais pobres do mundo (BANERJEE et al., 2004; MUKHERJEE; RAI, 2011). Ela foi produzida inicialmente por *Streptococcus haemolyticus,* uma proteína natural, portanto capaz de causar reações alérgicas (MEDVED et al., 1966; OLIVEIRA, 2001) .

Uroquinase ou ativador de plasminogênio (u–PA, EC 3.4.21.31) é uma molécula multifuncional que serve tanto com uma serino protease especifica de fibrinogênio ou como um indutor de sinal, que facilita a função da plasmina em processos imunológicos, tais como o recrutamento de células imune, geração de sinais anti–inflamatórios na cicatrização, e em condições patológicas na propagação de tumores, ela é expressa na forma de zimogênio, em que a ativação envolve a conversão proteolítica de uma pró-enzima de cadeia simples (MEDCALF, 2007; MUKHERJEE; RAI, 2011; STIE; FOX, 2012). Atualmente não é utilizado para o tratamento de IAM, entretanto é frequentemente utilizado para trombólise nas salas de hemodinâmica e para tratamento da embolia pulmonar grave (OLIVEIRA, 2001).

O ativador tecidual do plasminogênio (t-PA) atua como protease fibrinolítica endógena intravascular. Nos últimos anos têm se descoberto seu papel *in vivo* influenciando na resposta inflamatória (MEDCALF, 2007). Também tem se avaliado a sua administração na capacidade de reduzir a deposição de fibrina induzida pela endotoxina e mortalidade concomitante em coelhos, assim como, a comparação da eficácia clínica do recombinante t–PA e da estreptoquinase, entretanto essas investigações ainda não revelaram uma clara preferência por uma ou outra droga (BANERJEE et al., 2004; SCHOOTS et al., 2004). O t-PA não possui imunogenicidade, e tem uma especificidade razoável pela fibrina e não tem resistência ao PAI-1 (OLIVEIRA, 2001).

Apesar do seu intenso uso, muitos dos agentes fibrinolíticos têm apresentado algumas desvantagens como: meia-vida curta, reações alérgicas, baixa especificidade pela fibrina, um alto custo, fontes restritas e excessiva complicação hemorrágica, principalmente dentro do trato intestinal (KIM et al., 2011; HUANG et al., 2013; ZHANG et al., 2013). Nesse sentido, pesquisas para busca de novas fontes de enzimas fibrinolíticas econômicas e seguras, que causem menos efeitos colaterais têm sido estudadas nas últimas décadas (NAVEENA; GOPINATH; et al., 2012b).

OLIVEIRA (2001) mostra algumas das propriedades que os novos candidatos a futuros agentes trombolíticos devem apresentar: reperfusão rápida, especificidade pela fibrina, baixa incidência de

sangramento sistêmico, baixa incidência de hemorragia intracraniana, resistência ao PAI-1, baixa taxa de reoclusão, não apresentar efeito na pressão arterial, não possuir antigenicidade e ter um custo razoável.

3. 4 Fontes de enzimas fibrinolíticas

O CheongGukJang (pasta de soja sem sal, de origem coreana) e o Natto (um alimento tradicional japonês, feito de soja e consumido a mais de 2000 anos) ambos são fermentados com o auxilio de bactérias da espécie *Bacillus,* foram uma das primeiras fontes de enzimas fibrinolíticas, tendo o *Bacillus subtilis natto*, como uma bactéria isolada a partir do Natto, e que produz a nattoquinase uma enzima fibrinolítica capaz de reduzir a formação de coágulos sanguíneos por hidrólise, diretamente nos trombos e também por atuar na conversão de plasminogênio em plasmina (OMURA et al., 2005; RADNAABAZAR et al., 2011; ZHANG et al., 2013).

Enzimas isoladas de animais vêm sendo estudadas nos últimos anos, sendo isoladas do veneno de cobra como o *Bothrops atrox* (CINTRA et al., 2012), *Agkistrodon saxatilis* (KOH et al., 2001) e *Bothrops colombiensis* esta última, uma espécie de cobra envolvida na maioria dos acidentes ofídicos da Venezuela, também têm demonstrado ação de degradar a fibrina diretamente e eficientemente, além de não possuírem uma ação tóxica e nem efeitos secundários como hemorragias, sendo investigado seu uso para o tratamento de doenças trombolíticas (GIRÓN et al., 2013). A lumbroquinase é uma enzima proteolítica que foi extraída primeiramente e encontrada na cavidade do corpo e nos órgãos digestivos de *Lumbricus rubellus* uma espécie de minhoca, esta enzima consegue dissolver coágulos de fibrina do sangue e inibir a ativação de plaquetas e sua agregação (MIHARA et al., 1991; LU; CHEN, 2010).

Atividade fibrinolítica também tem sido encontrada em poliquetas *Neanthes japonica* (WANG et al., 2011) e atividades fibrinogenolítica e anticoagulantes podem ser encontradas até mesmo em tecidos que revestem alguns animais, por exemplo, os ferrões de arraias marinhas como a das espécies *Dasyatis sephen* e *Aetobatis narinari* (KUMAR et al., 2011).

As plantas também têm sido alvos de investigações, em virtude de suas ricas fontes de compostos químicos versáteis promissores e com valores medicinais, sendo que nos últimos anos, proteases que afetam a coagulação e a fibrinólise foram isoladas e caracterizadas a partir do látex de *Euphorbia hirta* (PATEL et al., 2012) de folhas de *Spondias dulcis* (ISLAM et al., 2013) ou de ervas já utilizadas a centenas de anos na medicina popular do leste asiático, como a *Ganoderma lucidum* Vk12 (KUMARAN et al., 2011).

Ambientes marinhos também tem sido alvo de exploração, para produção de novas enzimas com atividades fibrinolíticas por se tratar de um ambiente novo, pouco explorado e rico em uma diversidade de micro–organismos. Nos últimos anos, várias enzimas foram identificadas e caracterizadas a partir de micro-organismo de ambientes marinhos, tais como: actinomicetos marinhos (NAVEENA; SAKTHISELVAN; et al., 2012), *Bacillus subtilis* HQS-3 uma bactéria marinha isolada do mar de lama de Beihai, sudeste da

China (HUANG et al., 2013) e *Bacillus subtilis* ICTF-1 (MAHAJAN et al., 2012). Além disso, essas enzimas originadas de micro–organismos marinhos são capazes de funcionar em condições que levam à precipitação ou desnaturação da maioria das proteínas de micro-organismos não comuns nesse habitat, e podem ter uma menor ou nenhuma toxicidade ou efeitos secundários quando utilizados para aplicações terapêuticas (MAHAJAN et al., 2012).

As bactérias da espécie *Bacillus,* são as maiores produtoras de enzimas fibrinolíticas em virtude de metabolicamente serem mais diversificadas do que fungos, além de crescerem mais rápido em comparação aos mesmos e serem mais facilmente passíveis de manipulações genéticas (BAJAJ et al., 2013), vários trabalhos tem demonstrado seu uso (JEONG, 1997; CHANG et al., 2012; SALES, 2013) Entretanto, *Bacillus* sp. produz enzimas que podem variar, sendo intracelulares ou extracelulares o que pode elevar os custos de produção em larga escala (MAHAJAN et al., 2012). Outras bactérias como os *Streptomyces* sp. também tem demonstrado uma boa atividade fibrinolítica (CHITTE et al., 2011; BHAVANI et al., 2012; MEDEIROS E SILVA et al., 2013).

Diferentemente os fungos tem a capacidade de produzir proteases com atividade fibrinolítica de forma extracelular, entre esses se destacam os fungos fitopatogênicos como o *Paecilomyces tenuipes* (KIM et al., 2011) e o fungo filamentoso *Aspergillus oryzae* KSK-3 (SHIRASAKA; NAITOU, 2012). No trabalho de ARBIND; JAGDEEP (2011) foi apresentado uma variedade de enzimas fibrinolíticas produzidas a partir de fungos (Tabela 2).

Tabela 2. Enzimas fibrinolíticas produzidas por fungos (Adaptada de ARBIND; JAGDEEP, 2011).

Nome da proteína	Espécie	Tipo de atividade	Referência
FP Protease	*Fusarium* sp. BLB	Serino protease	SUGIMATO et al. (2007)
Enzima fibrinolítica	*Fusarium oxysporium* N.R.C 1	Metalo protease	ABDEL-FATTAH et al. (1993)
Enzima fibrinolítica	*Fusarium semmitectum*	Não definida	BUCKLEY et al. (1981)
Enzima FU – P	*Fusarium* sp.	Serino metalo proteases	BIN et al. 2009
Enzima fibrinolítica	*Rhizopus chinensis* 12	Sulfuril–metalo proteases	KIRK et al. (2001)
PoFE	*Pleurotus estreutus*	Quimiotripsina–metalo proteases	WOL-SUK et al. 2010
Enzima fibrinolítica	*Pleurotus eryngii*	Serino protease	OHM, R.A .

			(2010)
Enzima fibrinolítica	*Schizophyllum commune* BL 23	Metalo protease	PANDEE et al. (2008)
Mushroquinase	*Schizophyllum commune*	Metalo protease	PARK et al. 2010
Enzima fibrinolítica	*Schizophyllum commune*	Metalo protease	LU et al. (2010)
Enzima fibrinolítica	*Bionecteria sp.*	Serino protease	ROVATI et al. (2010)
FVP – 1	*Flammulina velutipes*	Metalo protease	PARK et al. (2007)
TSMEP1 TSMEP2	*Tricholoma saponaceum*	Metalo protease	KIM et al. (2001)
Enzima fibrinolítica	*Rhizomucor miehei*	Não definida	ALI et al. (2008)
GFMEP	*Grifola frondosa*	Metalo protease	NONAKA et al. (1997)
FFP1	*Fomitella fraxinea*	Serino protease	LEE et al. (2006)
FFP2	*Fomitella fraxinea*	Metalo protease	LEE et al. (2006)
Nova enzima fibrinolítica	*Aspergillus fumigates*	Metalo protease	LARCHER et al. (1992)
AMMEP	*Armillaria mellea*	Metalo protease	KIM et al. (2001)
CMase	*Cordyceps militaris*	Metalo protease	KIM et al. (2006)
Enzima fibrinolítica	*Cordyceps militaris*	Serino metalo proteases	CUI et al. (2008)
Enzima fibrinolítica	*Oidenderon flavum*	Metalo protease	THARWAT et al. (2006)
Enzima fibrinolítica	*Albatrellus confluens*	Serino metalo proteases	KIM, J.H (2008)
Enzima fibrinolítica	*Perenniporia fraxinea*	Metalo protease	KIM et al. (2008)

3.5 Produção de enzimas por FES

A FES é um processo fermentativo produtor de uma grande variedade de biomoléculas, entre essas as enzimas proteásicas (RAVIKUMAR et al., 2012). A FES tem um enorme potencial para a produção de enzimas, sendo um sistema melhor do que a fermentação submersa (FS), pois apresenta pontos de vista tanto econômicos quanto ambientais vantajosos (IANDOLO et al., 2011), embora não seja a tecnologia de fermentação industrial mais amplamente utilizada devido a alguns fatores como: dificuldades na concepção

de biorreatores, devido a homogenicidade, superaquecimentos e limitações relacionadas à quantidade de inóculo utilizando esporos, particularmente no caso de processos que envolvam micro–organismos como os fungos (CASTRO et al., 2011). A FES envolve todos os processos que utilizam o material insolúvel em água para o crescimento microbiano, na ausência de água livre (Figura 3), estas condições são especialmente adequadas e tendem a favorecer o crescimento de fungos filamentosos, devido às necessidades de umidade mais baixas em comparação com as bactérias (YEGIN et al., 2011; RAVIKUMAR et al., 2012).

BARRIOS–GONZÁLEZ (2012) explana que talvez a maior desvantagem da FES, em relação à FS, é o pouco conhecimento da fisiologia dos fungos e outros micro-organismos utilizados nessa fermentação, necessitando que os pesquisadores e empresas tenham mais atenção a sua parte biológica, já que a maioria das pesquisas se detém aos estudos de otimização de produção das biomoléculas (temperatura, umidade, aeração, entre outros) e esquece-se de estudar sobre o crescimento do fungo.

A FES tem sido utilizada há séculos, sendo inicialmente utilizada na produção de alimentos fermentados em países orientais (OMURA et al., 2005; RADNAABAZAR et al., 2011; ZHANG et al., 2013). Nos últimos 20 anos, este tipo de fermentação tem atraído e renovado seu interesse e credibilidade em muitas corporações industriais, pois os custos nos processos industriais atuais são bastante elevados para a produção de várias biomoléculas, especialmente enzimas (HOLKER; LENZ, 2005; RAHARDJO et al., 2006; THANAPIMMETHA et al., 2012).

Sendo o processo de FES preferido à FS, devido a uma série de vantagens: que incluem uma maior concentração e produtividade, alto rendimento do produto requerido, facilidade no processo de *downstream* e requisitos de energias mais baixos tendo uma redução dos custos operacionais e com o uso de máquinas mais simples para fermentação quando comparados com a FS, além de um baixo débito de águas residuais, tempo de fermentação mais curto, diminuição ou ausência de degradação de enzimas por proteases indesejáveis, utilização de substratos de baixo custo, além de evitar fenômenos que influenciam negativamente a produção de biomoléculas como a ausência e/ou menor repressão catabólica, e inibição de biossíntese de aminoácidos e amônia (CASTRO, et al., 2011; YEGIN et al., 2011; RAVIKUMAR et al., 2012; BARRIOS-GONZALEZ, 2012).

Há muitos casos em que as enzimas produzidas em FES diferem das produzidas em FS, pois essas enzimas apresentam diferentes características como: temperatura ótima mais elevada ou estabilidade ao pH, diferentes parâmetros cinéticos, ou mesmo enzimas que não foram segregadas para o meio líquido, são segregadas em meio sólido, além do produto gerado ser muitas vezes obtido mais rápido, embora possam ser até mesmo produzidas pelo mesmo micro–organismo (ACUNA-ARGUELLES et al., 1995; HÖLKER; LENZ, 2005; BARRIOS-GONZÁLEZ, 2012).

Um exemplo de maior produção de enzima por FES quando comparado a FS pode ser visto nos estudos de HIGASHIO e YOSHIOKA (1982) onde utilizando estirpes de fungos como *Mucor racemosus* para a produção de protease coagulante do leite na FES atingiu uma produção de 6500 U/g de substrato

sólido, o que corresponde a 600-1000 U/mL enquanto que na FS utilizando o mesmo micro–organismo obteve uma atividade enzimática entre 600 e 800 U/mL. DUTTA (2008) utilizando *Penicillium citrinum* para a produção de celulase em FES comparando com a FS também apresentou melhores resultados de produção da enzima quando utilizado o primeiro método mencionado de fermentação.

VINIEGRA-GONZÁLEZ (2003) mostrou nos seus estudos que a taxa máxima de crescimento específico, nível de biomassa máxima, produção de enzima/biomassa e a taxa de produção secundária ou de decomposição, revelou uma produtividade da enzima usando um sistema de FES maior do que na FS em todos os parâmetros estudados.

Pode-se utilizar resíduos agrícolas e alimentares para a produção industrial de enzimas, principalmente pertencentes à classe de enzimas ligninolíticas, como as lacases, a manganês peroxidase (MnP) e lignina peroxidase (LIP), e de enzimas de hidrólise de carboidratos, tais como as amilases, as xilanases , as celulases, pectinases e sobretudo as proteases (IANDOLO et al., 2011).

Atualmente existem dois tipos de sistemas de FES que são separados de acordo com a natureza da fase sólida utilizada, podendo ser: FES em substratos sólidos naturais, ou FES em suportes inertes impregnados, embora essa última não apresente resultados de produção tão satisfatórios quando comparados ao método clássico (BARRIOS-GONZÁLEZ, 2012).

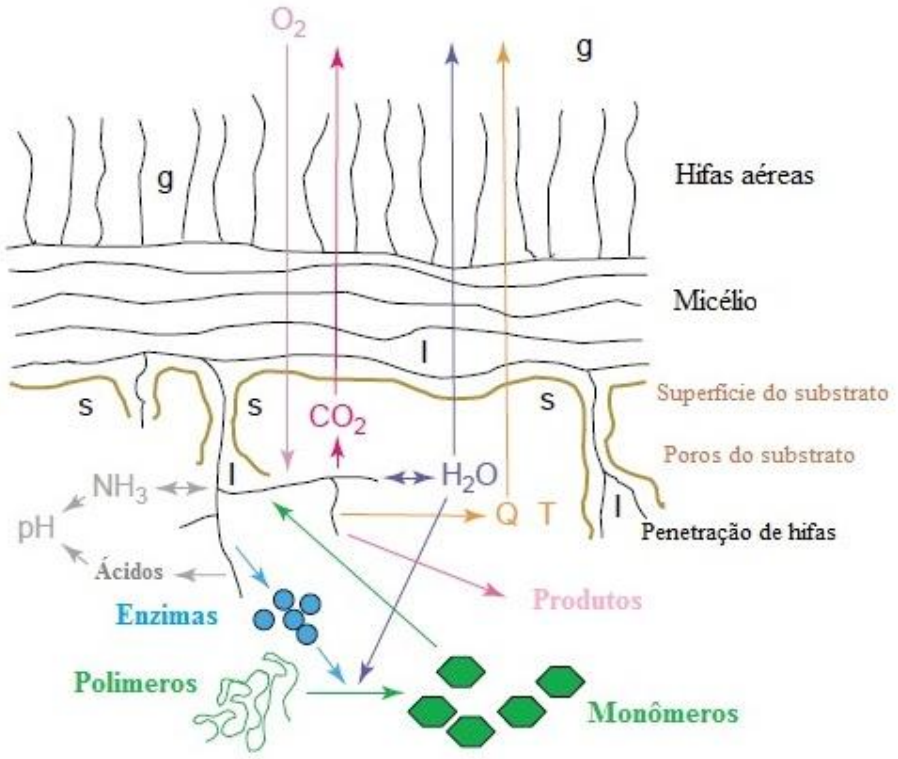
No esquema da Figura 3, podem ser observados alguns dos processos que ocorrem em microescala durante a fermentação em estado sólido (FES). Após a esporulação, as hifas dos fungos se desenvolvem em um tapete de micélio (preto), que se espalha sobre a superfície das partículas que constituem o substrato sólido (castanho). A partir do tapete de micélios, as hifas aéreas sobressaem para o espaço gasoso, ao passo que outros penetram no substrato por crescimento em poros cheios de líquido. Em níveis normais de umidade, os espaços vazios entre as hifas aéreas são mais susceptíveis de serem preenchidos com gás (g), ao passo que os espaços vazios no interior vão sendo cobertos por um tapete de micélio e dentro do substrato são preenchidos com líquido (l) (HOLKER; LENZ, 2005).

As atividades metabólicas mostradas ocorrem principalmente perto da superfície do substrato e no interior dos poros, no entanto, as regiões expostas do micélio (por exemplo, as hifas aéreas) mostram também o metabolismo e pode haver um transporte de substâncias a partir da penetração para as hifas aéreas. Enzimas hidrolíticas (azul claro), que são produzidos pelo micélio, difusa para a matriz sólida e catalisam a degradação de macromoléculas em unidades menores (verde). O_2 é consumido e CO_2, H_2O, calor e produtos bioquímicos interessantes são produzidos durante a fermentação. Assim, os gradientes desenvolvem dentro do biofilme, que, por exemplo, força a difusão de O_2 a partir da fase gasosa para as regiões mais profundas do biofilme (lilás) e CO_2 a difundir a partir destas regiões para a fase gasosa (vermelho). Desenvolvimento de calor (Q; laranja) leva a um aumento rápido da temperatura (T), que é um problema sério durante FES (HOLKER; LENZ, 2005).

O calor é, por conseguinte, removido do substrato não só através de condução, mas também por meio de evaporação, que é parte do complexo balanço de água no sistema (azul escuro). Ao lado de

evaporação, o equilíbrio de água inclui a absorção de água pelo micélio no decurso do crescimento, o consumo de água durante as reações de hidrólise e de produção de água através da respiração. Como outro fator importante, o pH local, pode ser alterado devido à liberação de ácidos de carbono e da troca de amônia (cinza). Os produtos bioquímicos de interesse que são libertados para a matriz sólida e os espaços cheios de líquidos durante a fermentação pode absorver o sólido e pode ter que ser extraída para uso posterior no final do processo. Todos estes e muitos outros fenômenos podem influenciar fortemente o desempenho do processo durante FES (HOLKER; LENZ, 2005).

Figura 3. Ilustração sobre o crescimento dos fungos na superfície do substrato na FES (Fonte: HOLKER; LENZ, 2005).



3.6 Fatores que influenciam na produção de enzimas por FES

Para a produção, sobretudo de enzimas fibrinolíticas muitas tentativas em laboratórios têm sido feitas para melhorar a expressão dessas enzimas, sendo importantes nos processos industriais para determinar uma melhor atividade e estabilidade de enzimas que determine uma viabilidade econômica e uma aplicação da enzima mais rentável (RAVIKUMAR et al., 2012; ABO-ELMAGD; HOUSSEINY, 2012).

Algumas estratégias como a seleção de um meio ideal de cultura, otimização das condições ambientais, superexpressão por linhagens geneticamente modificadas, modificação química no sítio ativo,

introdução de pontes de dissulfeto, imobilização, estabilização entrópica, teor de umidade inicial, pH e porosidade do substrato, temperatura da cultura, presença de nutrientes e a quantidade de inóculo adicionada são fatores determinantes na produção da biomolécula de interesse (PENG et al., 2005; GOHEL; SINGH, 2013).

Dentre esses parâmetros, a temperatura de cultivo, o teor de água e o pH do meio de crescimento mostram como sendo as variáveis constantes mais significativas e também mais complexas no processo de biorreação, sendo a temperatura a que mais propicia a existência de uma ligação entre a síntese de enzimas e seu metabolismo energético (ITO et al., 2011; BHUNIA et al., 2012).

O baixo teor de umidade utilizado na FES possibilita que esse tipo de fermentação só possa ser efetuado por um número limitado de micro-organismos, principalmente leveduras e fungos, embora algumas bactérias também viessem sendo utilizadas. Além disso, o mínimo teor de água presente inibe ou diminui o crescimento de bactérias e leveduras contaminantes (HÖLKER; LENZ, 2005; COUTO; SANROMÁN, 2006).

O tamanho do substrato também tem sido um fator importante para a produção da enzima pelo micro–organismo, já que o tamanho do mesmo influencia no crescimento microbiano, pois geralmente, partículas de substrato menores fornecem uma maior área de superfície para o micro–organismo, entretanto resulta em uma aglomeração do substrato, ocasionando num crescimento limitado. Por outro lado, partículas maiores propiciam uma melhor aeração e uma superfície limitada para o crescimento microbiano, estudos devem então ser feitos para ver essa relação e o melhor tamanho de partícula do substrato de acordo com o micro–organismo estudado (COUTO; SANROMÁN, 2006).

A FES também pode ser realizada principalmente por culturas mistas, que regulam internamente o crescimento e a formação de determinado produto, o que poderia ser difíceis de alcançar de outra forma ou utilizando uma única cultura (HÖLKER; LENZ, 2005; PUROHIT et al., 2006).

3.7 Resíduos agroindustriais utilizados na FES

Um critério importante para a produção de enzimas por FES é a seleção do substrato, pois com o aproveitamento dos mesmos, pode–se agregar valor a estes produtos e reduzir os custos de produção das enzimas (SILVA et al., 2009; NAVEENA; GOPINATH; et al., 2012b). A indústria de processamento de alimentos, por exemplo, gera uma grande quantidade de resíduos por ano em todo o mundo, causando um problema sério de deposição (GÓMEZ et al., 2005), vale ressaltar que é importante ao pensar em larga escala na produção de uma biomolécula, que o substrato utilizado em questão tenha um baixo custo e uma ampla disponibilidade (COUTO et al., 2006).

Vários substratos têm sido utilizados na FES como: bagaço de cana, resíduos de banana, raízes de canola, farelo de trigo, resíduos de uva, palha de trigo, madeira (IANDOLO et al., 2011), sabugo de milho (CHA et al., 2010), farelo de soja, casca de café (PANDEY et al., 2000), palma doce ou forrageira

(*Nopalea coccinellifera*) (CARVALHO et al., 2012), torta de babaçu (*Orbygnia* sp) (CASTRO et al., 2010), casca e farelo de arroz (SILVEIRA; FURLONG, 2007). A seleção dos componentes do meio é geralmente crítica para a produção fermentativa, sobretudo de enzimas fibrinolíticas (PENG et al., 2005). A utilização de resíduos de baixo custo pode reduzir o custo de produção das enzimas entre 40 a 60% (DHILLON et al., 2012). Além disso, a reciclagem dos resíduos agroindustriais reabastece os solos com nutrientes, sendo importante do ponto de vista ambiental e sustentável (VASSILEV et al., 2007).

Substratos à base de cereais, tais como farelo de arroz, farelo de trigo, flocos de aveia, descascados ou em pérolas de cevada, promovem uma fonte adequada de nutrientes para o crescimento de fungos por conter elevado teor de carbono, e níveis adequados de nitrogênio orgânico, servindo não só como fontes de nutrientes necessários ao crescimento do micro–organismo, mas também fornece compostos que podem induzir a produção de determinada biomolécula (HALTRICH et al., 1997; CERTIK; ADAMECHOVA, 2009).

Os farelos de arroz e de trigo são frequentemente utilizados como substrato, para a fermentação em estado sólido visando à produção de insumos para a indústria de alimentos, devido à sua abundância e serem ricos em nutrientes como proteínas, lipídios, vitaminas e compostos funcionais (COELHO et al., 1999; MOREIRA; FURLONG, 2007). Além de esses farelos serem pouco utilizados na alimentação humana, pela indisponibilidade biológica e características organolépticas pouco agradáveis (MOREIRA; FURLONG, 2007).

A soja é outra matéria-prima de custo acessível e apresenta em sua composição, importantes compostos químicos: lipídios, carboidratos, minerais, vitaminas, fibras, polissacarídeos, lecitina e isoflavonoides, além de ser uma rica fonte de proteína vegetal constituindo um excelente meio nutricional para o crescimento e desenvolvimento de micro-organismos ao longo da FES (AGRAWAL et al., 2004; PARIS, et al., 2012).

De acordo com SOCCOL & VANDENBERGUE (2003), o Brasil tem um potencial biotecnológico para utilizar seus resíduos agroindustriais, não só por ser um país agrícola que se destaca internacionalmente por suas exportações, mas também por existir grupos de pesquisa que geram grandes esforços no desenvolvimento de bioprocessos industriais, baseados na FES com expectativas que no futuro possam produzir pelo menos alguns dos produtos mais utilizados no mercado, tais como: enzimas, pigmentos, aromas, polissacarídeos, biopesticidas, ácidos orgânicos, entre outros, utilizando resíduos agro-industriais como substrato.

3.8 *Mucor subtillissimus*

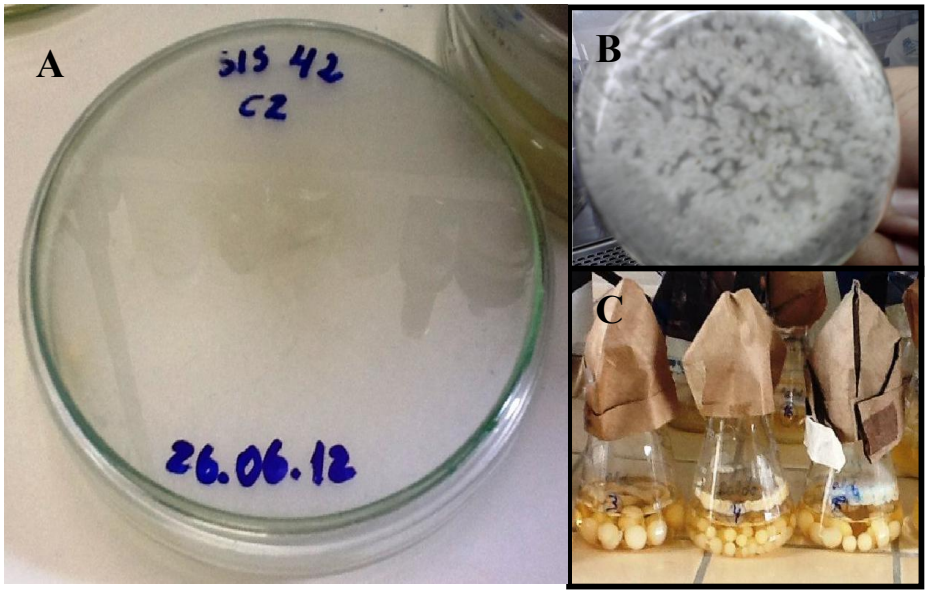
Os fungos filamentosos têm se destacado por serem ideais para a FES, uma vez que eles crescem na natureza em substratos sólidos tais como madeira, caule, raiz, folhas de plantas e na ausência de água livre, e também têm o poder de intrusão para penetrar profundamente nos espaços intercelulares, e intracelulares

da matriz do substrato para o melhor aproveitamento dos nutrientes presentes (MUKHERJEE; BANERJEE, 2011; BARRIOS-GONZÁLEZ, 2012). Dentre esses fungos filamentosos, o gênero *Mucor,* pode se apresentar sobre a forma leveduriforme ou podendo formar um pseudomicélio ou constituir hifas, que podem agrupar-se ou justapor-se (ESPOSITO; AZEVEDO, 2010; YIKE, 2011).

Estima-se que cerca de 60% das enzimas comercializadas, se originam a partir de fungos (ERJAVEC et al., 2012). Proteases extracelulares são facilmente produzidas e isoladas a partir de culturas fúngicas, sendo a expressão destas, reguladas em resposta a fonte de carbono, fonte de nitrogênio e o pH extracelular (YIKE, 2011). Representantes desse gênero *Mucor*, são utilizados em muitos países da Ásia e da África para a fermentação de alimentos à base de soja e outros produtos agrícolas ricos em lipídios (ZHANG et al., 2013).

A espécie *Mucor subtillissimus* pertence ao filo Zygomycota, classe Zygomycetes, ordem Mucorales, gênero *Mucor*. Essa ordem é um grupo filogeneticamente antigo de fungos, a qual pertence também os gêneros *Rhizopus*, *Rhizomucor*, *Lichtheimia corymbifera*, *Cunninghamella*, *Absidia* e *Blakeslea* (ESPOSITO; AZEVEDO, 2010; PHULPIN-WEIBEL et al., 2013). O reconhecimento de Mucorales pode ser feito facilmente, observando apenas as características macroscópicas, devido à formação de colônias de lã (Figura 4) com pequenos pontos (esporângio) que aparecem em alguns dias e bem distribuídos (SHARIFIA, 2007).

Figura 4. Crescimento do *Mucor subtillisssimus:* (A) *M. subtillisssimus* crescido em meio Czapek. (B) *M. subtillisssimus* crescidos na FES utilizando farelo de trigo como substrato, (C) *M. subtillisssimus* crescidos na FS.



O gênero *Mucor* contém cerca de 3000 espécies, que são amplamente distribuídas no solo, podendo ser isolados das superfícies de plantas e vegetais em decomposição, e em fezes de herbívoros, sendo um zygomycota dimórfico que pode alternar seu modo de crescimento entre leveduriforme e filamentoso

dependendo do estímulo ambiental, esses fungos desempenham um papel importante sendo responsáveis pela colonização primária do substrato (ALVES; TRUFEM, 2002; INOKUMA et al., 2013; KARIMI; ZAMANI, 2013). Além de apresentar um excelente crescimento em substratos à base de cereais (CERTÍK et al., 2013).

Os representantes de *Mucor* reproduzem-se, assexuadamente, por meio de aplanósporos formados internamente em esporângios e clamidósporos; Sexuadamente, pela fusão de dois gametângios geneticamente compatíveis, pertencentes ou não ao mesmo micélio e dando origem ao zigósporo (ALVES et al., 2002).

O gênero *Mucor* é utilizado biotecnologicamente na produção de ácidos orgânicos (INOKUMA et al., 2013), etanol (VINCHE et al., 2012). lipases (ALVES et al., 2002; ULKER; KARAOĞLU, 2012), proteases (SOMKUTI; BABEL, 1968; ESCOBAR; BARNETT, 1993; ALVES et al., 2005; LUIZ et al., 2008) e proteases coagulante do leite (AYHAN; TANYOLAC, 2001; SATHYA et al., 2010; YEGIN et al., 2011, 2012). Sendo o gênero *Mucor*, bons produtores de proteases aspárticas em culturas sólidas. (YEGIN et al., 2011).

Qualquer processo de produção de biomoléculas independente do tipo de fermentação requer uma fase de operação e produção do produto de interesse, e uma segunda fase relativa à recuperação e a purificação desse produto. Enzimas produzidas por fungos filamentosos, geralmente são produzidas de forma extracelular o que facilita a etapa do processo de recuperação (YEGIN et al., 2011).

3.9 Sistema de Duas Fases Aquosas

Métodos de purificação como o sistema de duas fases aquosas (SDFA) e cromatografias são as mais utilizadas e adequadas para a pré-purificação e separação adequada entre o produto e outros "contaminantes". As indústrias necessitam de etapas que propiciem uma afinidade mais rápida, e que seja eficaz e essencial para o desenvolvimento do produto (ERJAVEC et al., 2012). Neste sentido, o SDFA um tipo de extração liquido–liquido, que desde meados de 1950, iniciado por Albertsson (MONTEIRO – FILHO, 2010) vem sendo utilizado como alternativa aos métodos tradicionais de purificação.

O SDFA pode ser formado pela adição de dois (ou mais) polímeros, ou um polímero e uma solução salina especifica, que acima de certas concentrações críticas e temperaturas, resultam nas duas fases aquosas imiscíveis, onde a biomolécula migra preferencialmente para uma das fases enriquecidas com o polímero ou para o sal presente (MARINI et al., 2011; ASENJO; ANDREWS, 2012; SALES et al., 2013). Essa migração para uma determinada fase ocorre: dependendo do tamanho e/ou conformação da molécula, hidrofobicidade e das interações eletroquímicas e específicas feitas entra a molécula e o polímero (MARINI et al., 2011; ASENJO; ANDREWS, 2012).

Com relação ao particionamento de proteínas especificamente, os fatores que mais influenciam na sua migração para uma das fases são: a massa molar do PEG, a concentração do polímero presente no

SDFA, a resistência iônica do sal, temperatura, pH e hidrofobicidade (PERICIN et al., 2009; ASENJO; ANDREWS, 2012).

Dentre as vantagens do SDFA destacam-se: a clarificação simultânea, concentração e fracionamento de um extrato bruto, curto tempo de processamento, baixo consumo de energia, ambiente compatível para a biomolécula devido à alta quantidade de água presente no sistema (mais de 80% de água em ambas as fases), facilidade de ampliação de escala, e elevada área interfacial de contato de operação (YEGIN et al., 2011; RAJA et al., 2012; COELHO et al., 2013).

Diversos estudos mostram a utilização do SDFA, composto pelo polietilenoglicol (PEG) um polímero, que é utilizado em uma das fases e têm inúmeras vantagens em virtude da sua alta disponibilidade, seu baixo custo, propiciar a recuperação da atividade proteica, ser uma substância atóxica, e não causar distúrbios ao meio ambiente por ser biodegradável (SARMENTO et al., 1994). Além desse polímero, ter sido aprovado para o consumo interno pela FDA (Food and Drug Administration) (LI; KAO, 2003).

O sistema PEG/SAL permite uma maior seletividade, propriedades físicas favoráveis no que se refere a sua viscosidade, a sua rápida separação, diferença de densidade entre as fases, e um menor custo, sendo o fosfato, sulfato e citrato os sais comumente utilizados (XIE et al., 2011; PADILHA et al., 2011; RAJA et al., 2012).

Diversas biomoléculas e partículas podem ser particionadas pelo SDFA: proteínas, vírus, organelas, fragmentos de membrana (COELHO et al., 2013), metabólitos de cianobactérias (CHAVEZ-SANTOSCOY et al., 2010), lectinas (PORTO et al., 2011; SOARES et al., 2011), proteases queratinolíticas (BACH et al., 2012), colagenolíticas (LIMA et al., 2011), α-amylase (PORFIRI et al., 2011), ascorbato oxidase (PORTO et al., 2010), fitase (NEVES et al., 2012), lipase (SHOW et al., 2012) e enzimas fibrinolíticas (SALES et al., 2013; CHANG et al., 2012; MEDEIROS E SILVA et al., 2013) demonstrando a sua variedade de aplicações industriais.

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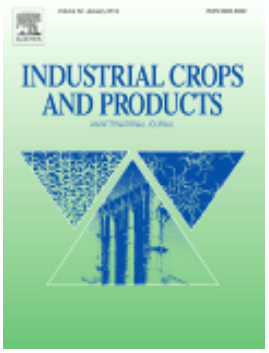
CAPÍTULO I



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PRODUCTION AND CHARACTERIZATION OF NEW FIBRINOLYTIC PROTEASE FROM *Mucor subtillissimus* SIS42 IN SOLID-STATE FERMENTATION

Impact factor – 2.468

PRODUCTION AND CHARACTERIZATION OF NEW FIBRINOLYTIC PROTEASE FROM *Mucor subtillissimus* SIS42 IN SOLID-STATE FERMENTATION

Thiago Pajeú Nascimento[1], Amanda Emmanuelle Sales[2], Camila Souza Porto[2], Romero Marcos Pedrosa Brandão[2], Galba Maria Campos Takaki[3], Tatiana Souza Porto[2,4], Ana Lúcia Figueiredo Porto[2,*]

[1]Center for Biological Sciences, Federal University of Pernambuco, 50670-901 Recife, PE, Brazil

[2]Department of Morphology and Animal Physiology, Federal Rural University of Pernambuco, 52171-900 Recife, PE, Brazil

3Center for Research in Environmental Sciences, Catholic University of Pernambuco, 50050-900 Recife, PE, Brazil

[4]Academic Unit of Garanhuns, Federal Rural University of Pernambuco, 55296-901 Garanhuns, PE, Brazil

[*]e-mail: analuporto@yahoo.com.br

ABSTRACT

Fibrinolytic enzymes have received attention owing to their medicinal potential for thrombolytic diseases these are becoming a leading cause of morbidity and mortality worldwide. Various natural enzymes purified from animal, plant and microbial sources have been extensively studied. The aim of our study was to produce fibrinolytic protease by *Rhizopus arrhizus* var. *arrhizus* SIS30 and *Mucor subtillissimus* SIS42 filamentous fungi species isolated from soil of Caatinga-PE, Brasil, by solid-state fermentation using agroindustrial residues: passion fruit peel, corncob, cassava peel, soybeans, *Malpighia emarginata* seed, wheat bran and citrus pulp. A 2^4 full factorial design was used to evaluate the main effects and interactions of the amount substrate, moisture and temperature over the fibrinolytic activity production and protease activity. The best producer of enzymes was *Mucor subtillissimus* SIS42 using 3g wheat bran, moisture (50%) at a temperature of 25°C for 72 hours with a fibrinolytic activity and activity protease of 144.58 U/mL and 48.33 U/mL respectively. This enzyme was very stable at 45ºC for 150 min. The optimum temperature was 45ºC. The protease activity was enhanced by K^+, Ca^+ and Mn^+, in opposition Cu^+ showed strong inhibition. The high specificity to chromogenic substrate and strongly inhibited by PMSF clearly indicates that is a chymotrypsin-like serine protease. According to these results, the enzyme seems to be an interesting alternative as a possible candidate for thrombolytic therapy.

Key words: *Mucor subtillissimus*, fibrinolytic protease, solid-state fermentation, thrombolytic activity.

1. INTRODUCTION

According to data provided by the World Health Organization (WHO) in 2000, heart diseases were responsible for 29% of the total mortality rate in the world. About 17.3 million people die each year of cardiovascular disease victims. It is estimated that in 2030 the number of deaths could reach 23.6 million (Mine et al., 2005). Cardiovascular diseases, including acute myocardial infarction, ischemic heart disease, valvular heart disease, peripheral vascular disease, arrhythmias, high blood pressure and stroke (Mukherjee et al., 2012; Simkhada et al., 2010). These problems are usually caused by accumulation of fibrin (blood clots) that adheres to the wall of the blood vessels leading to thrombosis (Montriwong et al., 2012).

Various thrombolytic agents have been used in the therapeutic treatment of thrombosis, but due to their high cost and hemorrhagic side effects, have been sought for new sources. Especially for fibrinolytic enzymes produced by microorganisms that have the potential to inhibit blood coagulation which are able to degrade the fibrin, beyond them are more advantageous in view of large scale production. Between microorganisms stand out bacteria of the genus *Bacillus*, cyanobacteria, fungi, and *Streptomyces* (Banerjee et al., 2013; Chang et al., 2012; Lu et al., 2010a, 2010b; Medeiros e Silva et al., 2013; Shirasaka et al., 2012).

Filamentous fungi have been shown to be a good choice for the production of fibrinolytic enzymes because they are good producers and their enzymes are easily extracted, since most of them are produced extracellular (Germano et al., 2003). The order Mucorales comprised the genus *Rhizopus*, *Mucor*, *Rhizo-Mucor*, *Cunninghamella* and *Absidia* are known in literature by demonstrate a potential for production of proteinases with milk clotting activity. The genus *Mucor* is known as producer of extracellular proteases, amylases and lipases (Yegin et al., 2011).

Among the types of fermentation for enzyme production by filamentous fungi the solid-state fermentation (SSF) is the most suitable since they the microrganisms grow in nature in solid substrates such as wood, stem, root, plant leaves, and in the absence of free water. The SSF is increasingly gaining more interest for enzyme production because it is a low-cost technology, easy recovery of biomolecules formed, and higher yields when compared to submerged fermentation. SSF is an alternative to the use of agro industrial substrates source avoiding impacts to environment, being an economically viable method and clean environmental point of view (Pandey et al., 2000; Banerjee et al., 2005; Certik et al., 2009).

Other advantages of SSF are the presence of phenomena that helps prevent negative influence the biosynthesis of enzymes, and cases of catabolite repression by glucose or inhibition of the biosynthesis of amino acids and ammonia (Bansal, et al., 2012; Soccol and Vandenberghe, 2003; Gombert et al., 1999; Yegin et al., 2011). The present study was evaluated the production of fibrinolytic protease by *Rhizopus arrhizus* var. *arrhizus* SIS30 and *Mucor subtillissimus* SIS42 in solid-state fermentation using agroindustrial residues.

2. MATERIAL AND METHODS

2.1 Microorganisms

In this study, was used two species of filamentous fungi *Rhizopus arrhizus* var. *arrhizus* SIS30 and *Mucor subtillissimus* SIS42 both isolated from soil Caatinga, Serra Talhada, PE-Brazil.

2.2 Medium for maintaining and sporulation

These strains were maintained periodically on Czapek medium at 7 days for sporulation. The sterilization of the medium was performed by autoclaving at 121°C, 1 atm pressure for 20 minutes. The strains were incubated in BOD (Body Oxygen Demand) at 30°C for 7 days.

2.3 Preparation of inoculum

The spores were collected by nutrient solution comprised of 0.5% (w/v) Yeast extract, 1% (w/v) Glucose, 0.01% Tween 80 (w/v); diluted in sodium phosphate buffer 245mM and pH 7.0 previously sterilized, the spores were counted in Neubauer chamber to a final concentration of 10^7 spores/mL. Erlenmeyer flasks 125mL were inoculated with fungal spores into the autoclaves substrates for producing of enzymes.

2.4 Selection substrates for the SSF

Rhizopus arrizus var. *arrizus* SIS30 and *Mucor subtillissimus* SIS42 were inoculated in Erlenmeyer flasks 125 mL containing 5g of substrates (moisture of 40%) in BOD at 30°C for 72 hours. The substrates used for were: passion fruit peel, corncob, cassava peel, soybeans, *Malpighia emarginata* seed, wheat bran and citrus pulp, sieved for whom possessed granulometry between 0.6 to 2.0mm. These substrates were dried in at 65°C until complete dehydration and then stored in plastic containers.

2.5 Production of fibrinolytic protease by SSF

The fermentations were carried out in Erlenmeyer flasks 150mL containing of different substrates autoclaved for 20 min at 121°C. These substrates were moistened as needed with nutrient solution and water to reach the desired moisture. To producing fibrinolytic proteases, was done using full factorial design (2^3) to determine the influence of the parameters: amount of substrate, moisture and temperature (Table A.1). After inoculation with microorganism, the flasks were incubated in BOD according to the factorial design.

Table A.1 - Levels of the variables studied in 2^3 complete factorial designs for production the fibrinolytic proteases.

Variables	Levels		
	Low (-1)	Central (0)	High (+1)
Substrate Amount (g)	3	5	7
Temperature (ºC)	25	30	35
Moisture (%)	30	40	50

2.6 Enzyme extraction

Extraction of the enzyme was performed after 72 hours of fermentation. Were added 7.5 ml of sodium phosphate buffer pH 7 (245mM) per 1g of substrate, these flasks were placed in an orbital shaker at 150 rpm for 90 min at room temperature. After this period, the contents were centrifuged at 3500 rpm for 10 min, and the supernatant was used for determination of the biochemical activity.

2.7 Protease activity

The determination of protease activity was done by the method of Ginther (1979). One units of enzyme activity was defined as the amount of enzyme that produced an increased in the optical density of 0.1 per hour at 440 nm. The experiments were done in duplicate.

2.8 Fibrinolytic activity

The fibrinolytic activity was determined using the spectrophotometric method described by Wang et al (2011). The supernatant diluted was collected and the absorbance at 275 nm was measured. In this assay, 1 unit (fibrin degradation unit, FU) of enzyme activity is defined as a 0.01 per–minute increase in absorbance at 275 nm of the reaction mixture.

2.9 Protein determination

The protein determination method was made by Bradford (1976) using Coomassie Brilliant Blue G-250 and bovine serum albumin as a standard. Each experiment was performed in triplicate and the average value was then calculated after correction of the corresponding blank.

2.10 Amidolytic activity

Amidolytic activity was measured using the synthetic substrates: (S7388 Sigma) N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide–Chymotripsin substrate, (G8148 Sigma) Gly-Arg-p-nitroanilide-dihydrochloride-urokinase and plasmin substrate. The determination method was described KIM et al. (1996).

2.11. Effect and stability temperature on protease activity

The temperature effect was determined by incubating the crude extract at temperatures ranging between 10 and 100°C for 60 min. To determine the stability to temperature, aliquots were withdrawn every 30 min for a period of 3 hours. These aliquots were submitted to the determination of protease activity.

2.12 Effect of metal ions on protease activity

The crude extract was evaluated in the presence of ions. These ions are described as inhibitors or activators of protease activity. The effect of ionic solutions was evaluated at a concentration of 2.5mM; 5mM and 10mM. The crude extract was exposed to the following ions: zinc (Zn^+), magnesium (Mg^+), copper (Cu^+), ferrous (Fe^+), calcium (Ca^+), magnesium (Mg^+), sodium (Na^+), potassium (K^+), and cobalt (Co^+)·and incubated at 37°C for 60 min. The ions were dissolved in Tris–HCl pH 7.75 with 0.15 M NaCl.

2.13 Effect of inhibitors in protease activity

To evaluate the effect of inhibitors on enzyme activity, the crude extract was exposed to the following inhibitors: PMSF (fluoride-methylphenylsulfonyl $C_7H_7FO_2S$), 2-mercaptoethanol (2-hydroxy-1-ethanethiol-C_2H_6SO), ethylenediaminetetraacetic acid (EDTA-acetic-$C_{10}H_{16}N_2O_8$), Pepstatin A and Iodoacetic Acid. These inhibitors were incubated for 60 min at 37°C, they were dissolved in according to the protocol provided by Sigma, and the concentration of the solutions was standardized at 5mM.

2.14 Statistical analysis

The effects were evaluated by an analysis of variance with a significance level of 95% to make estimates of the main and second-order effects a linear, absolute value of the factors in relation to the response variables studied. Statistical analysis of the experimental design was performed using the software Statistical 8.0 (Statsoft Inc., Tulsa, OK, USA).

3. RESULTS AND DISCUSSION

3.1 Selection of substrate for production of fibrinolytic protease

The production of fibrinolytic protease by *Rhizopus arrizus* var. *arrizus* SIS30 and *Mucor subtillissimus* SIS42 was carried out using 7 agroindustrial residues used as substrates, where not showed growths of the strains when used does: passion fruit peel, citrus pulp and *Malpighia emarginata* seed. This phenomenon may be due low rates of protein present in these substrates, which can be seen in the studies presented by Oliveira et al. (2002), Aguiar et al (2010); Henrique et al. (2003). Already the substrates that have higher protein content in their constitution as soybeans and wheat bran were those with a higher protease production from fungi studied, as can be seen in Table A.2. In runs were obtained fibrinolytic

activity of 47 U/mL using soybeans and 78 U/mL using wheat bran by *Mucor subtillissimus* SIS42 and the strain *Rhizopus arrhizus* var. *arrhizus* SIS30 was obtained 39 U/mL and 58 U/mL when grown in wheat bran and soybean respectively.

The maximum protease production was observed with wheat bran used as the substrate. Similar to studies by Ravikumar et al. (2012) that for to produce protease, using SSF from *Pleurotus sajor-caju* used different products agricultural, and showed that of all the substrates tested, wheat bran was the best substrate for the production of protease obtained 35 U/mL.

Table A.2. Production of fibrinolytic proteases by *Rhizopus arrizus* var. *arrizus* SIS30 and *Mucor subtillissimus* SIS42 using different substrates in SSF.

Substrates	FA (U/mL)		U/gdb (FA)		PA (U/mL)	
	Mucor	*Rhizopus*	*Mucor*	*Rhizopus*	*Mucor*	*Rhizopus*
Corncob	10.81	10.47	162.15	157.05	9.87	11.76
Cassava peel	18.24	16.42	273.60	246.3	16.10	14.54
Soybean	47.00	39.03	705.01	585.06	27.06	23.87
Wheat Bran	78.00	58.05	1170.05	870.07	38.08	30.77

FA - Fibrinolytic Activity; U/gdb - Unit gram of the dry basis; PA – Protease activity

3.2 Production of fibrinolytic protease by SSF

Considering the results presented for production fibrinolytic protease using wheat bran as substrate from *M. subtillissimus* SIS42 were performed factorial designs 2^3 according to the variables shown in Table A.1 using wheat bran as substrate. One of the advantages of using wheat bran as the substrate, for SSF is that these grains are rich in nutrients such as proteins, lipids, vitamins and functional compounds, and are not used as food, due the unavailability biological and organoleptic unpleasant (Silveira and Furlong., 2007).

Besides, wheat bran serves as sources of nutrients necessary for microorganism growth, and also provides compounds, which can induce the production of determined biomolecules (Haltrich et al., 1996 Certik; Adamechiva, 2009). Studies by Alves (2002, 2005) shown protease production by genus *Mucor*, where the first work was about the production of proteases by 12 species studied, and the largest producer was *Mucor racemosus* Fres. f. *chibinensis*. In the second study, of 56 isolates of *Mucor* starting from dung of herbivores, 82% showed production of enzymes with protease activity. Sathya (2009) also obtained extracellular protease produced by *Mucor circinelloides* using agroindustrial residues through SSF.

Determination of activity protease and fibrinolytic activity from *M. subtillissimus* SIS42 was performed with 72, 96 and 120 hours of fermentation. The highest values of enzyme production occurred

with 72 hours of fermentation using wheat bran as substrate. Soares et al. (2013) using wheat bran as substrate for protease production by the nematophagous fungus *Monacrosporium sinense* (SF53) by solid-state fermentation, obtained values of activity of 38.0 U/mL at 211 hours of incubation time. These results were presented below in this work, but, obtained with less fermentation time (72 hours) and the protease activity of 48.33 U/mL.

Table A.3 shows the values of the protease activity and fibrinolytic activity using factorial design, the best conditions for the production of the enzyme was 3g substrate amount, 50% moisture and temperature of 25°C. Some authors have reported that enzyme production by *Mucor* spp. in SSF using wheat bran mixture was optimal cultivation at 30°C (Yegin et al., 2011; Tubesha; Al-Delaimy, 2003). Studies by Agrawal et al. (2004) shows that most fungi have an optimum temperature of protease production by SSF between 28°C to 30°C, unlike the optimal conditions obtained by our studies that show a better enzyme production at temperature of 25°C.

Table A.3. Production of fibrinolytic proteases by *Mucor subtillissimus* SIS42 using 2^3 factorial design.

	Variables			Wheat Bran			
Runs	Substrate amount (g)	Moisture (%)	Temperature (ºC)	PA (U/mL)	U/gds	FA (U/mL)	U/gds
1	3	30	25	36.20	440.74	106.66	799.95
2	7	30	25	27.23	316.62	84.16	631.20
3	3	50	25	48.33	801.93	144.58	1084.35
4	7	50	25	47.90	644.97	84.58	634.35
5	3	30	35	26.26	223.29	38.33	287.47
6	7	30	35	23.36	218.72	75.83	568.72
7	3	50	35	45.83	668.72	90.83	681.22
8	7	50	35	38.66	569.64	32.08	240.60
9 C	5	40	30	35.56	456.67	79.16	593.70
10 C	5	40	30	36.20	465.69	80.41	603.07
11 C	5	40	30	22.43	253.73	80.00	600.00
12 C	5	40	30	29.16	367.56	80.83	606.22

U/gdb - Unit gram of the dry basis; PA –Protease activity; FA – Fibrinolytic activity; C – Central Points

The statistical analysis showed that all variables were significant (Fig. A.1). However, the variable temperature was more statically significant showing a negative effect. The decrease of the temperature indicated the greater of the enzyme production by microorganism. The temperature is essential in regulate and in the enzyme synthesis (Calik et al. 2001; Ito et al., 2011; Bhunia et al., 2012).

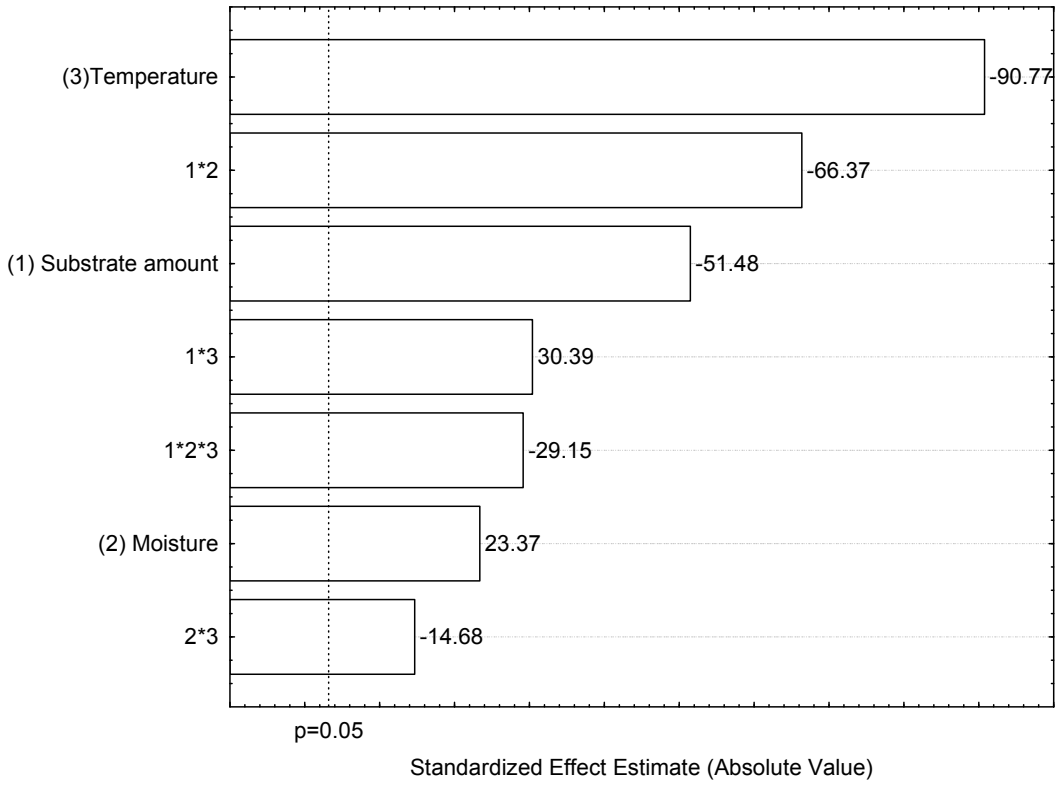

Fig.A.1. Pareto chart of variables effects in fibrinolytic activity (U/mL). PA – Protease activity

However, the moisture demonstrated a positive effect (Fig. A.1). The high level moisture tends to increased enzyme production, may be due a rise the substrate porosity, consequently a better oxygen transfer favoring the development of microorganism (Delabona et al., 2013). In this work, the moisture presented (50%) was found in accordance with that reported by Chutmanop et al. (2008) that used to produce proteases by *Aspergillus oryzae* (Ozykat-1) using wheat bran and rice bran as substrate. Thanapimmetha et al. (2011) also showed similar results, the data indicated that the optimum moisture content for the protease production in SSF in this study was at 45% in optimization of protease production in solid-state fermentation using *Jatropha curcas* as residue.

3.3 Amidolytic activity

The crude extract produced by *M. subtilissimus* exhibited highest activity against N-succinyl-Ala-Ala-Pro-Phe-pNa, a substrate for chymotrypsin, suggesting that it is a chymotrypsin-like protease. Sugimato et al. (2007) using specie of *Fusarium* sp. BLB to produce a protease with fibrinolytic activity was also characterized as serine protease. Similar results were presented by *Cordiceps militaris* (Choi et al., 2011), *Perennipiria fraxinea* (KIM et al., 2008) and *Armillaria mella* (LEE et al., 2005).

3.4 Effect and stability to temperature on activity protease

The temperature is one of the most critical parameters that have to be controlled in bioprocess (Sathya et al., 2009). As shown in Fig. A.2 *M. subtillissimus* SIS42 exhibited an optimum temperature of the enzyme protease at 45ºC. Proteases from fungi show similar results for optimum temperature such as 45ºC for *Colletotrichum gloeosporioides* (Dunaevsky et al., 2007), *Rhizopus chinenesis* 12 (Kirk et al. 2001), *Schizophyllum commune* (Lu et al. 2010a) and *Myceliophthora* sp. (Zanphorlin et al., 2011), 50ºC for *Trichoderma reesei* QM9414 (Dienes et al., 2007) and *Fusarium culmorum* (Pekkarinen et al., 2002).

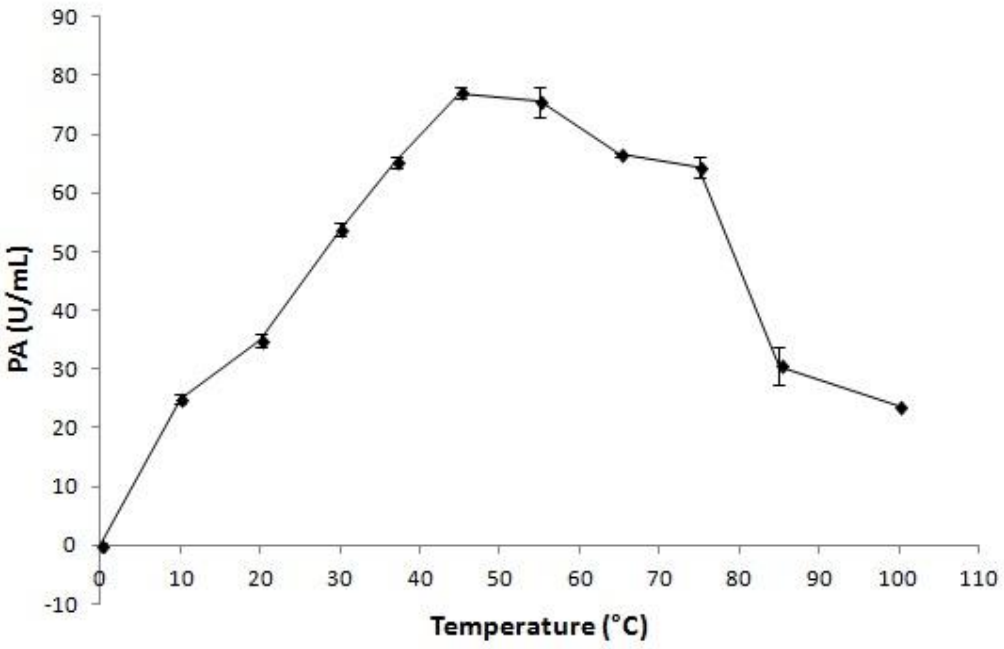

Fig. A.2. Optimum temperature of protease produced by *Mucor subtillissimus* SIS42.
PA – Protease activity

The protease from *M. subtillissimus* SIS42 *M.* retained 75.52% its original activity after submit to 45°C for 120 min (Fig.A.3), it was more stable than the enzyme reported by Zanphorlin et al. (2010) where this enzyme in SSF maintained 95% of the maximum activity at 60°C. No activity protease was detected after heating the enzyme at 100°C for 15 min.

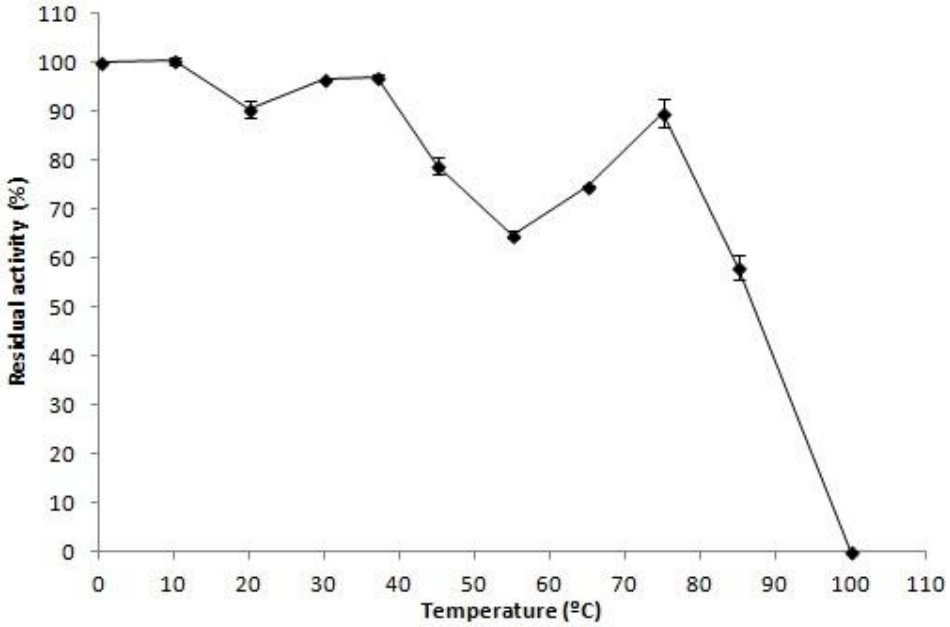

Fig. A.3. Stability of protease by *Mucor subtillissimus* SIS42 at 45°C after 120 min.

3.5 Effect of metal ions on protease activity

It was found that the addition of ions K^{+}, Mn^{2+}, Ca^{2+}, increased protease activity at all concentrations used, while the reduction was observed when Zn^{2+},Co^{2+} were added at concentrations of 5 mM and 10 mM already the addition of Cu^{2+} ions had reduced enzyme activity (Table A.4). Similar results were obtained by Ravikumar et al. (2012), where the protease from *Pleurotus sajor-caju* enhanced the enzyme activity with addition the Ca^{2+}.

The protease isolated from *Ulocladium botrytis* by solid-state fermentation from Heba et al. (2012) were inhibited in the presence of Zn^{+} or Cu^{2+} and increased significantly in the presence of Ca^{2+},Mg^{2+}, and K^{+}. This increase of protease activity by metallic ions was due the binding link between enzyme and substrate, combining with both and so holding the substrate in the active site of the enzyme. Also can be due to changes in electrostatic, bonding that would change the structure of enzymes (Roy et al. 1990; Heba et al., 2012).

Table A.4. Effect of metal ions on protease activity

Ions	Residual activity (%)		
	2.5 mM	5 mM	10 mM
Control	100.0	100.0	100.0
K^+	130.0	133.9	106.7
Ca^+	123.1	124.8	116.1
Mn^+	109.3	104.3	110.4
Zn^+	108.4	98.7	85.8
Mg^+	103.8	102.9	96.5
Co+	101.1	91.9	84.5
Cu^+	85.7	78.2	64.5
Fe^+	101.3	80.7	107.1
Na^+	89.8	94.4	115.7

3.6 Effect of inhibitors in protease activity

The enzyme activity was significantly inhibited by PMSF (81%) and not by any other inhibitor (Table A.5). These results demonstrated that is a serine protease. Corroborating the results presented, by Germano et al. (2003) that using *Penicillium* sp. for protease production by SSF had 93% of the protease activity inhibited by PMSF. Zanphorlin et al. (2011) also obtained through of SSF by thermophilic fungus *Myceliophthora* sp an inhibited of protease by PMSF (100%) indicating that is it a serine-protease.

Table A.5. Effect of inhibitors on protease activity.

Inhibitors Control	Residual activity (%)
Control	100.0
EDTA	90.9
β-Mercaptoethanol	93.4
PMSF	18.4
Pepstatin A	98.9
Iodoacetic Acid	97.6

4. CONCLUSION

In conclusion, a new protease was produced by *Mucor subtillissimus* SIS42 using wheat bran as substrate in SSF. Inhibition studies and high specificity to chromogenic substrate clearly indicate that is a chymotrypsin-like serine protease. The enzyme exhibited stability over a wide range of temperature. Further studies will be made to the purification of the enzyme.

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CAPÍTULO II

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ARTIGO SERÁ SUBMETIDO – SEPARATION AND PURIFICATION TECHNOLOGY

PURIFICANTION OF FIBRINOLYTIC PROTEASE FROM *Mucor subtilissimus* SIS42 USING AQUEOUS TWO-PHASE SYSTEMS (PEG/ SULFATE)

Impact factor – 2.894

PURIFICANTION OF FIBRINOLYTIC PROTEASE FROM *Mucor subtillissimus* SIS42 USING AQUEOUS TWO-PHASE SYSTEMS (PEG/SULFATE)

Thiago Pajeú Nascimento[1], Amanda Emmanuelle Sales[2], Camila Souza Porto[2], Romero Marcos Pedrosa Brandão[2], Galba Maria Campos Takaki[3], Tatiana Souza Porto[4], Ana Lúcia Figueiredo Porto[2,*]

[1]Center for Biological Sciences, Federal University of Pernambuco, Cidade Universitária, Recife, Pernambuco, 50670-901, Brazil.

[2]Animal Morphology and Physiology Department, Federal Rural University of Pernambuco, Dois Irmãos, Recife, Pernambuco, 52171-900, Brazil.

[3]Nucleus of Research in Environmental Sciences (NPCIAMB), Catholic University of Pernambuco, Boa Vista, 50050-590 Recife, PE, Brazil.

[4]Academic Unit of Garanhuns, Federal Rural University of Pernambuco, Garanhuns, Pernambuco, 55292-901, Brazil.

[*]e-mail: analuporto@yahoo.com.br

ABSTRACT

A fibrinolytic protease was produced by *Mucor subtillissimus* SIS42 in solid-state fermentation. The partitioning in aqueous two-phase systems was composed of polyethylene glycol (PEG) and sodium sulfate. The influence of PEG molar mass, PEG concentration, sulfate concentration, on partition coefficient, yield and purification were investigated. A central composite rotary design was applied to optimize the purification. The best purification conditions (PF=10) and yields (102%) were achieved using an ATPS composed by 30.00% (w/w) PEG 6000 g/mol, 13.20% (w/w) Na_2SO_4. SDS–PAGE and fibrin zymography showed the molecular mass of the purified enzyme (94kDa). Among the synthetic substrates, the most sensitive was N-succinyl-Ala-Ala-Pro-Phe-qNA, and PMSF almost completely inhibited the purified enzyme activity. These results indicated that the purified fibrinolytic protease was a chymotrypsin-like serine protease.

Key words: *Mucor subtillissimus*, fibrinolytic protease, ATPS.

1. INTRODUCTION

The *Mucor subtilissimus* is a Zygomycota dimorphic, which can switch between yeast and filamentous growth modes depending on environmental stimuli received [1]. This fungus belongs to the order Mucorales further comprising genus, *Rhizopus Mucor*, *Rhizomucor*, *Cunninghamella* and *Absidia* known in the literature to demonstrate a potential for producing of protease milk clotting and the production of lipases, amylases and extracellular proteases [2].

Protease constitutes a large and complex group of enzyme, which plays an important nutritional and regulatory role in nature [3]. Those enzymes are considered chemically bio- supporting products, being the most important group industrial enzyme with a great variety of industrial and biotechnological applications accounting for about 60% of the total enzyme market in the world and 40% of the total worldwide enzyme sale [3, 4, 5]. Among these enzymes stand out fibrinolytic protease by being able to degrade fibrin, the major protein component of blood clots, the accumulation of this protein leads to thrombosis responsible for cardiovascular disease including myocardial infarction [6]. In recent years, several studies have been made to search for new sources of thrombolytic agents such as bacteria, algae, plants, worms, snake venom, insects, and fungi [6, 7, 8, 9, 10]. Filamentous fungi have been shown to be a good choice for the production of fibrinolytic enzymes because they are good producers and their enzymes are easily extracted, since most of them are produced extracellular [11].

Partitioning in aqueous two-phase system (ATPS) is a good method, which has proved to be a valuable tool for separating and purifying mixtures of biomolecules [12, 13, 14, 15] since many traditional methods generate chemical pollutants impairing the environment and are so difficult large-scale production. Therefore traditional techniques as chromatography and ammonium sulfate precipitation are slowly being replaced by cheaper methods of purification and a lower cost of production and operation. The ATPS have been shown to be a promising alternative to pre purifications, especially proteases [6, 12, 16, 17].

Aiming to improve the conditions of purification of these enzymes reducing costs and considerable amount of work and time some methods have been used, as the response surface methodology (RSM). This is a collection of mathematical and statistical techniques that are useful for modeling and analysis in applications where a response of interest (or output) is influenced by several factors. The objective of RSM is to optimize this response [18,19,20] which includes experimental design, model fitting, validation and optimization condition involving a minimum number of experiments for a large number of factors in improving in production or purification enzyme [8,21].

The objective of this study was to examine the optimal operation conditions of the purification process in order to maximize the purification of fibrinolytic protease from *Mucor subtilissimus* SIS 42 by ATPS by response surface methodology (RSM).

2. MATERIAL AND METHODS

2.1 Microorganisms

The filamentous fungi *Mucor subtillissimus* SIS42 isolated from soil Caatinga, PE-Brazil was used in this study.

2.2 Medium for maintaining and sporulation

This strain was sporulated, maintained periodically on Czapek medium for 7 days. The sterilization of the medium was performed by autoclaving at 121°C, 1 atm pressure for 20 minutes. The strain was incubated in BOD (Body Oxygen Demand) at 30°C for 7 days.

2.3 Preparation of inoculum

The spores were collected by nutrient solution comprised of 0.5% (w/v) Yeast extract, 1% (w/v) Glucose, 0.01% Tween 80 (w/v); diluted in sodium phosphate buffer 245mM and pH 7.0 previously sterilized, the spores were counted in Neubauer chamber to a final concentration of 10^7 spores/mL. Erlenmeyer flasks 125mL were inoculated with fungal spores into the autoclaves substrates for producing of enzymes.

2.4 Production of fibrinolytic proteases by solid-state fermentation (SSF)

The spore solution of *Mucor subtillissimus* SIS42 was inoculated, in Erlenmeyer flasks 125 mL containing 3g of wheat bran as substrates, moisture (50%) and incubated in BOD at 25°C for 72 hours.

2.5 Enzyme extraction

The extraction of the enzyme was performed after 72 hours of fermentation, were added 7.5 ml of sodium phosphate buffer pH 7 (245mM) per 1g of substrate, these flasks were placed in an orbital shaker at 150 rpm for 90 min at room temperature. After this period, the contents were centrifuged at 3500 rpm for 10 min, and the supernatant was used for determination of the biochemical activity.

2.6 Fibrinolytic activity

The fibrinolytic activity was determined using the spectrophotometric method described by Wang (2011) [23]. Briefly, 0.4 mL of 0.72% fibrinogen was placed in a test tube with 0.1 mL of 245mM phosphate buffer pH 7 and incubated at 37°C for 10 min, 0.1 mL of diluted enzyme solution was added, and incubation continued at 37°C. This solution was again mixed after 20 and 40 min. At 60 min, 0.7 mL of 0.2M trichloroacetic acid was added, and mixed. The reaction mixture was centrifuged at 15,000 xg for 10 min. Then, 1 mL of the supernatant was collected and the absorbance at 275 nm was measured. In this assay, 1 unit (fibrin degradation unit, FU) of enzyme activity is defined as a 0.01 per minute increase in

absorbance at 275 nm of the reaction solution. Each experiment was performed in triplicate and the average value was then calculated after correction of the corresponding blank.

2.7 Protein determination

The protein determination method was performed by Bradford (1976) [24], using Coomassie Brilliant Blue G-250 and Bovine Serum Albumin. Each experiment was performed in triplicate and the average value was then calculated after correction of the corresponding blank.

2.8 Binodal curve

The PEG concentrated solution (400, 4000 and 8000g/mol) was 30% (w/v) and it was prepared by dissolving PEG in distilled water. The sodium sulfate (Na_2SO_4) solution was 30% (w/v) and it was prepared by dissolving the salt in distilled water. The concentrations of binodal curves were determined by mixing appropriate volumes of sodium sulfate with PEG using the titration method described by Albertsson [25].

2.9 Preparation of the aqueous two-phase systems (ATPS)

Aqueous two-phase systems (ATPS) were prepared in 15 mL graduated tubes with different concentrations of sulfate sodium salts mixture solutions and 50% (w/w) PEG solutions at 25ºC±2 according to statistical design described in Table 1. The amount of 2g crude extract and water was added to a final amount of 10g with pH 6.5 at 37ºC. After addition of all components of the system and vortex shaking for 1.0 min, the two-phases were separated by settling for 60 min. Then, the top and bottom phases were measured and analyzed separately. The both phases were assayed for protein and fibrinolytic activity determinations.

Table 1. Experimental design 2^3 for fibrinolytic protease purification using PEG/Sodium sulfate (ATPS).

Variables	Levels		
	Low (-1)	Central (0)	High (+1)
M_{PEG} (g/mol)	4000	6000	8000
C_{PEG} (% w/w)	18	24	30
$C_{Na_2SO_4}$ (% w/w)	15	20	25

M_{PEG} – PEG molar mass; C_{PEG} – PEG concentration; $C_{Na_2SO_4}$ – Sulfate sodium concentration

2.10 Factorial design

The influence of the variables PEG molar mass (M_{PEG}), PEG concentration (C_{PEG}), and sodium sulfate concentration ($C_{Na_2SO_4}$) on the response variables, partition coefficient (K), yield (Y) and purification factor (PF) were analyzed according to factorial design 2^3, with four replications at the central points

(Table 1) to allow estimation of pure experimental error [26]. The values selected for these variables were chosen according to the binodal curves (Figure 1).

2.11 Determination of partition coefficient and yield in ATPS

The activity partition coefficient of fibrinolytic proteases is calculated as the ratio of the fibrinolytic activity in the top phase to that of the bottom phase Eq. (1). Where, FA is the fibrinolytic activity (U/mL). The subscripts "t" and "b" represent the top and bottom phases, respectively.

$$K_{FA} = \frac{FA_t}{FA_b}$$

The activity yield (Y_{FA}) was determined as the ratio of total activity in the top phase or bottom phase to that in initial fibrinolytic proteases solution and expressed as percentage Eq.(2). Where FA_t or FA_b, are the fibrinolytic activity in the top phase and bottom phase, respectively and FA_i initial fibrinolytic proteases solution.

$$Y_{FA} = \left(\frac{FA_t . V_t}{FA_i . V_i} \right) \times 100$$

The purification factor (PF) was calculated as the ratio of the specific activity (U/mg) in the top phase (SA_t) and bottom phase (SA_b) to the specific activity (U/mg) of the initial crude extract (SA_i) Eq. (3).

$$PF = \frac{SA_t}{SA_b}$$

2.12 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a 12% polyacrylamide running gel according to the method of Laemmli [27]. The molecular mass was calibrated using a molecular mass marker (Low-Range Rainbow Molecular Weight Markers - GE Healthcare) as a standard. Protein bands were detected by staining with Coomassie brilliant blue R-250.

2.13 Fibrin zymography

Fibrinolytic activity was analyzed using a fibrin zymography gel. Fibrin zymography was carried out as described by Kim et al. [28]. Fibrinogen and thrombin were mixed with 12% polyacrylamide gel solution, and the mixture was electrophoresis on a fibrin gel. The molecular mass was calibrated with a molecular mass marker as a standard. After electrophoresis, the gel was washed with 2.5% Triton X-100

for 1 h, rinsed three times with distilled water, and incubated in reaction buffer (0.1 M glycine, pH 8.4) at 37ºC for 18 h. The staining and distaining procedures were similar to those of SDS-PAGE.

2.14 Effect of inhibitors in protease activity

To evaluate the effect of inhibitors on enzyme activity, the purified enzyme was exposed to the following inhibitors: PMSF (fluoride-methylphenylsulfonyl $C_7H_7FO_2S$), 2-mercaptoethanol (2-hydroxy-1-ethanethiol-C_2H_6SO), ethylenediaminetetraacetic acid (EDTA-acetic-$C_{10}H_{16}N_2O_8$), Pepstatin A and Iodoacetic Acid. The inhibitors were incubated for 60 min at 37°C, they were dissolved in according to the protocol provided by Sigma, and the concentration of the solutions was standardized at 5mM.

2.15 Amidolytic activity

Amidolytic activity was measured using the synthetic substrates: (S7388 Sigma) N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide–Chymotripsin substrate, (G8148 Sigma) Gly-Arg-p-nitroanilide-dihydrochloride-urokinase and plasmin substrate. The determination method was descrited KIM et al. (1996) [29].

2.16 Response surface methodology

To establish the optimum conditions for purification of fibrinolytic protease in ATPS, response surface methodology (RSM) was used. A two-factor central composite design was applied. The variables chosen were concentration levels of PEG 6000 g/mol and the concentration of Na_2SO_4. The design was centered on the variable values where the highest purification factor of fibrinolytic activity was previously achieved based on design factorial 2^3 ATPS. The values were coded according to the chosen design . The coded values for the independent variables were -1.4142 (lowest level), -1, 0, +1, and +1.4142 (highest level) (Table 2).

The complete design consisted of 12 experimental points which included four replications at the central point. The 12 samples were prepared in random order. In each experiment, the following parameters were calculated: fibrinolytic activity in PEG phase (FA/mL), fibrinolytic activity in Na_2SO_4 phase (FA/mL), partition coefficient, activity yield (top and bottom phase) and purification factor (top and bottom phase).

2.17 Statistical analysis

The effects were evaluated by an variance analysis with a significance level of 95% to make estimates of the main and second-order effects a linear, absolute value of the factors in relation to the response variables studied. Statistical analysis of the experimental design was performed using the software Statistical 8.0 [22].

Table 2. Response surface design used for optimizing the purification of fibrinolytic protease in PEG 6000/Sodium sulfate two-phase system.

Variables	Range and levels				
	-1.4142	-1	0	+1	+1.4142
$C_{PEG6000}$(% w/w)	15.60	18.00	24.00	30.00	32.40
C_{Na2SO4} (% w/w)	9.36	10.00	11.6	13.20	13.84

C_{PEG} – PEG concentration; C_{Na2SO4} – Sulfate sodium concentration.

3 RESULTS AND DISCUSSION

3.1 Binodal curve (PEG/Sodium sulfate)

For purification using ATPS, binodal curves were constructed to obtain the minimum concentration of polymer and salt needed to form an ATPS, where were established using the methodology proposed by Albertsson [16].

The binodal curve of PEG 4000, 6000 and 8000 - sodium sulfate is shown in Figure 1, where the top phase contained 46.5% (w/w) PEG 4000 (g/mol) and the bottom phase of the system contained 10.3% (w/w) of sodium sulfate.

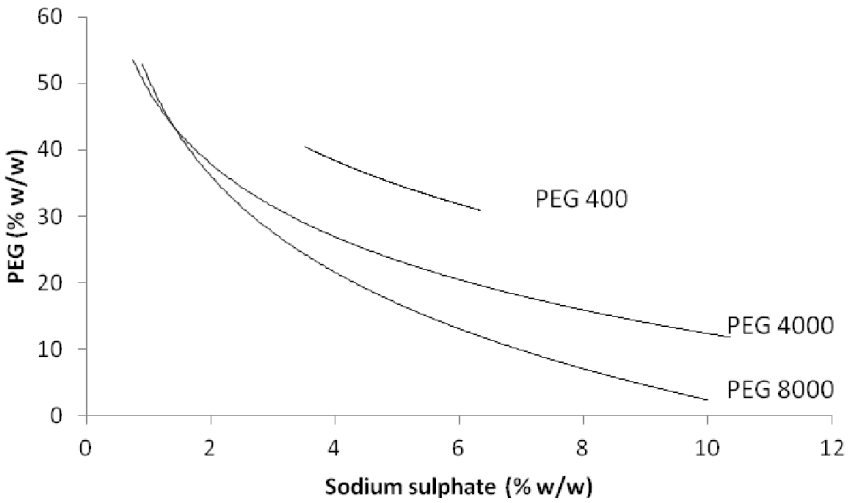

Figure 1. Binodal curve for the system PEG/Sodium sulfate pH 6.5 with water in different PEG molar mass and temperature at 27°C ± 2°C.

3.2 ATPS in the partition coefficient fibrinolytic protease.

The experimental results for the partition coefficient (K) the fibrinolytic protease in APTS using PEG/Na_2SO_4 are presented in Table 3. The most runs of the factorial design occurred enzyme partitioning

to the bottom phase (K_{FA} ranged 0.26 to 0.86). This partition of fibrinolytic protease was preferentially to the bottom phase (salt-rich phase) according to the studies Sales et al. [31] that evaluated the partition of fibrinolytic proteases by the integrated process of production and purification from *Bacillus* sp. UFPEDA 485 and in all assays, the enzyme preferentially partitioned to the bottom phase.

The maximum values of the partition coefficient (K_{FA}) were 2.32 and 2.25 observed in runs 7 and 8, respectively. This increase in the partition coefficient the fibrinolytic protease indicates that these runs migrated to the top phase, which was more hydrophobic due to the predominance of PEG, causing a decreased affinity of the enzyme for salt phase [12, 30]. Such phenomenon may be explained by the effect of salting out, where in the biomolecules was directed to another stage in consequence of the large amount of salt in the bottom phase [6, 30].

The parameter C_{PEG} was the most contributed for the partition of the enzyme. A positive effect of C_{PEG}, C_{Na2SO4} and lower for M_{PEG} was observed in Figure 1. Medeiros e Silva et al. [6] also evaluated the partition of fibrinolytic proteases from *Streptomyces* sp. DPUA1576 using PEG–phosphate salts in ATPS displayed positive (C_{PEG}) and negative (M_{PEG}) effects, indicating that the increase on C_{PEG} and a decrease on M_{PEG} causes an improvement on enzyme partition to top PEG rich phase.

Table 3. Results of full factorial design (2^3) the fibrinolytic protease partition.

Runs	M_{PEG} (g/mol)	C_{PEG} (% w/w)	C_{Na2SO4} (% w/w)	FA^t (U/mL)	FA^b (U/mL)	K_{FA}	$Y_{FA}{}^t$	$Y_{FA}{}^b$	PF^t	PF^b
1	4000	18	10	11.66	44.16	0.26	18.96	73.31	1.05	0.89
2	8000	18	10	24.16	47.08	0.51	35.10	81.41	1.59	0.96
3	4000	30	10	19.16	9.58	2.00	40.43	9.61	1.03	0.81
4	8000	30	10	12.08	15.83	0.76	25.07	16.42	0.58	1.39
5	4000	18	13.2	20.83	28.75	0.72	28.81	49.71	1.74	0.68
6	8000	18	13.2	14.58	37.50	0.38	20.17	64.84	0.73	1.16
7	4000	30	13.2	17.50	15.0	2.32	169.13	34.97	3.50	2.03
8	8000	30	13.2	11.25	5.0	2.25	22.56	5.18	0.68	0.63
9 (C)	6000	24	11.6	23.75	57.08	0.81	128.71	130.36	2.55	3.21
10 (C)	6000	24	11.6	20.83	52.91	0.83	121.30	124.50	2.61	3.68
11 (C)	6000	24	11.6	20.41	57.08	0.59	70.20	106.51	1.59	3.29
12 (C)	6000	24	11.6	22.08	55.00	0.56	78.86	115.62	2.05	4.51

M_{PEG} – PEG molar mass; C_{PEG} – PEG concentration; C_{Na2SO4} – Sulfate sodium concentration; K_{FA} - Partition coefficient of fibrinolytic proteases; $Y_{FA}{}^t$ - Activity yield in the top phase; $Y_{FA}{}^b$ - Activity yield in the bottom phase; PF^t - Purification factor in the top phase; PF^b - Purification factor in the bottom phase; FA^t - Fibrinolytic activity in the top phase; FA^b - Fibrinolytic activity in the bottom phase; (C) - Central points.

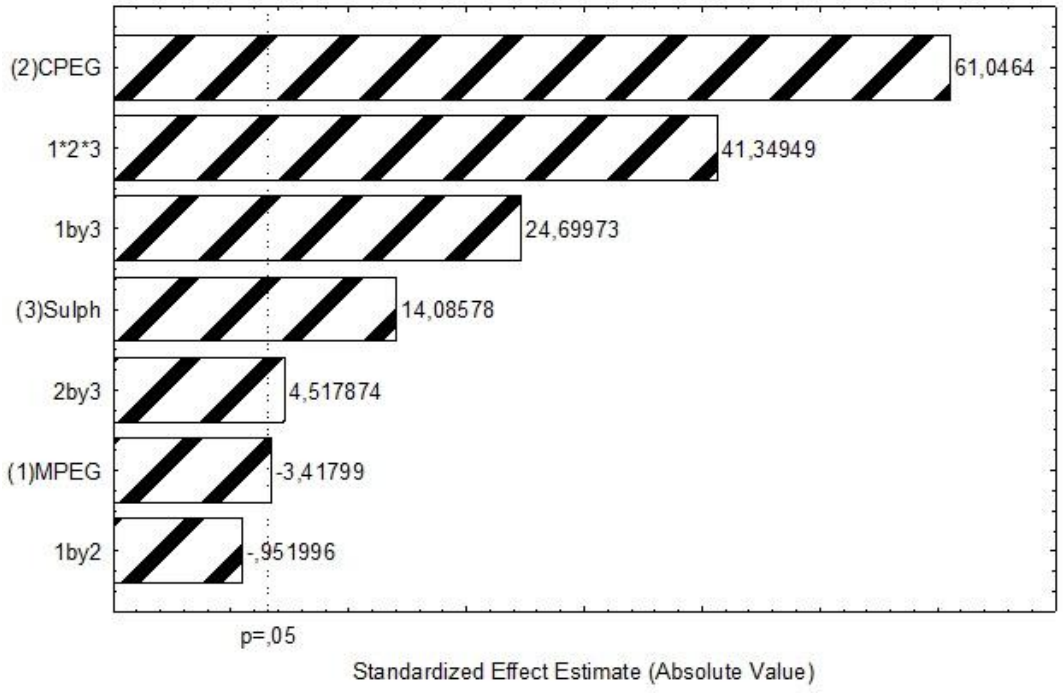

Figure 2. Effects of independent variables on the partition coefficient (K) of the fibrinolytic protease.

This work, corroborates with observed by [6], which polymers using higher PEG molar mass (8000g/mol) for a partition in ATPS fibrinolytic protease creates a repulsive effect on fibrinolytic protease partition, leading to a decrease in the coefficient partition.

3.3 Effect of variables using ATPS in yield and purification factor of fibrinolytic protease

The results of activity yield demonstrate that the C_{PEG} negative effect was the most significant. The interaction between C_{PEG} and C_{Na2SO4} was positive, which indicates that simultaneous decreases in both variables led to improved protease yield. The highest recovery value $Y_{FA}{}^{b}$ (106.51 at 130.36%) in bottom phase (central points) was obtained with M_{PEG} 6000 (g/mol), C_{PEG} 24.00% (w/w) and C_{Na2SO4} 11.60% (w/w). This high recovery, more than 100% in the bottom phase are associated, with removal of contaminants and inhibitors during the purification system, which enhances the enzymatic activity [33, 34].

The purification factor in the bottom phase had values of purification PF^{b} (3.21 at 4.51) occurred also in central points; these values are in agreement studied of [35] that obtained values between 0.5 and4.0 of purification factor by fibrinolytic protease from *Auricularia polytricha* and using ATPS (PEG/potassium phosphate).

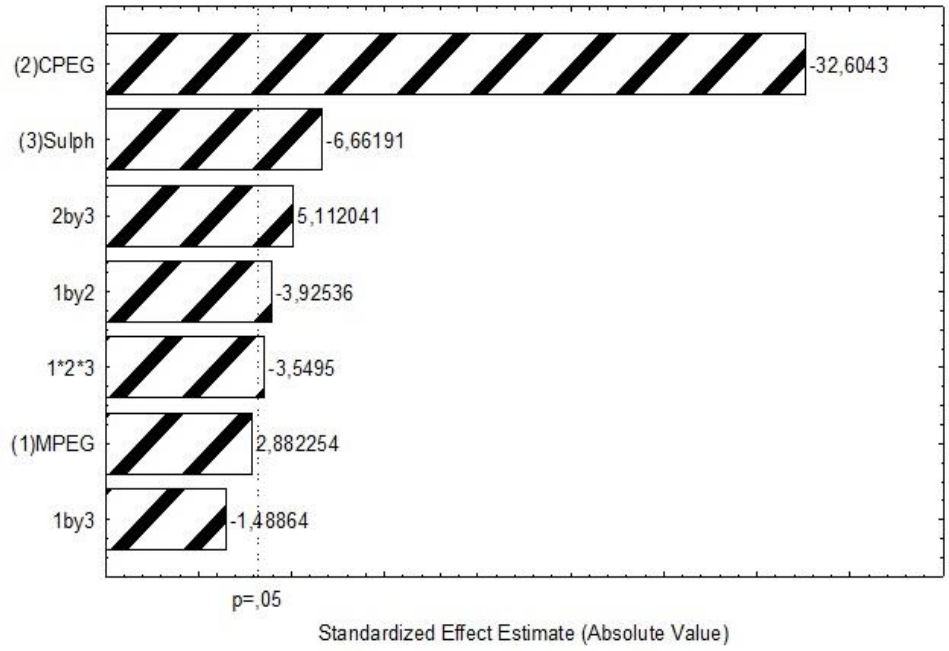

Figure 3. Effects of variables on activity yield of the fibrinolytic protease in bottom phase.

3.4 SDS-PAGE and fibrin zymography

SDS-PAGE and fibrin zymography were employed to verify the enzyme purity (Fig 5). The molecular mass of the fibrinolytic enzyme from *Mucor subtilissimus* SIS 42 was found to be 94 kDa, using bottom phase (central points) in ATPS. The molecular mass of the purified enzyme was according with the value of Novel fibrinolytic enzyme (66-97kDa) from culture supernatant of *Schizophyllum commune* BL23 [35] and fibrinolytic enzyme (80-173kDa) produced by *Bionectria* sp. isolated from Las Yungas rainforest [36].

Although these values confirmed by SDS-PAGE are larger than the majority of known fibrinolytic enzymes, have reported a fibrinolytic enzyme by fungus of molecular mass ranging of 27kDa to 32 kDa from *Fusarium* sp. BLB [37], *Rhizopus chinensis* 12 [38], *Pleurotus ostreutus* [39], *Schizophyllum commune* [40].

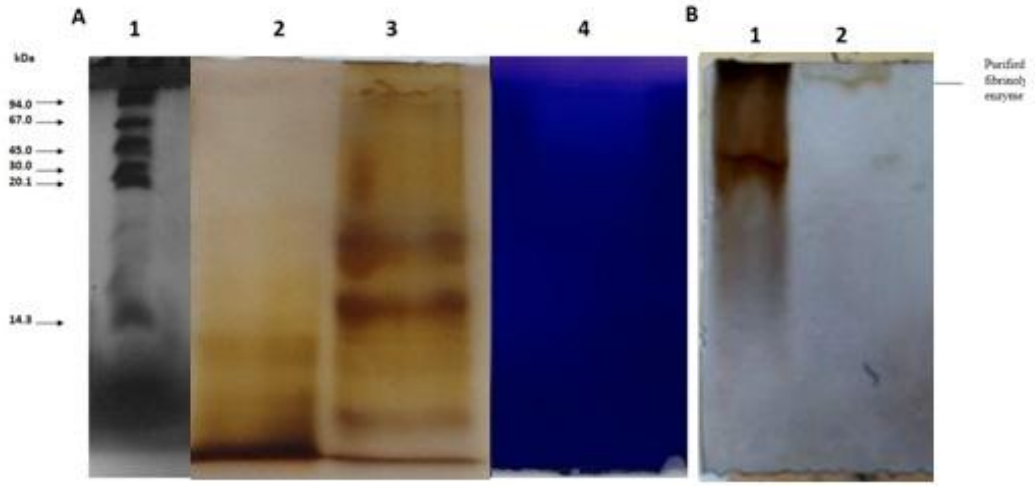

Figure 4. (A) Molecular mass determination of fibrinolytic enzyme using SDS-PAGE (10%) and fibrin zymography. Lane 1 is the protein molecular mass marker; Lane 2, crude extract; Lane 3, crude extract precipitated; Lane 4, fibrin zymography of crude extract. (B) Purity of fibrinolytic enzyme as determined by PAGE (12%). Lane 1 is crude extract; Lane 2 is purified sample from bottom phase of aqueous two-phase system.

3.5 Amidolytic activity and effect of inhibitors in protease activity

The amidolytic activity of fibrinolytic protease was investigated. The most sensitive substrate for purification enzyme was N-succinyl-Ala-Ala-Pro-Phe-pNa, a substrate for chymotrypsin, suggesting that it was a chymotrypsin-like protease. So, fibrinolytic protease was considered as an enzyme, similar to other fibrinolytic enzymes produced from *Bacillus subtilis*-fermented [41].

The effect of various inhibitors on protease activity was summarized in Table 4. The fibrinolytic protease was completely inhibited by PMSF, which is a well-known inhibitor of serine protease. These results corroborate with [42] that produced fibrinolytic enzyme by *Bacillus amyloliquefaciens* DC-4 the type of serine protease.

Table 4. Effect of inhibitors on protease activity.

Inhibitors Control	Residual activity (%)
Control	100.0
EDTA	100.0
β-Mercaptoethanol	97.7
PMSF	0.0
Pepstatin A	91.3
Iodoacetic Acid	100.0

3.16 Response surface methodology

According to the analysis of variance, f-value for the overall regression model was significant at 5% level and the lack of fit is insignificant indicating that the first-order model with interaction is very adequate in approximating the response surface of the experimental design (Table 5). The coefficient of determination (R^2) was 0.8584.

Table 5. Analysis of variance for the experimental results of the central composite design.

Factor	SS	DF	MS	F	P
C_{PEG} (L)	30.3801	1	30.3801	47.7621	0.0062
C_{PEG} (Q)	7.5192	1	7.5192	11.8214	0.0412
Sulf $_{Na}$ (L)	9.4769	1	9.4769	14.8991	0.0307
Sulf $_{Na}$ (Q)	2.8499	1	2.8499	4.4805	0.1245
C_{PEG} (L) by Sulf $_{Na}$ (L)	15.3275	1	15.3275	24.0972	0.0161
Lack of fit	9.2865	3	3.0955	4.8666	0.1131
Pure error	1.9082	3	0.6360		
Total SS	79.1095	11			

In Table 6 was showed estimated effects for partition coefficient of fibrinolytic protease (K_{FA}),yield of enzyme in bottom-phase ($Y_{FA}{}^{b}$) and purification factor in bottom phase (PF^{b}) on fibrinolytic protease purification process using central composite design. The fibrinolytic activity obtained during purification in Na_2SO_4 phase varied between 34.58 and 94.16 (U/mL), accordingly three-dimensional graphs were generated for the pair-wise combination of the two factors (Figure 3) about partition coefficient (K_{FA}) of the enzyme. The variables C_{PEG} and Na_2SO_4 concentration contribute for partition of fibrinolytic protease. Only the variable Sulf Na (Q) was not significant in the enzyme partition.

In this work, the activity maximum of fibrinolytic protease was estimated to 94.16 U/mL in bottom phase. The C_{PEG} and Na_2SO_4 concentration levels, does not contribute to increasing fibrinolytic activity. Diverging from those presented by Ashipala et al [7], that used central composite rotable design (CCRD) to determine the best conditions for the production and purification of fibrinolytic enzyme, by *Bacillus subtilis* DC-2 in ATPS (PEG 4000/Na_2SO_4) and clearly showed that the fibrinolytic activity is dependent mainly on PEG 4000 concentration, Na_2SO_4 concentration and pH.

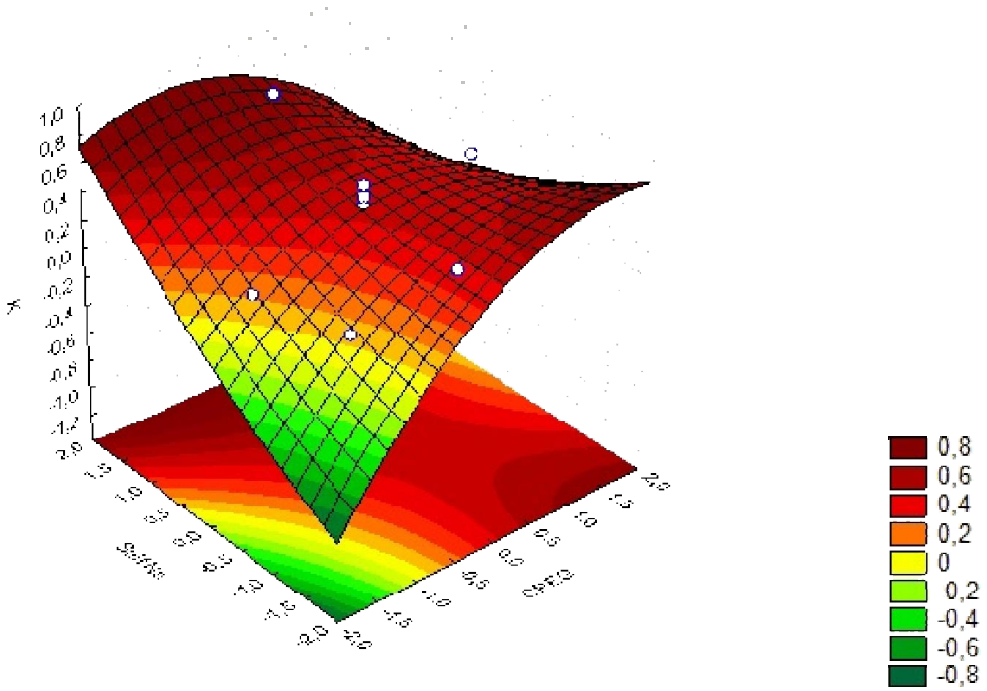

Figure 5. Three-dimensional contour plots showing interactive effects of C_{PEG} and Na_2SO_4 concentration in partition coefficient of the fibrinolytic protease.

It can be observed in Table 7, the variables: C_{PEG} (Q), $Sulf_{Na}$ (L) C_{PEG} (L) and the interations between C_{PEG} (L) and $Sulf_{Na}$ (L) were statistically significant at 95% confident level and with positive effect on purification factor in bottom phase. The C_{PEG} (30.00%), C_{Na2SO4} (13.20%) were most promising, for the higher purification factor in bottom phase PF^b=10 and 102% of yield (Table 6). This value of purification was higher the obtained by factorial design, conducted at the beginning of this work (PF= 3.21 at 4.51) and higher that the results obtained by Medeiros et al. [6] where purified fibrinolytic protease produced *Streptomyces* sp. DPUA1576 using PEG-phosphate aqueous two-phase systems (PF=1.51). Using ATPS Pericin et al. [43] purified protease–aspergillopepsin I in PEG/NaH_2PO_4 and obtained PF=5.76.

Table 6. Central composite design matrix with measured and responses of fibrinolytic activity.

Runs	C_{PEG} (%)	C_{Na2SO4} (%)	FA^b (U/mL)	K_{FA}	$Y_{FA}{}^b$	PF^b
1	18.00	10.00	94.16	0.049	177.18	2.04
2	18.00	13.20	71.66	0.436	137.84	2.28
3	30.00	10.00	93.33	0.442	78.05	1.94
4	30.00	13.20	76.25	0.246	102.02	10.01
5	15.60	11.60	84.16	0.129	175.96	2.16
6	32.40	11.60	51.66	0.452	47.53	7.79
7	24.00	9.36	57.50	0.370	74.53	1.34
8	24.00	13.84	34.58	0.795	56.40	1.61
9	24.00	11.60	90.00	0.403	143.00	3.83
10	24.00	11.60	89.58	0.465	127.36	3.60
11	24.00	11.60	87.91	0.546	99.33	2.06
12	24.00	11.60	88.33	0.388	117.91	3.43

C_{PEG} – PEG concentration; C_{Na2SO4} – Sulfate sodium concentration; K_{FA} - Partition coefficient of fibrinolytic proteases; $Y_{FA}{}^b$ - Activity yield in the bottom phase; PF^b - Purification factor in the bottom phase; FA^b - Fibrinolytic activity in the bottom phase.

Table 7. Estimated effects for partition coefficient (K_{FA}), yield ($Y_{FA}{}^b$) and purification factor (PF^b) on fibrinolytic protease purification process using central composite design.

Variables	K_{FA}	$Y_{FA}{}^b$	PF^b
C_{PEG} (Q)	-4.08	0.51*	3.43
C_{PEG} (L) by Sulf $_{Na}$ (L)	-4.06	1.73*	4.90
Sulf $_{Na}$ (L)	3.90	0.79*	3.85
C_{PEG} (L)	3.24	-6.13	6.91
Sulf $_{Na}$ (Q)	1.06*	2.69*	-2.11

*Significant effects $p< 0.05$

4 CONCLUSION

The purification conditions of fibrinolytic protease production by *Mucor subtlillissimus* SIS42 in ATPS were optimized using central composite rotary design. In this study, the experimental results clearly showed high purification of the enzyme in the bottom-phase rich in sodium sulfate. This enzyme was characterized as chymotrypsin-like protease. This work demonstrating the potential application of ATPS

processes for the recovery of fibrinolytic protease, as a first step in the development of a biotechnological process with commercial application.

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CONCLUSÃO

Uma nova protease fibrinolítica foi produzida pelo fungo filamentoso *Mucor subtillissimus* SIS42, com todas as condições de produção otimizadas, utilizando farelo de trigo como substrato através de fermentação em estado sólido. Utilizando um central composto para a purificação da protease fibrinolítica por sistema de duas fases aquosas PEG/Sulfato de sódio, obteve a otimização das condições de purificação da enzima. Sendo esta, caracterizada como uma serino protease semelhante a quimiotripsina. Desta forma, este trabalho demonstra um potencial para a produção e purificação de enzimas fibrinoliticas por via fermentativa com um maior rendimento e pureza. Espera-se que com os resultados obtidos, possa em etapas futuras permitir a purificação por métodos cromatográficos e a realização de testes *in vitro* para aplicação dessas enzimas na terapia trombolítica.

ANEXOS

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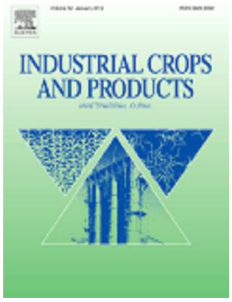
INDUSTRIAL CROPS AND PRODUCTS

An International Journal



AUTHOR INFORMATION PACK

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DESCRIPTION

Industrial Crops and Products, an International Journal, publishes academic and industrial research on **industrial** (non-food) **crops** and **products**, containing both crop-oriented and product-oriented research papers - a platform where agricultural research meets industrial R&D.

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Molecular biology; Protein detection methods; Food safety; Food contaminants and Protein toxins
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Catalytic conversion of fats and oils

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medicinal and nutraceuticals; antioxidants; waxes; resins; latices; guayule and phytochemicals of the plants of the semiarid lands

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Chemistry; quality and biological activity of natural plant products (herbs, spices, aromatic, medicinal plants and specialty crops used in nutrition and cosmetics) and their derivatives (essential oils, polyphenols, fatty acids, triterpenes); Plant tissue culture and micropropagation of herbs, spices, aromatic, medicinal plants

S. Korkut, Duzce University, Duzce, Turkey

Fibres and fibre compounds; natural fibres-based composites; waxes; resins; gums; rubber and other polymers; composites and reconstituted products; energy and chemicals from forest biomass; non-wood forest products; adhesives for wood; bonding strength; contact angles; adhesion by chemical bonding; mechanical properties of adhesives; surface roughness/morphology; wood-based composite materials and their applications.

O. Koul, Insect Biopesticide Research Centre, Jalandhar, India

Biopesticides; plant natural products; insect toxicology and physiology; Insect plant interactions and chemical ecology.

J. Labidi, Universidad del Pais Vasco (Basque Country), San Sebastian, Spain

biorefinery processes; pulp and paper; lignin; process simulation/optimization

M-P. Laborie, Albert-Ludwigs-Universität Freiburg, Freiburg, Germany

particle boards; wood; wood adhesive; nanocellulose; cellulosic composites; adhesion; interface properties; bio-based adhesives

D. Lachenal, Grenoble INP - Pagora, St. Martin d'Hères Cedex, France

pulping; lignin; bleaching; biorefinery from lignocellulosics

L. Lazzeri, Research Institute for Industrial Crops, Bologna, Italy

Crops for soil remediation; biofumigation; oilseeds

A. Monti, University of Bologna, Bologna, Italybiofuels; biomass; bioenergy crops

D. Pasquini, Universidade Federal de Uberlândia (UFU), Uberlandia MG, Brazil

vegetal macromolecules; cellulosic fibers; composites; nanocellulose; nanocomposites; polymers from renewable sources

R. Pavela, Crop Research Institute, Prague, Czech Republic

botanical insecticides

W.B. Phippen, Western Illinois University, Macomb, IL, USA

oil seed crops; plant breeding; genetics; agronomy; GC oil analysis

D. Pioch, CIRAD, Montpellier Cedex 5, France

oleaginous, oleochemistry (especially palms); extraction of active compounds, especially forest products (water and supercritical CO2 based); biorefinery (cascade extraction of a range of useful compounds, from a single biomass, including destructuration of lignocellulose; Ultra and microfiltration for separating fractions

A. Pizzi, Université Henri Poincaré (Nancy I), Epinal Cedex 9, France

particle boards; wood; wood adhesive

Y. Popineau, Institute National de la Recherche Agronomique, Nantes, France

D.A. Ravetta, Museo Egidio Feruglio, Trelew, Chubut, Argentina

new crops; arid lands; resins; specialty oils, secondary metabolites; eco-physiology

C. Regnault-Roger, Universite de Pau et des Pays de l'Adour, Pau Cedex, France

natural Insecticides; essential oils; plant chemistry

R. Roseberg, Oregon State University, Klamath Falls, OR, USA

soil science; agronomic aspects of crop production

H. Ruiz, Autonomous University of Coahuila, Saltillo-Coahuila, Mexico

renewable energy, specifically in biorefinery process and bioethanol production of second generation using lignocellulosic materials (agricultural residuos), hydrothermal process (autohydrolysis), simultaneous saccharification and bioethanol fermentation and modeling of enzymatic hydrolysis.

A.J.D. Silvestre, Universidade de Aveiro, Aveiro, Portugal

extractives; GC-MS

R.C. Sun, Beijing Forestry University, Beijing, China

Celulose chemistry; cellulose derivatives; biorefinery; fibres

W. Thielemans, University of Nottingham, Nottingham, UK

lignin; surface modification; composites; starch and cellulose nanoparticles; self assembly

D. Turley, National Non-Food Crops Centre, York, England, UK

non food crops in general; economic aspects; processing; rural strategies; agronomy of non-food crops; biofuels and bioenergy applications; bio-based materials

GUIDE FOR AUTHORS

INTRODUCTION

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The scope of the journal covers a vast range of crops and research disciplines. Crops should contain significant renewable resources such as:

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SEPARATION AND PURIFICATION TECHNOLOGY

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ANEXOS OPCIONAIS

RESULTADOS COMPLEMENTARES

Tabela A. Efeito de surfactantes na atividade proteásica utilizando farelo de trigo como substrato na FES por *Mucor subtillissimus* SIS42.

Surfactantes	Atividade resídual (%)			
	0.5%	1.0%	1.5%	2.0%
Controle	100%	100%	100%	100%
SDS	48,17	35,98	46,51	52,32
Tween 20	84,49	52,32	84,49	84,41
Tween 80	89,30	86,23	81,92	83,93

Figura A. Efeitos simultâneos da umidade e da quantidade de resíduo sobre a atividade fibrinolítica, da protease produzida por *Mucor subtillissimus* SIS42 na fermentação em estado sólido utilizando farelo de trigo como substrato.

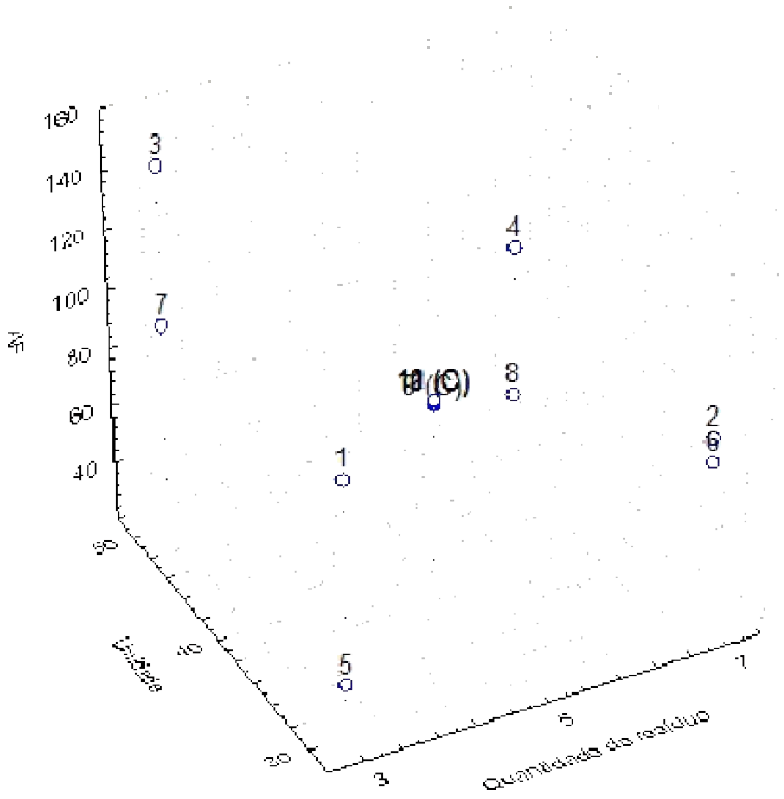


Tabela B. Efeito de inibidores na atividade proteásica após a purificação por SDFA utilizando PEG/Sulfato de Sódio e farelo de trigo como substrato.

Inibidores	Atividade residual (%)
Controle	100.00
EDTA	100.00
β-Mercaptoetanol	97.70
PMSF	0.00
Pepstatina A	91.34
Ácido Iodoacético	100.00

Tabela C. Efeito de íons metálicos na atividade proteásica após a purificação por SDFA utilizando PEG/Sulfato de Sódio.

Íons	Residual activity (%)		
	2.5 mM	5 mM	10 mM
Control	100%	100%	100%
K^+	93,86	93,86	100,00
Ca^+	119,09	107,50	102,27
Mn^+	109,31	107,50	100,90
Zn^+	89,77	76,36	30,00
Mg^+	102,50	92,73	110,91
Co+	65,68	72,50	61,59
Cu^+	66,13	57,95	54,09
Fe^+	75,68	65,59	52,95
Na^+	91,59	92,67	98,40

Figura B. Efeito da temperatura na atividade proteásica da enzima purificada por SDFA utilizando PEG/Sulfato de Sódio após 30 min.

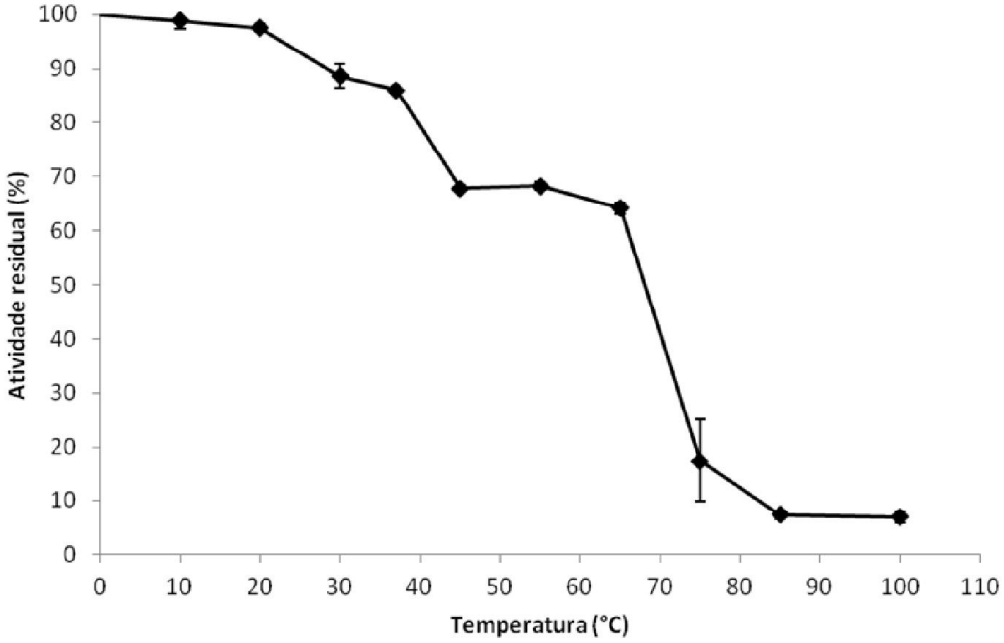


Tabela B. Produção de protease com atividade fibrinolítica produzida por *Mucor subtillissimus* SIS42 usando grão de soja como substrato através de um planejamento fatorial 2^3.

Ensaios	Quantidade de substrato (g)	Umidade (%)	Temperatura (ºC)	Atividade Proteásica (U/mL)	Atividade Fibrinolítica (U/mL)
1	3	30	25	55,33	140,00
2	7	30	25	41,26	97,91
3	3	50	25	49,46	119,16
4	7	50	25	48,06	76,25
5	3	30	35	16,86	52,91
6	7	30	35	30,40	62,91
7	3	50	35	45,40	106,60
8	7	50	35	35,66	74,58
9	5	40	30	18,60	50,83
10	5	40	30	14,13	50,41
11	5	40	30	19,60	50,83
12	5	40	30	19,66	54,58

Figura C. Efeitos das variáveis no planejamento fatorial 2^3 para a produção da protease fibrinolítica produzida pelo *Mucor subtillissimus* SIS42 por FES utilizando grão de soja como substrato.

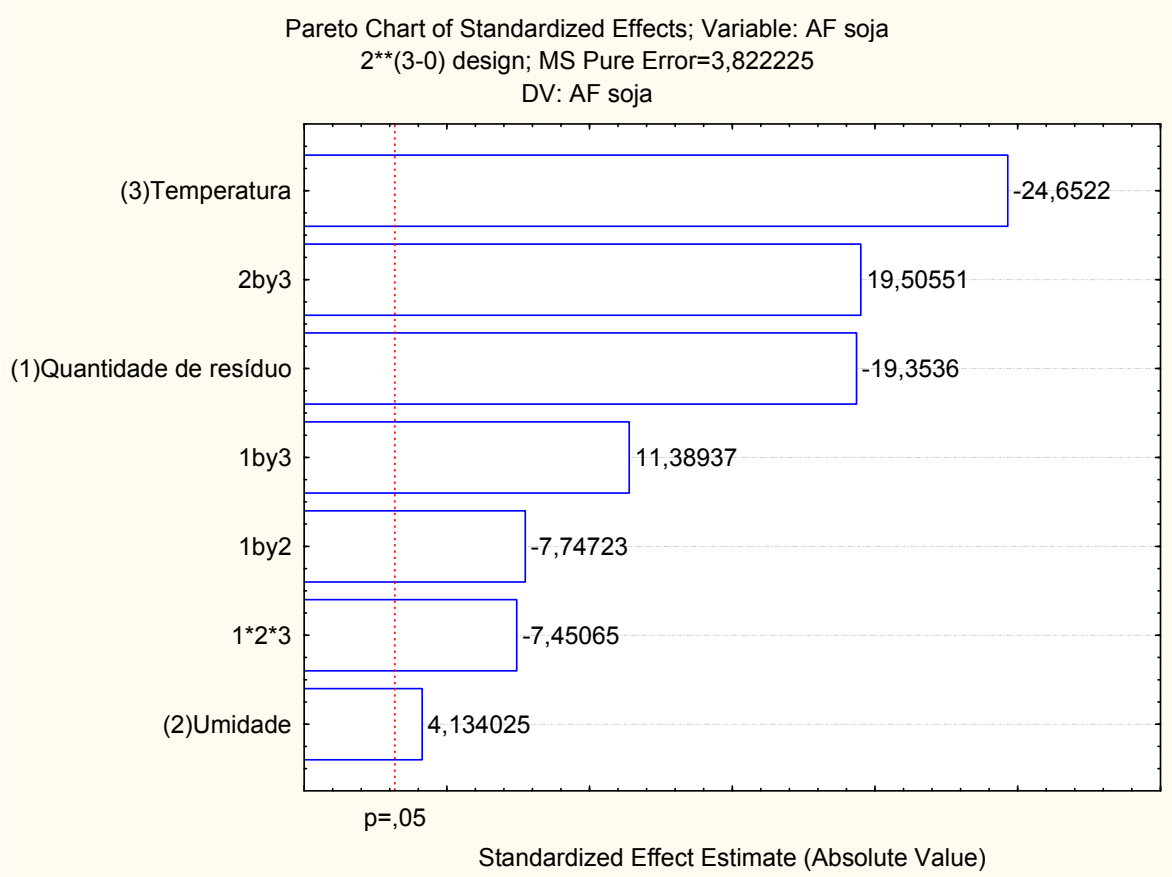
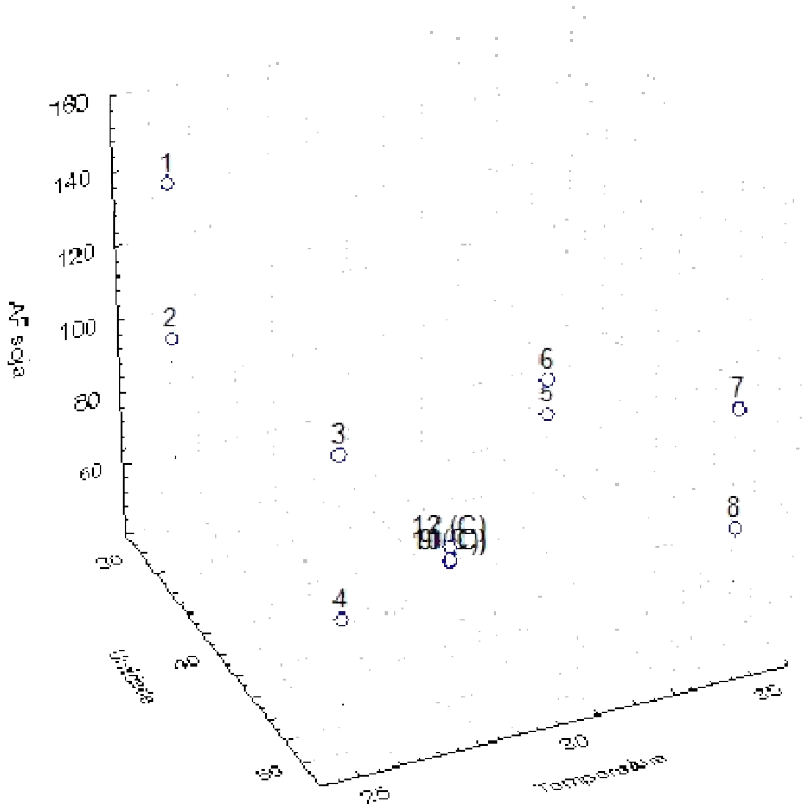


Tabela C. Teste de toxicidade do extrato bruto produzido por *Mucor subtillissimus* SIS42 por FES utilizando farelo de trigo como substrato, frente a *Artemia salina.*

Concentração	Tempo			
	24 horas		48 horas	
	Vivos (%)	Mortos (%)	Vivos (%)	Mortos (%)
Controle (0%)	100,00	0,00	100,00	0,00
100%	50,00	50,00	0,00	100,00
50%	63,33	36,66	23,33	76,66
25%	80,00	20,00	40,00	60,00
12,5%	93,33	6,66	43,33	56,66
6,25%	93,33	6,66	83,33	16,66
3,12%	90,00	10,00	73,33	26,66
1,57%	100	0	100,00	0,00

Figura D. Efeitos simultâneos da umidade e da temperatura, sobre a atividade fibrinolítica da protease produzida por *Mucor subtillissimus* SIS42, na fermentação em estado sólido utilizando grão de soja como substrato.



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Production of fibrinolytic protease by fungal strains for Solid-state Fermentation using agricultural products

Nascimento, T. P.[1,4]; Sales, A. E.[1]; Porto, C.S.[1]; Campos-Takaki, G.M.[2]

Porto, T.S[1,3] ;Porto, A.L.F[1]

[1] DMFA, UFRPE, PE, Brazil, [2]NPCIAMB, UNICAP, PE, Brazil, [3] UAG,UFRPE, PE, Brazil. [4]CCB, UFPE, PE, Brazil.

Utilization of agricultural by-products as low-cost substrates for microbial enzyme production resulted in an economical and promising process. Solid-state fermentation offers several advantages for enzyme production by fungal strains. Fibrinolytic enzymes are important in treatment of cardiovascular diseases. Intravascular thrombosis due to fibrin aggregation in arteries is one of the main causes of cardiovascular disease in humans. The purpose of this work is a comparative study of two fungal species according to their fibrinolytic protease production in solid-state fermentation (SSF) using different agro- industrial substrates. The fungal strains *Mucor subtilissimus* SIS42 and *Rhizopus arrhizus* var. *arrhizus* SIS30 was isolated by soil of the Caatinga, Pernambuco-Brazil. The fibrinolytic enzyme activities were performed using Fibrinolytic spectrophotometric assay using fibrin as substrate. The substrates used were the agro-industrial products: wheat bran, soybean, corncorb and cassava peel. However growth was observed only in cultures of wheat bran and soybean. The fibrinolytic activity was 201.17 U/g using soybean and 218.61 U/g using wheat bran by *Mucor subtilissimus* SIS42. While for the strain *Rhizopus arrhizus* var. *arrhizus* SIS30 was obtained 188.54 U/g and 170, 46 U/g, when fermented in wheat bran and soybean respectively. Further optimization experiments using *Mucor subtilissimus* SIS42 strain for the production of fibrinolytic protease may provide auspicious results in the future.

Word Keys: SSF, Fibrinolytic protease, fungal
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FIBRINOLYTIC ENZYME PRODUCED BY FILAMENTOUS FUNGI

Thiago Pajeú Nascimento[1], Amanda Emmanuelle Sales[1], Camila Souza Porto[1], Fabrício Bezerra de Sá[1], Galba Maria Campos Takaki[2], Tatiana Souza Porto[1,3], Ana Lúcia Figueiredo Porto[1,]

[1] Animal Morphology and Physiology Department, Federal Rural University of Pernambuco, Dois Irmãos, Recife, Pernambuco CEP 52171-900, Brazil.

[2] Nucleus of Research in Environmental Sciences (NPCIAMB), Catholic University of Pernambuco, Boa Vista 50050-590 Recife, PE, Brazil.

3 Academic Unit of Garanhuns, Federal Rural University of Pernambuco, Garanhuns, Pernambuco CEP 55292-901, Brazil.

Fibrinolytic proteases are enzymes that degrade fibrin, the main component of blood clots. The accumulation of this protein leads to thrombosis responsible for cardiovascular disease including myocardial infarction. A promising alternative to thrombolytic therapy has been the production of these enzymes by microorganisms which promotes low cost, high efficiency and capacity for large scale production. Among the microorganism, filamentous fungi has been gaining attention because they are capable of producing extracellular proteases and a source bit being exploited for the production of fibrinolytic enzymes. This study aimed to select species of filamentous fungi isolated from soil of Caatinga - Pernambuco - Brazil and assess their potential for production of protease fibrinolyticusing the medium for the fermentation of soy because it is a medium rich in proteins and low cost. Was used the medium 2% soybean. The fibrinolytic activity was performed by the spectrophotometric method and by degradation of the blood clot. Among the 36 isolates studied, 58% showed fibrinolytic activity above 100 U/mL. The microorganism with the higher activity in terms of enzyme production was *Mucor subtilissimus* SIS 42 who presented with 96 hours of fermentation fibrinolytic activity of 415 U/mL. Initially, the amount of protein present in the fermentation broth was 0.498 mg/ml and after 72 hours of fermentation 0.026 mg/mL showing the degradation of the protein exists in the production of proteases by the microorganism. At that time crude extract had a capacity of enzymatic degradation of the equine blood clots in 16.7%. The results are very attractive and are in agreement with the literature, these fungi are also little studied in the production of fibrinolytic proteases and are promising potential in the treatment of cardiovascular diseases.

Key-words: *Mucor subtilissimus*, fibrinolytic protease, thrombosis.

PRODUCTION FIBRINOLYTIC PROTEASE BY SOLID-STATE FERMENTATION USING A FACTORIAL DESIGN

T. P. Nascimento[1], A. E. Sales[2], C. S. Porto[2], G. M. Campos-Takaki[3], T. S. Porto[4] and A. L. F. Porto[2]

[1] Federal University of Pernambuco- UFPE
[2] Federal Rural University of Pernambuco-UFRPE
[3] Catholic University of Pernambuco-UNICAP.
[4] Academic Unit of Garanhuns – UAG/UFRPE

According to the World Health Organization (WHO) 17.3 million people die each year of cardiovascular disease victims. Various thrombolytic agents have been used in the therapeutic treatment of thrombosis, but due to their high cost and hemorrhagic side effects, have been sought new sources. This study describes the production of protease fibrinolyticby *Mucor subtillissimus* SIS 42 using solid-state fermentation through design factorial using soybean as substrate. The conditions for producing protease fibrinolyticwere used a full factorial design (23) to determine the influence of the parameters: substrate amount (3, 5 and 7g), moisture (30, 40 and 50%) and temperature (25, 30 and 35ºC). Extraction of the enzyme was performed using of sodium phosphate buffer 245mM (pH 7). The fibrinolytic activity was determined using the spectrophotometric method being performed with 72, 96 and 120 hours of fermentation. In run 1 which used 3g of soybean as the substrate, 30% humidity underwent a temperature of 25°C proved to have the best conditions for the production of fibrinolytic protease by the microorganism where the test with these conditions obtained an activity of 140.0 U/mL, 1878.35 U/gbs after 72 hours of fermentation. All variables and the relationships between them were significant, the temperature and amount of these substrates had a negative effect, as well as the relationship between them. However moisture demonstrated a positive effect, namely the greater the amount of moisture tends to use higher enzyme production possibly due to a rise of the porosity of the substrate having as a consequence a better transfer of oxygen thereby favoring the development of microorganism. The literature show that most fungi have an optimum temperature of protease production by SSF between 28°C to 30°C, which differs from that shown in this study, where the *M. subtillissimus* SIS42 has its best

production of enzymes at 25 °C for both the factorial design using soybean as the substrate. During this study, the fibrinolytic enzyme showed a high stability showed that the choice of buffer for enzyme extraction was essential for SSF, where one can recover the maximum enzyme produced by the microorganism and in some cases even enhances the ability of the specific activity of proteases. Studies de optimization the enzyme produced by *Mucor subtillissimus* SIS42 for application in process sanguine coagulation may provide promising results in the future.