UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO - UFRPE

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOCIÊNCIA ANIMAL – PPGBA

Produção, purificação parcial por Fermentação Extrativa em Sistema de Duas Fases Aquosas (SDFA) e caracterização bioquímica de enzimas fibrinolíticas do *Bacillus amyloliquefaciens* UFPEDA 485

Fabiana América Silva Dantas de Souza

Recife-PE, 2014

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Tese apresentada ao Programa de Biociência Animal da Universidade Federal Rural de Pernambuco, como pré-requisito parcial para obtenção do grau de Doutor em Biociência Animal. Área de Concentração: Biotecnologia.

Orientador (a): Prof. Drª. Ana Lúcia Figueiredo Porto (UFRPE)
Co-Orientador: Prof. Dr. José António Couto Teixeira (UMinho**)**

Fabiana América Silva Dantas de Souza

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,

O que sabemos é uma gota e o que ignoramos é um oceano.

(Isaac Newton)

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LISTA DE ABREVIATURAS E SÍMBOLOS

A - Adenina

AFi - Atividade Fibrinolítica na fase inferior

AFs - Atividade Fibrinolítica na fase superior

APC ou PCa - Proteína C

Arg – Arginina

BSA – Bovine Serum Albumin

C4b – Proteína de ligação da proteína S

D-Di - Dímero D

EC - Comissão de Enzimas

EDTA - Ácido etilenodiaminotetracético - $C_{10}H_{16}N_2O_8$

Fator Va - Ac-globulina ativado na cascata de coagulação

Fator VIIIa - Globulina anti-hemofílica ativada na cascata de coagulação

Fator Xa - Fator Stuart ativo na cascata de coagulação

Fator XII - Fator Hageman

FBN - Fibronectina

FDA - Food and Drug Administration

FE- Fermentação extrativa

FII - Protrombina

FOB - Free on board

FpA - Fibrinopeptídeos A, peptídeos N-terminais

FpB - Fibrinopeptídeos B, peptídeos N-terminais

G – Guanina

Glu - Glutamina

K - Coeficiente de Partição

KAL - Calicreína

kDa - Kilodaltons (peso molecular)

K_m - Constante de Michaelis-Menten

MSR - Metodologia de Superfície de Resposta

OMS - Organização Mundial de Saúde

PAI - 1 - Inibidor do ativador de plasminogênio t-PA

PAI - 2 - Inibidor do ativador de plasminogênio u-PA

PCa-PS - Complexo de proteínas C e S ativo

PCC - Planejamento Central Composto

PDF - Produtos de degradação da fibrina

PEG - Polietileno Glicol

pH - Potencial Hidrogeniônico

pI - ponto isoelétrico

pKAL - pré-calicreína tecidual

PM - Peso Molecular

PMGN - Plasminogênio

PMN - Plasmina

PMSF - fluoreto de fenilmetilsulfonil - $C_7H_7FO_2S$

*p*NA - *p*-nitroanilide

rpm - Rotação por Minuto

rpoB - Subunidade β da RNA polimerase

SDFA - Sistema de Duas Fases Aquosas

TNKase - tenecteplase

t-PA - Fator tecidual ativador de plasminogênio

u-PA - uroquinase ativador de plasminogênior

V_{max} - Velocidade Máxima da Reação

vvm - Volume de ar por Volume de meio por Minuto

RESUMO

Os distúrbios cardiovasculares é um problema frequente na medicina humana e veterinária, implicando muitas vezes em consequências fatais. As enzimas fibrinolíticas são uma alternativa promissora frente à indústria farmacêutica. Este trabalho objetivou selecionar uma bactéria do gênero *Bacillus* produtora de enzima fibrinolítica, otimizar a produção enzimática por fermentação convencional em frascos contendo meio de soja, à 37^{0}C e pH 7,2, com farinha de soja (1% a 3%), glicose (0,5% a 1,5%) e agitação (100 a 200 rpm) utilizando planejamento central composto, aumentar a produção enzimática em biorreator de 7L estudando a influência da aeração (0,5 a 1,5 vvm) e agitação (200 a 800 rpm), avaliar a produção e purificação integradas de protease fibrinolítica por fermentação extrativa (FE) em sistema de duas fases aquosas (SDFA) utilizando meio de soja com adição de polietileno glicol (PEG) e sulfato de sódio (Na_2SO_4), além de avaliar o potencial anticoagulante do extrato enzimático e determinar as características bioquímicas das enzimas fibrinolíticas produzidas nas fermentações convencional e extrativa. O *Bacillus amyloliquefaciens* UFPEDA 485 foi o melhor produtor de protease fibrinolítica. A condição ótima em frascos foi com 2% de farinha de soja e 1% de glicose em 48h, obtendo uma atividade fibrinolítica (AF) de 813 U.mL^{-1} e um percentual de degradação do coágulo sanguíneo *in vitro* de 62%. AF do extrato enzimático foi 91,52% inibida por PMSF (fluoreto de fenilmetilsulfonil) e 89,64% inibida por EDTA (ácido etilenodiaminotetracético), constatando a presença de serino-metalo proteases. Em biorreator, a melhor AF de 2.169 UmL^{-1} e atividade amidolítica (AA) de 1.587 µmol min^{-1} mL^{-1} foi a 800 rpm e 1,5 vvm em 12h. O substrato sintético de maior especificidade foi o N-succinil-Ala-Ala-Pro-Phe-*p*NA e os valores obtidos para K_m e V_{max} foram de 0,68 mM e 357,14 mmol min^{-1} mL^{-1}. A temperatura ótima foi de 37^{0}C e pH ótimo entre 7.0-8.0 para AA e 37ºC e pH 7.0 para AF. Após 9 meses à - 20^{0}C foram mantidas AF de 92,2% e AA de 95,8%. A melhor condição da FE foi com PEG 8000 (18% p/v) e Na_2SO_4 (13% p/v), obtendo AF de 835 U.mL^{-1} na fase sal. Nessas condições, AF aumentou na presença de $CaCl_2$ (440%), $MgCl_2$ (440%), $FeSO_4$ (268%) e KCl (268%) e foi 96,87%, inibida por EDTA. Fibrinogênio e trombina incubados com o extrato enzimático por 1h não formou coágulo de fibrina, evidenciando seu potencial anticoagulante. Logo, o *Bacillus amyloliquefaciens* UFPEDA 485 pode produzir enzimas com potencial para aplicação na terapia trombolítica e a FE permitiu uma purificação parcial da enzima, podendo ser uma alternativa para reduzir o custo de obtenção do produto.

Palavras-chave: Otimização; enzima fibrinolítica; anticoagulante; *Bacillus amyloliquefaciens;* fermentação extrativa; sistema de duas fases aquosas.

ABSTRACT

Cardiovascular disorders are a frequent problem in human and veterinary medicine, oftentimes causing fatal consequences. The fibrinolytic enzymes are a promising alternative for the pharmaceutical industry. The aim this work was to select a bacterium of the genus *Bacillus* producer fibrinolytic enzyme, optimize the enzyme production by fermentation conventional in flasks containing soyean medium at 37^0C and pH 7.2, with soybean flour (1% at 3%), glucose (0.5% at 1.5%) and agitation (100 at 200 rpm) using a central composite design, increase the enzyme production in 7L bioreactor studying the influence of the aeration (0.5 at 1,5 vvm) and agitation (200 at 800 rpm), evaluate the production process and purification integrated of fibrinolytic protease by extractive fermentation (EF) in aqueous two-phase system (ATPS) using soybean medium with added polyethylene glycol (PEG) and sodium sulphate (Na_2SO_4), as well as evaluate the anticoagulant potential of enzyme extract and determine the biochemical characteristics of the fibrinolytic enzymes produced at the conventional and extractive fermentations. *Bacillus amyloliquefaciens* UFPEDA 485 was the best producer of fibrinolytic protease. The optimum condition in flasks was with 2% soybean flour and 1% glucose in 48h, obtained a fibrinolytic activity (FA) of 813 $U.mL^{-1}$ and a percentage degradation of blood clots *in vitro* of 62%. FA of the enzyme extract was 91.52% inhibited by PMSF (fluoride methylphenylsulfonyl) and 89.64% inhibited by EDTA (ethylenediaminetetraacetic acid), confirming the presence of serine-metallo proteases. In bioreactor, the best FA of 2.169 $U.mL^{-1}$ and amidolytic activity (AA) of 1.587 µmol min^{-1} mL^{-1} was at 800 rpm and 1.5 vvm in 12 h. The synthetic substrate of larger specificity was N-succinyl-Ala-Ala-Pro-Phe-*p*NA and the values obtained for K_m and V_{max} were of 0.68 mM and 357.14 mmol min^{-1} mL^{-1}. The optimum temperature was of 37^0C and pH optimum between 7.0-8.0 for AA and 37^0C and pH 7.0 for FA. After 9 months at - 20^0C were maintained FA of 92.2% and AA of 95.8%. The best conditions of the EF was with PEG 8000 (18% w/v) and Na_2SO_4 (13% w/v), getting FA of 835 $U.mL^{-1}$ at the salt phase. In these conditions, FA increased in the presence of $CaCl_2$ (440%), $MgCl_2$ (440%), $FeSO_4$ (268%), KCl (268%) and was 96.87% inhibited by EDTA. Fibrinogen and thrombin incubated with enzyme extract by 1h not formed fibrin clot, demonstrating its potential anticoagulant. Therefore, the *Bacillus amyloliquefaciens* UFPEDA 485 can produce enzymes with potential for application at the thrombolytic therapy and EF allowed a partial purification of the enzyme and can be an alternative to reduce the cost of production of the product.

Keywords: Optimization; fibrinolytic enzyme; anticoagulant; *Bacillus amyloliquefaciens*; extractive fermentation; aqueous two-phase system.

INTRODUÇÃO

De acordo com a Organização Mundial da Saúde (OMS), as doenças cardiovasculares são responsáveis por 30% das mortes a cada ano. Estima-se que cerca de 23,6 milhões de pessoas morrerão por complicações causadas por distúrbios cardiovasculares até o ano de 2030 (DENG et al., 2010).

A trombose é desencadeada quando há um desequilíbrio no sistema hemostático e a formação destes coágulos sanguíneos é proveniente da interação entre fatores genéticos e/ou ambientais (CHRISTO et al., 2010; LILLICRAP et al., 2013). O tratamento da trombose visa prevenir a formação de coágulos utilizando anticoagulantes e, quando formados, dissolvê-los utilizando agentes fibrinolíticos. Contudo, os anticoagulantes como a heparina e varfarina e fibrinolíticos como ativadores de plasminogênio tecidual (t-PA) e uroquinase, dentre outros utilizados atualmente, causam diversos efeitos colaterais, além de requererem monitoramento constante devido ao alto risco de hemorragias. Desta forma, pesquisas vêm sendo direcionadas em busca de novos fármacos mais seguros e eficazes para manutenção do equilíbrio hemostático (AL-JUAMILY et al., 2013; BAJAJ et al., 2013; HEO et al., 2013).

As enzimas fibrinolíticas são proteínas que degradam coágulos de sangue e são consideradas uma alternativa promissora para auxiliar na terapia trombolítica. Estas enzimas podem ser provenientes de diversas fontes, inclusive microbianas (SHIRASAKA et al., 2012; CHOI et al., 2013; MUKHERJEE et al., 2013; PARK et al., 2013; BILHEIRO et al., 2013; PRIHANTO et al., 2013). Contudo, as enzimas fibrinolíticas extracelulares microbianas e principalmente as produzidas pelo gênero *Bacillus* têm se destacado pela sua facilidade de obtenção, alta atividade, propriedades fisiológicas adequadas ao sistema sanguíneo (pH 7,0 e temperatura 37°C) e elevada estabilidade (CHANG et al., 2012; HUANG et al., 2013).

A capacidade das espécies de *Bacillus* de produzir grandes quantidades de enzimas extracelulares e apresentar alta taxa de crescimento, que leva a ciclos de fermentação curtos, coloca este gênero entre os mais importantes produtores de enzimas industriais, além dos bioprodutos de algumas espécies de *Bacillus* serem considerados seguros na alimentação e na administração de drogas (SCHALLMEY et al., 2004; RATHAKRISHNAN et al., 2011).

Um fator relevante para a produção de proteases é a seleção de um agente indutor. Os meios de cultura à base de farinha de soja (substrato indutor na

produção de enzimas) são adequados em nível industrial por serem mais baratos e porque, em certos casos, se obtêm melhores rendimentos e aumento na produtividade. Isto se deve ao seu elevado nível protéico e uma variedade de moléculas orgânicas, em sua constituição, que evitam que a célula necessite sintetizá-las a partir de glicose e compostos inorgânicos (LONSANE, 1994). A farinha de soja, resíduo industrial proveniente do óleo de soja, rica em nitrogênio, tem demonstrado ser um excelente substrato para produção de enzimas fibrinolíticas (SALES et al., 2013; MEDEIROS e SILVA et al., 2013).

O custo do processo de produção e purificação de proteases é o principal obstáculo para a aplicação dessas enzimas nas indústrias. Para melhorar o rendimento da produção enzimática é necessário otimizar os processos e aumentar o escalonamento de produção dessas enzimas fibrinolíticas, consideradas de grande valor agregado para o mercado mundial (RAJ et al., 2012).

Contudo, para que um fármaco seja comercializado, é necessário que ele esteja puro. Diante desta necessidade, diversos métodos de purificação vêm sendo investigados e a bioconversão ou fermentação extrativa utilizando sistema de duas fases aquosas (SDFA) vem sendo considerada uma alternativa promissora na redução de etapas dos processos de purificação, reduzindo assim o custo para obtenção do produto final (DELOISA et al., 2009; NALINANON et al., 2009).

A fermentação extrativa é um processo de produção e recuperação simultânea. O SDFA é formado pela mistura de duas soluções aquosas de (polímeros-polímero ou polímero-sal) acima de certa concentração crítica, indicada por um diagrama de fases em que a formação de duas fases aquosas imiscíveis é observada. Em um comportamento ideal, espera-se que as células e os componentes do meio devam se concentrar em uma das fases do sistema, enquanto que a biomolécula de interesse deva preferir a fase oposta. Esse comportamento facilita a extração do produto, levando-o a uma purificação parcial e eliminando a influência de inibidores presentes no processo (SINHA et al., 2000; NG et al., 2013).

Considerando as desvantagens e o alto custo para obtenção dos fármacos utilizados atualmente para o tratamento de distúrbios vasculares, este trabalho busca produzir uma nova enzima com potencial para manutenção do controle hemostático, disponiblizando novas tecnologias à indústria farmacêutica.

OBJETIVOS

Objetivo geral

Selecionar uma espécie de *Bacillus* produtora de protease fibrinolítica, avaliar a produção enzimática em frascos agitados e biorreator por fermentação convencional, analizar o processo de produção por fermentação extrativa em sistema de duas fases aquosas (SDFA), bem como avaliar o potencial anticoagulante e determinar as características bioquímicas das enzimas fibrinolíticas produzidas pelo micro-organismo selecionado.

Objetivos específicos

- Selecionar uma espécie do gênero *Bacillus* com potencial em produzir enzimas fibrinolíticas.
- Otimizar as condições de produção da enzima fibrinolítica em frascos agitados por fermentação convencional, utilizando planejamento central composto estrela (PCCE) 2^3, avaliando a influência isolada e a interação das variáveis independentes concentração de farinha de soja (%), concentração de glicose (%) e agitação (rpm)
- Caracterizar bioquimicamente o extrato enzimático das condições otimizadas em frascos agitados, determinando pH e temperatura ótimos, estabilidade ao pH e à temperatura, efeito de inibidores e íons metálicos, além do efeito de degradação de coágulos de sangue *in vitro.*
- Avaliar a melhor condição de produção da enzima em biorreator de 7L, analisando a influência das variáveis independentes aeração (vvm) e agitação (rpm) com relação às variáveis respostas atividade amidolítica e fibrinolítica, utilizando planejamento fatorial completo 2^2.
- Caracterizar bioquimicamente o extrato enzimático com propriedade amidolítica, após fermentação convencional em biorreator, determinando efeito anticoagulante, especificidade do substrato, K_m e V_{max}, efeito de inibidores e íons metálicos, além da comparação entre as propriedades

amidolítica e fibrinolítica quanto à estabilidade por tempo de estocagem, pH e temperatura ótimos e estabilidade ao pH e à temperatura.

- Avaliar a melhor condição de produção e purificação integradas em frascos agitados por fermentação extrativa em sistema de duas fases aquosas (SDFA), analisando a influência das variáveis respostas massa molar do PEG (g/mol), concentração do PEG (%) e concrentação de sulfato de sódio (%) com relação às variáveis respostas coeficiente de partição (K), atividade fibrinolítica na fase superior (AFs) e atividade fibrinolítica na fase inferior (AFi) do sistema, utilizando planejamento fatorial completo 2^3.
- Caracterizar bioquimicamente o extrato enzimático com propriedade fibrinolítica, após fermentação extrativa em frascos agitados, determinando pH e temperatura ótimos, estabilidade ao pH e à temperatura, efeito de inibidores e íons metálicos.

CAPÍTULO I

1. REVISÃO BIBLIOGRÁFICA

1.1. O gênero *Bacillus* e sua importância na produção de enzimas de interesse industrial

O gênero *Bacillus* pertence à família *Bacillaceae*, é extremamente heterogêneo, tanto geneticamente quanto fenotipicamente. Estudos das regiões 16S, 23S e rpoB confirmam essa heterogeneidade e mostram que o gênero *Bacillus* pode ser dividido em muitos outros gêneros correlatos: *Alicyclobacillus, Amphibacillus, Aneurinibacillus, Brevibacillus, Filobacillus, Geobacillus, Gracilibacillus, Halobacillus, Jeotgalibacillus, Lysinibacillus, Marinibacillus, Paenibacillus, Salibacillus, Ureibacillus* e *Virgibacillus,* dos quais muitos destes constam nos registros da Fundação Oswaldo Cruz (SLEPECKY e HEMPHILL, 2006).

De acordo com a *List of Prokaryotic names with Standing in Nomenclature* do pesquisador J.P. Euzéby, há cerca de 265 espécies e 7 subespécies do gênero *Bacillus* spp. citadas, mas apenas 172 espécies aceitas. A partir do ano 2000, mais de 118 espécies foram incluídas no gênero e, nos últimos anos, foram incluídas novas espécies, tais como: *Bacillus beringensis* (YU et al., 2011); *Bacillus deserti* (ZHANG et al., 2011); *Bacillus zhanjiangensis* (CHEN et al., 2011); *B. berkeleyi* (NEDASHKOVSKAYA et al., 2012); *Bacillus daliensis* (ZHAI et al., 2012); *Bacillus eiseniae* (HONG et al., 2012); *Bacillus endoradicis* (ZHANG et al., 2012); *Bacillus iranensis* (BAGHERI et al., 2012); *Bacillus kochii* (SEILER et al., 2012); *Bacillus purgationiresistens* (VAZ-MOREIRA et al., 2012); *Bacillus cytotoxicus* (GUINEBRETIÈRE et al., 2013);

Os *Bacillus* spp. são extremamente atraentes industrialmente, devido a alta taxa de crescimento que leva a ciclos de fermentação curtos, capacidade para secretar proteínas extracelulares e pelo fato de os bioativos de algumas espécies geralmente serem considerados seguros na alimentação e na administração de

drogas intravenosas. Estima-se que o gênero *Bacillus* produza cerca de 50% do mercado total de enzimas, das quais, a maior parte são proteases. Além disso, já se conhece sobre a bioquímica, fisiologia e genética de algumas espécies do gênero, o que facilita ainda mais o desenvolvimento e uma maior exploração destes micro-organismos em processos industriais. Desta forma, com a caracterização do genoma do *B. subtilis* 168 e de algumas espécies relacionadas, o gênero *Bacillus* tem sido cada vez mais utilizado em tecnologias de bioprocessos à medida que avançamos na era genômica e proteômica (SCHALLMEY et al., 2004).

A maioria das enzimas utilizadas na produção industrial tem origem de bactérias do gênero *Bacillus* (RATHAKRISHNAN et al., 2011). A cada ano, novas enzimas de interesse industrial são produzidas por espécies deste gênero (Tabela 1) e esta diversidade enzimática desperta interesse por apresentar alta especificidade, expressiva atividade e estabilidade.

As proteases frequentemente produzidas por espécies de *Bacillus* possuem larga aplicação nas indústrias têxteis, de produtos de couro, de alimentos, produtos farmacêuticos e cosméticos, fabricação de cerveja, diagnóstico médico e na formulação de detergentes. Para uma enzima ser utilizada como um aditivo de detergente deve ser estável e ativa na presença de ingredientes típicos presentes nos detergentes, tais como surfactantes, agentes de branqueamento, amaciadores de tecidos, dentre outros (SATHYAVRATHAN e KRITHIKA, 2014).

A indústria farmacêutica tem grande interesse em diversas espécies do gênero *Bacillus* devido ao seu potencial em secretar enzimas para uso terapêutico, tais como, enzimas como a esfericase utilizadas no tratamento para bronquite crônica e pneumonia aguda e produzidas pelo *Bacillus sphaericus;* a ß-lactamase com fins para o tratamento de alergias agudas após administração de penicilina, sendo produzidas pelo *Bacillus cereus* (ZIMMER et al., 2009); a colagenase, enzima de grande importância na constituição da matriz extracelular do tecido conjuntivo, produzida pelo *Bacillus pumilus* (WU et al., 2010); e a peroxidase, produzida também por espécies do gênero *Bacilos,* que tem um papel importante na desintoxicação celular ao eliminar o peróxido de hidrogênio (RAJKUMAR et al., 2013).

Pesquisas recentes mostram que as proteases com potencial terapêutico, principalmente as proteases fibrinolíticas produzidas por *Bacillus* spp., apresentam uma importância bastante significativa na medicina e na indústria farmacêutica (AL-JUAMILY et al., 2013; BAJAJ et al., 2013).

Tabela 1. Enzimas de interesse industrial produzidas pelo gênero *Bacillus*

Espécie de *Bacilos*	Enzima	Aplicação Industrial	Referência
Bacillus circulans	Levansucrases	Alimentos; cosméticos; farmacêutica	OSEGUERA et al., 1996
Bacillus firmus	CGTase	Alimentos	GAWANDE et al., 1999
Bacillus circulans	Esterases	Alimentos; farmacêutica; papel e celulose; têxtil; bebidas: detergentes e agricultura	KADEMI et ai., 2000
Bacillus licheniformis	Tanase	Alimentos; cervejaria; farmacêutica; tratamento de efluentes	MONDAL et al., 2000
Bacillus thuringiensis	Quitinase	Agricultura; farmacêutica	REYES-RAMIREZ et al., 2004
Bacillus pumilus	Liase	Alimentos	KLUG-SANTNER et al., 2006
Bacillus subtilis	CotA lacase	Têxtil	DURÃO et al., 2008
Bacillus thuringiensis	Celulase e Chitinase	Hidrólise de resíduos agroindustriais; alimentos; bebidas; sacarificação	DUMAS et al., 2009
Bacillus cereus	Xilanase	Papel e cellulose; Alimentos	ROY e ROWSHANUL, 2009
Bacillus circulans	Endoglucanase	Hidrólise da fração amorfa da celulose	NIRMALA e SINDHU, 2011
Bacillus subtilis	Fitase	Alimentos; agricultura; ração animal	SHAMNA et al., 2012
Bacillus tequilensis	Lipase	Tratamento de efluentes; laticínios; alimentos; farmacêutico	BONALA e MANGAMOORI, 2012
Bacillus licheniformis	Colagenase	Couro; farmacêutica; medicina	BAEHAKI et al., 2012
Bacillus thuringiensis	Lacase	Efluente têxtil; papel	OLUKANNI et al., 2013
Bacillus amyloliquefaciens	Amilase	Alimentos; têxteis; detergentes; celulose	DEB. et al., 2013
Bacillus subtilis	Protease	Detergentes; alimentos; couro; medicina; farmacêutica; láctea: têxtil; bebidas	ZHU et al., 2013
Bacillus amyloliquefaciens	Protease fibrinolítica	Farmacêutica: medicina	HEO et al., 2013

1.2. Proteases: potencial terapêutico e aplicações atuais

As proteases (proteinases, peptidases ou enzimas proteolíticas) EC 3.4 crescem na indústria farmacêutica como uma promessa medicamentosa para vários tratamentos. A Food and Drug Administration (FDA) nos United States of America (U.S.A.) aprovou 12 terapias utilizando proteases, além de uma série de proteases completamente novas estarem em desenvolvimento clínico. Embora a utilização predominante dessas enzimas tenha se concentrado no tratamento de doenças cardiovasculares, estas também estão emergindo como agentes úteis no tratamento de fibrose cística, psoríase, sepse (infecção generalizada), hemofilia, inflamações recorrentes, desordens digestivas, hemorragia traumática, espasmos musculares, distúrbios na retina e outras doenças (CRAIK et al., 2011).

A primeira droga aprovada pelo FDA foi a u-PA (uroquinase tipo plasminogênio ativador) que inaugurou a era da terapia trombolítica à base de enzimas. A uroquinase derivada a partir da cultura de células de rim neonatal primário foi aprovada para a aplicação clínica em 1978 e permanece em uso pela sua capacidade de dissolver coágulos de sangue nos vasos sanguíneos e cateteres intravenosos (CRAIK et al., 2011), além de ter sido associada com a degradação de proteínas da matriz extracelular na invasão das células tumorais em metástase. Desse modo, esta protease também é alvo tanto para o tratamento como para diagnósticos do câncer (DASS et al., 2008).

Cascatas proteolíticas são responsáveis pela formação e dissolução de coágulos de sangue. Portanto, as enzimas que a compõem podem ser utilizadas para diversos benefícios terapêuticos (Figura 1).

O benefício da aplicação de proteases que atuam além do sistema de coagulação começou a surgir a partir de uma compreensão mais profunda da biologia de proteases e do domínio de novas tecnologias. Por exemplo, a anticoagulante PCa (proteína C ativada) pode ser notificada pelo seu efeito anti-inflamatório citoprotetor, enquanto que a pré-calicreína tecidual (pKAL) é um alvo para terapia gênica devido ao seu potencial anti-hipertensivo (CRAIK et al., 2011).

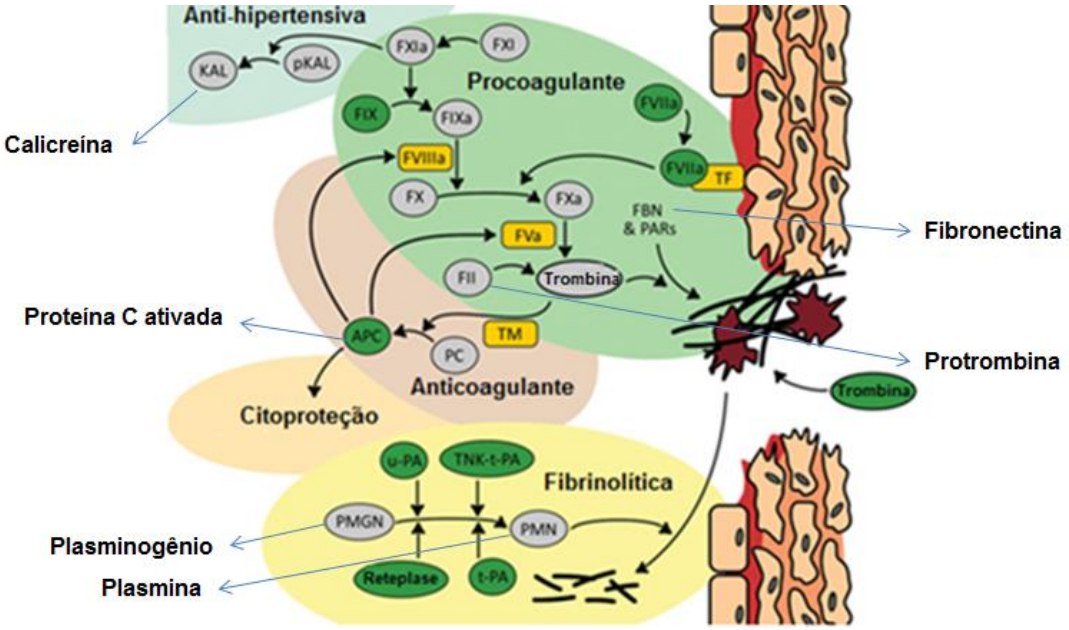

Figura 1. Proteases aplicadas com sucesso para terapias **pró-coagulante**: (FBN - fibronectina), (FII - protrombina); **anticoagulante**: (APC - proteína C ativada); **fibrinolítica**: (PMGN – plasminogênio), (PMN – plasmina), (TNKase ou TNK-t-PA - tenecteplase); **anti-hipertensiva**: (KAL – calicreína) e **citoprotetora**: (APC - proteína C ativada). Algumas proteases (mostradas em círculos verdes escuros) foram aprovadas para uso clínico. Cofatores de proteínas estão representados por retângulos alaranjados com arestas arredondadas (Fonte: Adaptado de CRAIK et al., 2011).

Atualmente várias proteases são utilizadas em diversos tipos de terapia, como por exemplo, a terapia genética para substituição de proteases constitutivas (KURSCHUS et al., 2010), aplicações médicas dermatológicas e cosméticas (CRAIK et al., 2011), tratamentos com toxinas botulínicas tipo B para paralisia e distonia cervical (TRUONG et al., 2009), proteases que auxiliam no processo digestivo (WOOLDRIDGE et al., 2009), dentre outras.

Como as proteases desempenham papéis fundamentais na fisiologia e fisiopatologia, estão disponíveis muitas opções para explorar a utilização dessas enzimas como agentes terapêuticos. A literatura relata 53 doenças hereditárias que são causadas por mutações nos genes de proteases que levam à perda de determinadas funções ou baixos níveis de expressão proteásica. Pesquisas futuras podem apresentar novas oportunidades para terapias de reposição de proteases para algumas dessas doenças (PUENTE et al., 2005).

O reconhecimento de que as proteases constituem uma classe de medicamentos seguros e eficazes estimula investigações para produção e aplicação de novos fármacos com a finalidade de melhorar as terapias atualmente aprovadas com essas enzimas. A engenharia de proteases é e continuará sendo utilizada com sucesso para modificar suas propriedades. Portanto, durante as últimas décadas as proteases proporcionaram resultados clínicos que sugerem um futuro promissor como uma classe terapêutica distinta, com diversas aplicações clínicas, destacando seu potencial em terapias vasculares (CRAIK et al., 2011).

O tratamento da trombose visa prevenir a formação de coágulos utizando anticoagulantes e, quando formandos, dissolvê-los utilizando agentes fibrinolíticos ou trombolíticos. Os medicamentos anticoagulantes que vêm sendo utilizados são a heparina, enoxaparina, varfarina e rivaroxabano e, para dissolver os trombos já formados na corrente sanguínea, são utilizados fibrinolíticos como uroquinase, pamiteplase, saruplase, estreptoquinase, anistreplase, monteplase, reteplase, duteplase, lanoteplase, alteplase, desmoteplase, estafiloquinase, tenecteplase e o ativador de plasminogênio tecidual (t-PA). Contudo, os anticoagulantes e fibrinolíticos utilizados causam diversos efeitos colaterais e requerem monitoramento constante devido ao alto risco de hemorragias, promovendo um desequilíbrio do sistema hemostático (KUMAR et al., 2011).

1.3. Proteases com propriedades anticoagulantes

A história da descoberta dos anticoagulantes é marcada pelo acaso. O efeito anticoagulante da heparina (administração intravenosa) foi descoberto por McLean em 1916, enquanto ele estava à procura de um pró-coagulante no fígado de um cão. Em 1941, foi registrada a primeira patente para um medicamento anticoagulante oral, o dicumarol, descoberto por cientistas da Universidade de Wisconsin. Esse medicamento recebeu melhorias em sua composição e, em 1946, foi desenvolvida a varfarina, anticoagulante oral da classe das cumarinas. A heparina, até a presente data, é o principal fármaco anticoagulante de ação rápida para o tratamento inicial de trombose venosa,

enquanto que os anticoagulantes orais só têm efeito depois de vários dias (GÓMEZ-OUTES et al., 2012).

Nos últimos anos, foram descobertos anticoagulantes capazes de inibir um único fator da cascata de coagulação, além de serem administrados em doses fixas indicadas para cada caso, não necessitando de monitoramento, oferecendo, assim, vantagens sobre anticoagulantes como a varfarina. Entre essas novas drogas, estão em uso a dabigatrana (ação direta sobre a trombina), a rivaroxabana e a apixabana (ação direta sobre o Fator Xa). A ação inibidora direta pelos novos fármacos anticoagulantes fornece uma resposta farmacocinética e farmacodinâmica mais previsível e consistente. No entanto, apesar dessas conquistas, os novos fármacos ainda oferecem desvantagens, tais como, dificuldade de eliminação da droga em pacientes com problemas renais, risco hemorrágico e tempo de meia vida curto do fármaco, podendo ocasionar trombose em caso de esquecimento da administração do medicamento (GÓMEZ-OUTES et al., 2012).

Diante do exposto, novas pesquisas vêm sendo realizadas na procura de anticoagulantes semelhantes aos anticoagulantes naturais fisiológicos, pois se acredita que, por esta via, os efeitos colaterais indesejados podem ser reduzidos (BERG et al., 2003; CHOI et al., 2013**.**

A Proteína C ativada é uma serino-protease natural do plasma sanguíneo que apresenta uma expressiva propriedade anticoagulante (Figura 2). Esta glicoproteína é sintetizada pelos hepatócitos e liberada na corrente sanguínea sob a forma de um zimogênio inativo. O zimogênio da proteína C é composto de duas cadeias peptídicas unidas por uma ligação dissulfeto (CRAIK et al., 2011).

A inibição da coagulação acontece pela ativação inapropriada da cascata de coagulação, ativando a via anticoagulante. As proteínas C e S inativadas se encontram livres no plasma e são dependentes da vitamina K. Para ativar a proteína C, a trombina produzida na cascata de coagulação se liga a trombomodulina (receptor de membrana), perdendo suas propriedades pró-coagulantes, transformando-se num potente ativador da proteína C (Figura 3). A proteína S se encontra inativada quando está complexada à proteína de ligação C4b, porém, quando livre, liga-se à proteína C, funcionando como um cofator e formando o complexo ativado PCa-PS (proteína C ativada e proteína

S) que tem a função de degradar os fatores Va (Ac-globulina ativado) e VIIIa (globulina anti-hemofílica ativada), limitando a produção de fibrina e impedindo a formação do coágulo sanguíneo (DAHLBACK e VILLOUTREIX, 2005).

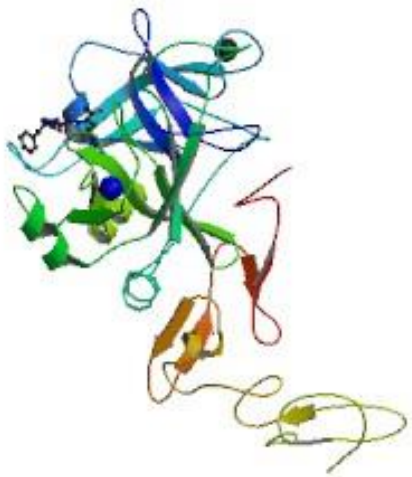

Figura 2. Estrutura cristalizada da proteína C humana ativada (PCa) complexada com D-Phe-Pro-Arg-Chloromethylketone (**PPACK**). (Fonte: Protein Data Bank: http://www.pdb.org/).

Além das propriedades anticoagulantes, outras propriedades são conferidas pela proteína C ativada, como função antiapoptótica e o auxílio no tratamento de diversas doenças inflamatórias, tais como a esclerose múltipla e artrite reumatóide e, em 2001, foi aprovada para o tratamento da sepse (CRAIK et al., 2011).

Alguns casos de doenças trombolíticas recorrentes estão relacionados com a resistência à proteína C ativada. Essa resistência ocorre devido a uma mutação do Fator V de Leiden, na qual acontece a substituição de uma guanina (G) por uma adenina (A) na posição 1691. Essa substituição resulta na troca da arginina 506 (Arg) do Fator V por uma glutamina (R506Q) (Glu). Com a substituição desse aminoácido, fica bloqueado o sítio onde a proteína C faz a clivagem natural do fator V, o que diminui a sua ação, aumentando a produção de trombina e consequentemente a formação de trombos (DUQUE e MELLO, 2003).

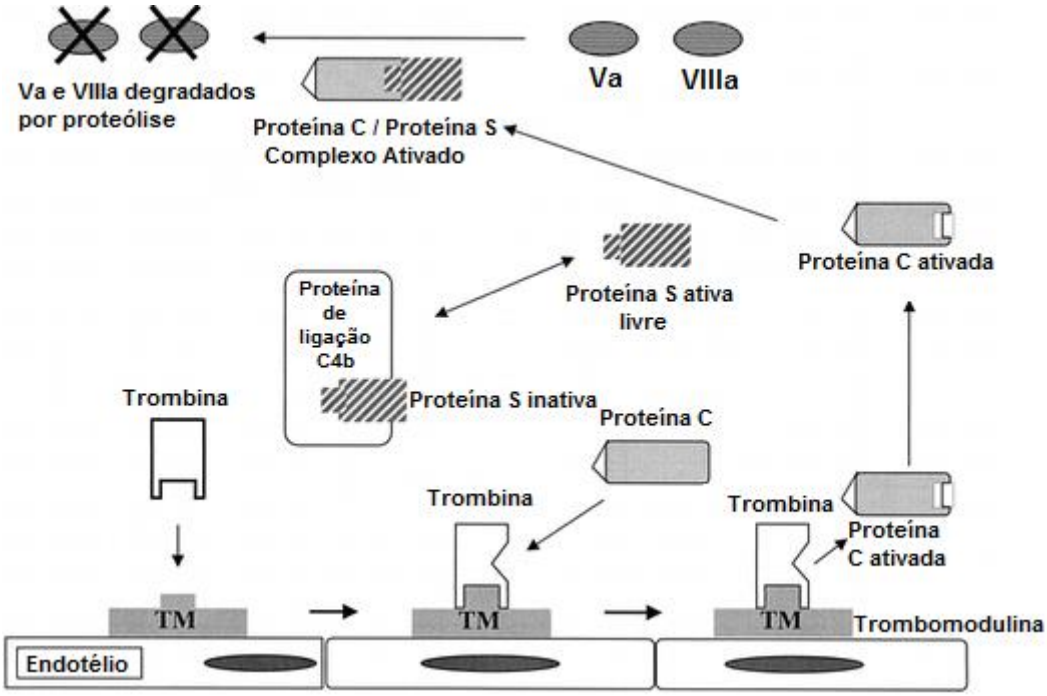

Figura 3. Sistema anticoagulante mostrando a ação da proteína C ativada (PCa) sobre os fatores de coagulação Va (Ac-globulina ativada) e VIIIa (globulina anti-hemofílica ativada).
(Fonte: Adaptado de http://www.pathologyoutlines.com/topic/coagulationproteinCS.html)

Metodologias utilizando substratos cromogênicos são bastante utilizadas para avaliar a atividade amidolítica de proteínas com propriedades semelhantes à proteína C ativada (BERG et al., 2003) e de outras proteínas relacionadas com o sistema hemostático (LU et al., 2010; CHOI et al., 2011; CHANG et al., 2012; CHOI et al., 2013; KIM et al., 2013)**.** A afinidade do sítio ativo dessas proteínas por determinados aminoácidos presentes nos peptídeos cromogênicos testados é indicada pela intensidade da cor após a reação, ou seja, quanto maior a afinidade da enzima pelo aminoácido ligado ao *p*NA (*p*-nitroanilide) e pelos outros aminoácidos que compõem o peptídeo cromogênico, maior é a liberação de *p*NA que gera uma intensidade da cor amarela no final da reação.

Pesquisas mostram a produção de enzimas com propriedade bifuncional (anticoagulante e fibrinolítica) (LU et al., 2010; HASSANEIN et al., 2011; CHOI et al., 2013). Essa característica conferida por tais enzimas gera expectativa e sugere que este potencial simultâneo atribua um maior controle hemostático ao ser administrado como fármaco.

1.4. Proteases com propriedades Fibrinolíticas

As enzimas fibrinolíticas ou trombolíticas são proteínas que degradam coágulos de sangue e normalmente são geradas nas células endoteliais dos vasos sanguíneos. As células endoteliais existem em todo o corpo: nas artérias, veias e no sistema linfático. Dessa forma, a baixa produção de enzimas fibrinolíticas pode levar ao desenvolvimento de coágulos sanguíneos em praticamente qualquer parte do corpo. Quando o corpo envelhece, a produção destas enzimas começa a diminuir, tornando o sangue mais propenso à coagulação. Esse mecanismo pode conduzir a diversas complicações envolvendo o sistema cardiovascular que podem levar à invalidez ou até mesmo à morte (VERSTEEG et al., 2013).

O mecanismo da coagulação sanguínea tem por finalidade a formação de fibrina insolúvel, formada a partir do fibrinogênio pela ação da trombina (BAKER et al., 2011). O fibrinogênio é uma glicoproteína plasmática essencial no processo hemostático e para a formação do coágulo. A concentração desta proteína no plasma sanguíneo normalmente está entre 1,5 e 4,0g/L, mas pode apresentar níveis mais elevados em determinadas condições, como por exemplo na gravidez. Estruturalmente, o fibrinogênio humano é composto por dois domínios globulares externos D, que estão ligados por meio de um domínio central E (Figura 4). Cada domínio D é composto de três cadeias polipeptídicas (α, β e ɣ) que juntas formam uma configuração em espiral. As massas moleculares das cadeias do fibrinogênio humano são 64, 55 e 47 kDa, para as cadeias α, β e ɣ, respectivamente. A clivagem do fibrinogênio pela trombina ocorre em sequências específicas de aminoácidos presentes no final das cadeias polipeptídicas, removendo os peptídeos N-terminais (fibrinopeptídeos A (FpA) e B (FpB)) e expondo os locais de polimerização. A polimerização ocorre através de interações não covalentes da cadeia polipeptídica exposta nos locais de ligação complementares presentes no domínio D de uma molécula vizinha (LEVY et al., 2012).

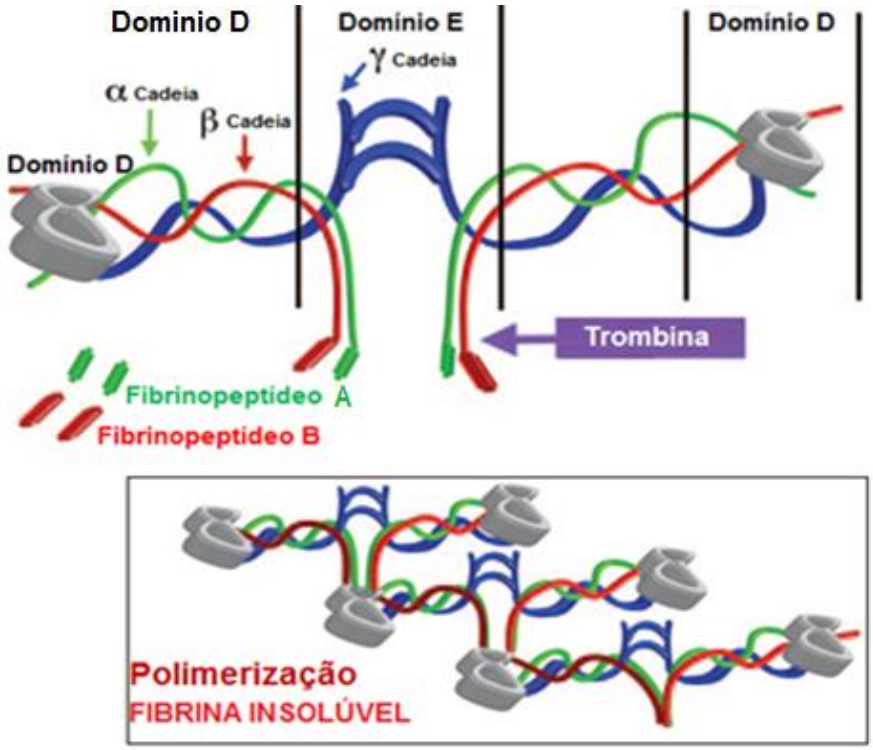

Figura 4. Estrutura do fibrinogênio contendo o domínio central E, os domínios globulares externos D com suas respectivas cadeias polipeptídicas (α, β e ɣ) e a clivagem dos fibrinopeptídeos A (FpA) e B (FpB) por ação da trombina, expondo os locais de polimerização para interações com as moléculas vizinhas e formação de fibrina insolúvel (Fonte: Adaptada de LEVY et al., 2012).

Os coágulos de sangue são compostos principalmente pela fibrina, uma proteína fibrosa que diminui o fluxo sanguíneo, aumentando a viscosidade do sangue. Essa proteína, quando reticulada, obstrui os vasos, interrompe o fornecimento de oxigênio e eleva a pressão arterial. Assim, as proteases fibrinolíticas são importantes porque mantêm o fluxo sanguíneo saudável, auxiliando no sistema de compensação circulatória do corpo (HARRIS et al., 2013).

No organismo estão presentes vinte enzimas que auxiliam no processo de coagulação do sangue, mas apenas uma enzima do corpo pode quebrar o coágulo, a plasmina (Figura 5A). A descoberta da primeira enzima fibrinolítica, a plasmina, aconteceu de maneira gradual com a colaboração de vários pesquisadores: Tillet e Garner (1933) descobriram a fibrinolisina estreptocócica; Milstone (1941) verificou que a ação da fibrinolisina estreptocócica dependia de uma globulina humana que ele chamou de fator

lítico e Kaplan (1944) constatou que esse fator lítico plasmático era uma protease ativada pelo fator estreptocócico; Christensen (1945) denominou o fator estreptocócico de estreptoquinase, o precursor plasmático do plasminogênio, sendo a protease ativa chamada de plasmina (DUQUE e MELLO, 2003).

Outra enzima fibrinolítica de importância é a Nattokinase (Figura 5B), que foi descoberta em 1980 pelo Dr. Hiroyuki Sumi, enquanto trabalhava como pesquisador durante sua graduação em química fisiológica da Faculdade de Medicina da Universidade de Chicago. O Dr. Sumi pesquisou por muito tempo enzimas trombolíticas, testou 173 alimentos naturais e encontrou o que procurava quando o Natto (alimento tradicional japonês feito de soja e fermentado pelo *Bacillus subtilis natto*) foi deixado em contato com um trombo artificial de fibrina em uma placa de Petri incubada a 37ºC (temperatura próxima à corporal). O trombo em torno do natto foi degradado completamente ao final de 18 horas. A enzima foi chamada de nattokinase, que significa "enzima em natto" (SUMI et al., 1987; MERUVU e VANGALAPATI, 2011).

O sistema fibrinolítico pode ser ativado pelas vias intrínseca e extrínseca. Quando ativado pelo mecanismo intrínseco, há formação de calicreína pelo endotélio lesado. A calicreína juntamente com o cininogênio de alto peso molecular e o Fator XII (Fator Hageman) ativado agem sobre o plasminogênio ativando-o em plasmina. Quando ativado pela via extrínseca, o endotélio vascular sintetiza ativadores de plasminogênio tipo tecidual (t-PA) e tipo uroquinase (u-PA), que nesta via também tem a função de converter o plasminogênio inativo em plasmina ativa, que degrada o coágulo gerando os produtos de degradação da fibrina (PDF): fragmento X, o primeiro a ser formado, é o maior deles, podendo ainda ser lentamente coagulado pela trombina; os fragmentos Y, E, e o dímero D (D-Di) têm pesos moleculares menores e não podem sofrer ação da trombina (LIMA et al., 2006).

Os mecanismos de ação e inibição do sistema fibrinolítico são desencadeados de acordo com a necessidade harmônica do sistema hemostático. Nesse processo, o endotélio vascular controla a síntese dos ativadores de plasminogênio ou pode ocorrer também a ação dos inibidores do sistema fibrinolítico (PAI-1 e PAI-2) que atuam sobre os ativadores de plasminogênio tipo tecidual (t-PA) e tipo uroquinase (u-PA) ou ainda pela ação de inibidores α

$_2$ - antiplasmina, α_2 - macroglobulina e α_1 - antitripsina, que agem inibindo diretamente a plasmina formada, como mostra na Figura 6 (LIMA et al., 2006; MACEDO, 2009).

(A) **(B)**

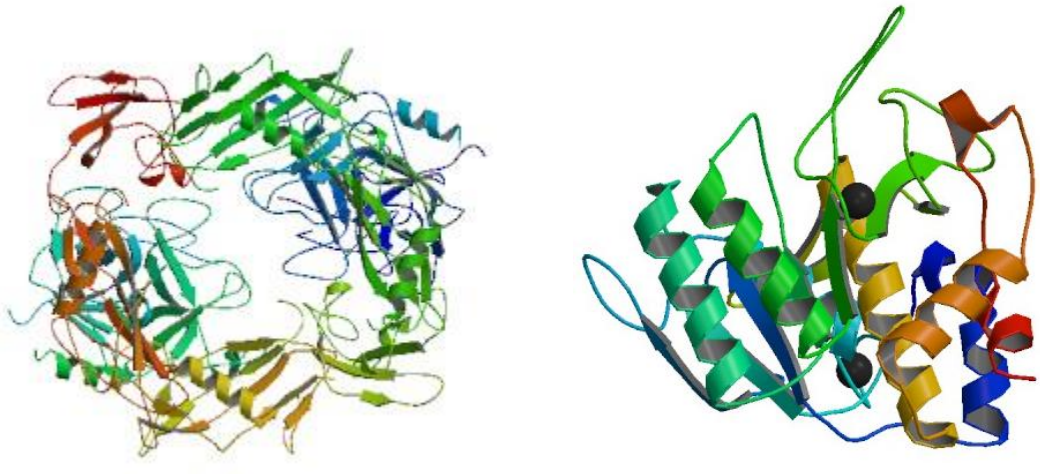

Figura 5. (A) Complexo do domínio catalítico da plasmina humana com estreptoquinase e (B) Estrutura cristalizada da Nattokinase (enzima fibrinolítica) do *Bacillus subtilis natto* (Fonte: Protein Data Bank: http://www.pdb.org/).

Todos esses mecanismos envolvendo a hemostasia são cada vez mais estudados e novos fatos vêm sendo elucidados, gerando novas teorias e apresentando novos conceitos para ativação das proteases que compõem o sistema hemostático. Na nova teoria, após a lesão vascular, as plaquetas aderem ao local danificado e através de interações entre receptores plaquetários com ligantes extracelulares e proteínas solúveis, o dano vascular é induzido por exposição do fator tecidual subendotelial, gerando vestígios de trombina transitória com vários efeitos sobre outros fatores de coagulação e plaquetas. Os íons de cálcio e os fosfolipídios são cofatores necessários para todas as reações que controlam a hemostasia. Esse novo modelo hemostático elucida o fato que as propriedades da trombina vão além da formação do coágulo de fibrina, relatando que a trombina tem efeito direto sobre os outros constituintes da coagulação, plaquetas e células endoteliais, além de participar

da sua própria regulação negativa influenciando diretamente os mecanismos da via anticoagulante e o sistema fibrinolítico (VERSTEEG et al., 2013)

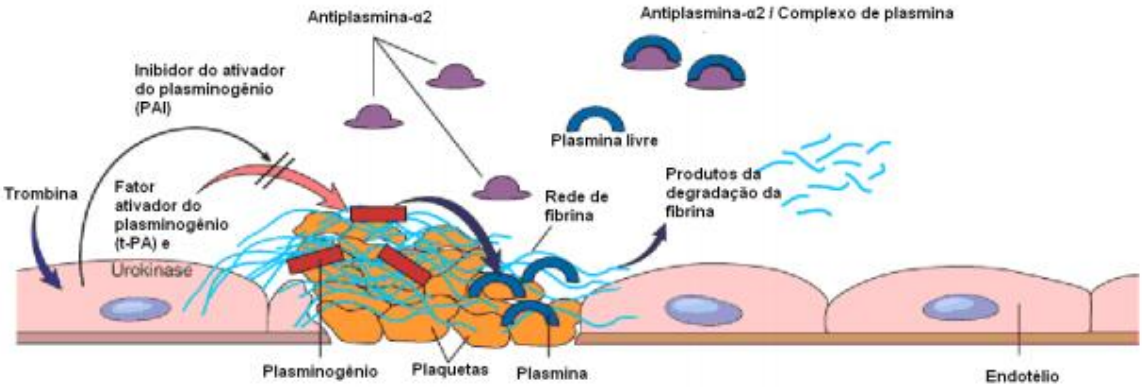

Figura 6. Mecanismo de ação do sistema fibrinolítico (Fonte: MACEDO, 2009).

Atualmente, embora a terapia trombolítica seja amplamente adotada como uma estratégia de primeira linha no tratamento de distúrbios cardiovasculares, ainda existe necessidade de trombolíticos com melhor farmacocinética e farmacodinâmica. Esforços estão concentrados no desenvolvimento de formulações de drogas com propriedades semelhantes à plasmina e à nattokinase, que degradam diretamente o coágulo sanguíneo. Diante desse fato, pesquisadores descobriram enzimas fibrinolíticas provenientes de diversas fontes, inclusive microbianas.

1.4.1. Obtenção e comercialização de enzimas fibrinolíticas

Ao longo dos anos, diversas fontes de enzimas fibrinolíticas foram descobertas. Dentre essas fontes, potentes proteases fibrinolíticas são produzidas em processos fermentativos envolvendo micro-organismos, principalmente por várias espécies do gênero *Bacillus*, dentre estas: *Bacillus firmus* (SEO e LEE, 2004); *Bacillus polymaxa* (MAHMOUD et al., 2011); *Bacillus subtilis* (NGUYEN et al., 2013); *Bacillus licheniformis* (AL-JUAMILY et al., 2013); *Bacillus cereus* (BAJAJ et al., 2013); *Bacillus amyloliquefaciens*

(HEO et al., 2013). Contudo, outras pesquisas mostram que enzimas fibrinolíticas podem ser provenientes de diversas fontes (Tabela 2).

Enzimas microbianas têm se destacado como um dos principais produtos biotecnológicos. As enzimas terapêuticas começaram a ser utilizadas na década de 80 e vêm ganhando cada vez mais importância na indústria biotecnológica. Uma ampla gama de enzimas, de diferentes fontes e para diversos usos terapêuticos pode ser encontrada no mercado. O Brasil tem grande potencial para obtenção de novos fármacos enzimáticos, por ter uma enorme quantidade e variedade de produtos naturais e uma notável biodiversidade microbiana disponível para transformação dos mesmos em produtos de maior valor agregado. Enzimas fibrinolíticas são exemplos de valiosos bioprodutos, devido sua eficiência, alta especificidade e grande potencial para uso terapêutico. Dessa forma, a relevância do uso de enzimas como medicamento se dá pelo fato de que pequenas quantidades desses catalisadores biológicos podem produzir efeitos bastante significativos em condições fisiológicas (ZIMMER et al., 2009).

O mercado de enzimas está divido de duas formas: enzimas industriais (para indústria de alimentos e ração animal) e enzimas especiais (terapêuticas, para diagnóstico, para pesquisa e para química quiral) (MONTEIRO e SILVA, 2009).

O uso da engenharia enzimática aliada à tecnologia do DNA recombinante e expressão heteróloga de enzimas vão ter um avanço significativo na próxima década, sendo importante no desenvolvimento de novos produtos industriais produzidos por via enzimática. Contudo, as enzimas não podem ser consideradas a única ferramenta para o avanço nos processos industriais, é necessário também, o conhecimento da bioquímica, fisiologia e a genética dos micro-organismos. Logo, a contribuição de novas áreas da biologia, como a proteômica, genômica e metabolômica, será de fundamental importância para a aplicação de micro-organismos em escala industrial e o desenvolvimento de tecnologias mais eficazes (MONTEIRO e SILVA, 2009).

Tabela 2. Fontes de enzimas fibrinolíticas

FONTE	REFERÊNCIA
Algas (*Costaria costata*)	KIM et al., 2013
(*Codium fragile*)	CHOI et al., 2013
Fungos (*Bionectria* sp.)	ROVATI et al., 2010
(*Candida Guilliermondii)*	RASHAD et al., 2012
(*Aspergillus oryzae*)	SHIRASAKA et al., 2012
Bactérias (*Streptomyces* sp.)	BAJAJ e SHARMA, 2011
(Pseudomonas aeruginosa)	RAJ et al., 2012
(Halophilic Lactic Acid Bacteria)	PRIHANTO et al.,2013
(Escherichia coli)	KOTRA et al., 2013
(Yersinia pestis)	KORHONEN et al., 2013
(Streptomyces sp.)	MEDEIROS e SILVA et al., 2013
(Paenibacillus sp)	VIJAYARAGHAVAN e VINCENT, 2014
Cobras (*Daboia russelii russelii*)	MUKHERJEE et al., 2013
(*Trimeresurus malabaricus*)	KUMAR et al., 2013
Poliquetas (*Neanthes japonica*)	DENG et al, 2010
(*Cirriformia tentaculata*)	PARK et al., 2013
Insetos (*Yellow Mealworm*)	HUANG et al., 2012
(*Eupolyphaga sinensis*)	WANG et al., 2012
Cogumelos (*Schizophyllum commune)*	PARK et al, 2010
(Ganoderma lucidum)	KUMARAN et al., 2011
Vegetais (*Artocarpus heterophyllus* latex)	SIRITAPETAWEE et al., 2012
(Euphorbia hirta latex)	PATEL et al., 2012
(Carica candamarcensis fruta)	BILHEIRO et al., 2013
Arraias marinhas (*Dasyatis sephen*)	KUMAR et al., 2011
(Aetobatis narinari)	
Minhocas (*Lumbricus rubullus*)	YONG-GANG et al*.,* 2010
(*Lumbricus bimastus)*	

O uso endovenoso de enzimas microbianas requer purificação elevada e preservação da atividade enzimática, evitando a desnaturação protéica e a proteólise. Várias questões devem ser consideradas para o uso de enzimas terapêuticas, dentre elas, alta atividade e estabilidade em pH fisiológico, baixa taxa de eliminação do organismo, baixa resposta imunológica e independência de cofatores exógenos. Além do mais, quando micro-organismos são utilizados como fonte destas biomoléculas, é importante utilizar cepas não patogênicas para evitar a presença de toxinas (ZIMMER, 2009).

A busca por substâncias que dissolvem coágulos de sangue é uma das principais prioridades para as indústrias farmacêuticas. Genentech em colaboração com investigadores da Universidade de Lueven (Bélgica) conseguiram clonar o gene que produz t-PA (RIJKEN e COLLEN, 1981) e diversas empresas estão trabalhando para produzir t-PA a partir de rDNA com mostra a Tabela 3.

A eficácia bioquímica e a viabilidade comercial de t-PAs produzidos a partir de tecnologias envolvendo rDNA continuam a ser demonstradas em diversas pesquisas realizadas por Universidades do mundo inteiro, além do sucesso proveniente de sua comercialização. O investimento diferenciado nas instituições de pesquisas em países diversos reflete na qualidade das enzimas fibrinolíticas produzidas. (Fonte: https://www.princeton.edu). Diante desse fato, é notória a necessidade de patrocínio para um maior avanço nas pesquisas envolvendo enzimas fibrinolíticas brasileiras.

É preocupante o atraso na produção de enzimas no Brasil, apesar da nossa vocação para a biotecnologia e potencial para produção e uso de biocatalisadores. No contexto mundial, a situação do Brasil é incomparável em relação à quantidade e à variedade de recursos naturais disponíveis para serem transformados, por tecnologia enzimática, em produtos úteis e de maior valor agregado. O Brasil possui uma notável biodiversidade microbiana a ser estudada, por uso direto ou após melhoramento genético, visando à produção de enzimas por processos fermentativos (Fonte: http://www.redetec.org.br).

Table 3. Empresas e Universidade envolvidas no desenvolvimento e comercialização de enzimas fibrinolíticas

Enzima	Empresas/Universidades	Descrição do Projeto
Estreptoquinase	Hoechst-Roussel (F. R. G.)	Produção por bactéria
	KabiVitrum (Sweden)	Produção por bactéria
Uroquinase	Abbott Laboratories (U. S.)	Extração por cultura de células renais
	Genex (U. S.)lMitsui Toatsu Chemicals, Inc. (Japão)	Produção por rDNA
	Genentech (U. S.)/Grunenthal (F. R.G.)	Produção por rDNA
Ativador de Plasminogênio Humano	Genentech/Universidade de Leuven (Bélgica)	Produção por rDNA
	Mitsubishi Chemical Industries, Inc. (Japão)/Kyowa Hakko Kogyo (Japão)	Produção por rDNA
	Biogen S.A. (Switz.)/Fujtsawa (Japão) Integrated Genetics (U.S,)	Produção por rDNA
	Toyobo Pharmaceutical (Japão) Chiron (U. S.)	Produção por rDNA
	Collaborative Resarch (U.S.)/Green Cross (Japão)	Extração por cultura de células renais
Agentes Fibrinolíticos e Anticoagulantes	Genentech/Yamanouchi Ltd. (Japão)	Desenvolvimento de estirpes de micro-organismos que produzem agentes fibrinolíticos
	Genex/Yamanouchi Ltd.	

(Fonte: https://www.princeton.edu)

Tabela 4. Preço comercial de alguns agentes fibrinolíticos

Fonte de obtenção da enzima	Enzima	Preço/Kg	Pedido mínimo	Prazo de entrega	Capacidade de fornecimento	País	Empresa/fornecedores
Extrato da soja (Natto) fermentado pelo *Bacillus subtilis natto*	Nattokinase	FOB: US $ 180-220 /Kg	5 Kg	20 dias	20 toneladas/ semana	Taiwan	Contek Life Science Co., Ltd.
Extrato da soja	Nattokinase	FOB: US $ 99-429/Kg	20 Kg	20 dias	20 toneladas/mês	Taiwan	Contek Life Science Co., Ltd.
Extrato da soja	Nattokinase	FOB: US $ 1-2000/Kg	25 Kg	5 dias	10 tonaladas/mês	China	Shaanxi Kingsci Biotechnology Co., Ltd.
Extrato de minhoca	Lumbrokinase	FOB: US $ 1550-3200/Kg	5 Kg	10 dias	1000 kiloampere/ quarto	China	Beijing Herbal Health Biotech Limited Liability Company
Extrato de minhoca	Lumbrokinase	FOB: US $ 1000-2000/Kg	1 Kg	3 dias	50 Kg / mês	China	Shaanxi Sciphar Hi-Tech Industry Co., Ltd.
Pó liofillizado de *Streptococcus* beta-hemolítico	Estreptoquinase	FOB: US $ 1-10/Kg	1 Kg	7 dias	1000 Kg/ano	India	Swapnroop Drugs and Pharmaceuticals

(Fonte: http://portuguese.alibaba.com/manufacturers/fibrinolytics.html)

Apesar das diversas fontes encontradas, as enzimas fibrinolíticas extracelulares microbianas e principalmente as produzidas pelo gênero *Bacillus* têm se destacado pela sua facilidade de obtenção/extração, expressiva atividade, propriedades fisicoquímicas compatíveis ao sistema sanguíneo (temperatura 37°C e pH 7,0) e elevada estabilidade (MUKHERJEE et al., 2012).

1.4.2. Propriedades bioquímicas das enzimas fibrinolíticas produzidas pelo gênero *Bacillus*

A diversidade de características bioquímicas das enzimas fibrinolíticas produzidas pelos *Bacillus* (Tabela 5) tem atraído cada vez mais o interesse de pesquisadores do mundo inteiro. De acordo com o mecanismo catalítico, a maioria das enzimas produzidas por espécies de *Bacillus* são caracterizadas como serinoproteases; contudo, podemos encontrar metalo proteases e até mesmo serino-metalo proteases. Na maioria dos casos o pH ótimo destas enzimas é encontrato entre a faixa neutra e alcalina e temperatura ótima a partir de 37^0C, podendo chegar até a 60^0C. Quanto ao peso molecular podemos encontrar uma ampla faixa de 18 kDa até 33 kDa, e alguns trabalhos relatam pesos moleculares de 46kDa (DEEPAK et al., 2010) e 65 kDa (CHOI et al., 2010). O ponto isoelétrico se encontra em torno de 5.8 a 9.2.

Outra importante propriedade que tem sido bastante estudada é a sequência de aminoácidos que constitui os peptídeos das enzimas fibrinolíticas. Como já se sabe, as principais características dos aminoácidos são em decorrência do tipo de cadeia lateral. Dessa maneira, fica claro que a atividade biológica de um dado peptídeo, bem como suas características físico-químicas, estão intimamente ligadas à sequência e aos tipos de aminoácidos que o constituem. O perfil sequencial dos aminoácidos das extremidades N-terminal ou amino terminal das cadeias peptídicas da maioria das enzimas fibrinolíticas produzidas por espécies de *Bacillus*, quando comparado, possuem em boa parte dos casos analisados, sequenciamentos idênticos (Tabela 6)

Tabela 5. Propriedades bioquímicas das proteases fibrinolíticas do gênero *Bacillus*

Micro-organismo	**Mecanismo catalítico**	**Temperatura e pH ótimos**	***PM**	****pI**	**Referências**
Bacillus subtilis IMR-NK1	Serinoprotease	55ºC e 7.8	31.5 kDa	8.3	CHANG et al., 2000
Bacillus amyloliquefaciens CD-4	Serinoprotease	48ºC e 9.0	28 kDa	8.0	PENG et al., 2003
Bacillus subtilis DC33	Serinoprotease	55ºC e 8.0	30 kDa	8.7	WANG et al., 2006
Bacillus Subtilis	Serinoprotease	60°C e 9.0	29.93 kDa	6.35	CHANG et al., 2012
Bacillus sp. AS-S20-I	Serinoprotease	37ºC e 7.4	32.3 kDa	5.8	MUKHERJEE et al., 2012
Bacillus subtilis HQS-3	Serino-metalo protease	45-50ºC e 8.0	26 kDa	9.0 - 9.2	HUANG et al., 2013
Bacillus subtilis K42	Metalo protease	40ºC e 9.4	20.5 kDa	-	HASSANEIN et al., 2011
Bacillus polymaxa NRC-A	Serinoprotease	40ºC e 9.5	18 kDa	-	MAHMOUD et al., 2011
Bacillus subtilis ICTF-1	Serinoprotease	50ºC e 9.0	28 kDa	-	MAHAJAN et al., 2012

* Peso molecular (PM); * * ponto isoelétrico (*pI*)

Tabela 6. Comparação do perfil amino terminal das cadeias peptídicas das enzimas fibrinolíticas produzidas por espécies de *Bacillus*

Bacilos	**Sequência do aminoácido N-terminal da enzima fibrinolítica**	**Referência**
B. subtilis IMR-NK1	AQPVPNGRTAIKA	CHANG et al., 2000
B. amyloliquefaciens DC-4	AQSVPYGVSQIKAPALHS	PENG et al., 2003
B. firmus NA-1	IVGGYEQZAHSQPHQ	SEO et al., 2004
Bacillus subtilis QK02	AQSVPYGISQIKAPALHSQG	KO et al., 2004
B. pumilus TYO-67	AQTVPYGIPQIKAD	TAKAHASHI et al., 2004
Bacillus subtilis CD33	AQSVPYGIPQIKAPA	WANG et al., 2006
Bacillus subtilis A26	AQSVPYGISQI	AGREB et al., 2009
B. amyloliquefaciens DJ-5	AQSVPYGISQIKAPA	CHOI et al., 2009[a]
Bacillus subtilis A1	QTGGSIIDPINGYN	YEO et al., 2011
Bacillus subtilis	AQSVPYGISQIK posição 1–12 VAVIDSGIDSSHPDLNVR posição 28–45 YPSTIAVGAVNSSNQR posição 171-186	NGUYEN et al., 2013

Apesar de todas as pesquisas e potenciais mostrados por estas novas enzimas que vêm sendo descobertas, a principal problemática ainda é minimizar o custo de produção e aumentar a escala em nível industrial. Para isso, estudos de otimização de bioprocessos e escalonamento da produção se fazem cada vez mais necessários.

1.4.3. Otimização e escalonamento da produção de enzimas fibrinolíticas

O custo do processo de produção de proteases é um dos principais obstáculos para a aplicação bem sucedida dessas enzimas nas indústrias. Para as enzimas fibrinolíticas, muitas tentativas em laboratórios têm sido realizadas para melhoria das mesmas, incluindo a seleção de um meio de

cultura ideal, a otimização das condições ambientais e nutricionais e, até mesmo, modificações genéticas de linhagens (JO et al., 2011).

Para as condições de fermentação, a seleção dos componentes do meio de cultivo é geralmente um fator crítico na produção de enzimas fibrinolíticas. Uma vez que os micro-organismos possuem diversas características fisiológicas, é necessário encontrar as condições favoráveis para a produção de acordo com cada espécie microbiana envolvida no processo. Para melhorar o rendimento da produção enzimática é necessário otimizar o processo e aumentar o escalonamento de produção dessas enzimas fibrinolíticas (AVHAD e RATHOD, 2014).

Embora as estratégias tradicionais para otimização dos processos de produção sejam relativamente simples, é frequente não encontrar a condição ótima para a resposta desejada. Em busca do melhor resultado, é comum a utilização de ferramentas estatísticas que auxiliem na análise de interações mútuas entre os fatores e as estimativas do valor exato da variável resposta em tempo mínimo com um número reduzido de ensaios. Entre outros, o planejamento central composto (PCC) é uma das ferramentas estatísticas utilizadas para auxiliar na otimização dos processos fermentativos, e a Análise de Metodologia de Superfície de Respostas (MSR) é utilizada para modelar, analisar e, finalmente, encontrar a condição ótima para a obtenção da resposta de interesse. Assim, cada vez mais pesquisas mostram a otimização da produção de enzimas fibrinolíticas utilizando PCC e MSR (LIU et al., 2005; DEEPAK et al., 2010; MAHAJAN et al, 2010; KANAGASABAI et al., 2013).

Um importante fator que aumenta em muitas vezes o rendimento e a atividade enzimática é a produção de enzimas em larga escala, realizada através de fermentações em biorreatores de tanque agitado aumentando o escalonamento do produto de interesse. Os reatores biológicos proporcionam em geral um aumento no rendimento do bioativo produzido e melhora a relação entre o desenvolvimento microbiano e o ambiente ao qual ele é submetido, gerando melhores resultados de obtenção do produto. Na produção em biorreatores, alguns fatores são essenciais para a obtenção dos resultados desejados, como por exemplo, a agitação e a aeração, parâmetros essenciais nos processos fermentativos. A agitação influencia a mistura e transferência de calor e massa, sendo um parâmetro importante para o crescimento e

desempenho de células microbianas. A aeração também é um fator chave nos processos aeróbios, devido ao seu impacto sobre a oferta de oxigênio. Em geral, a transferência de oxigênio depende da taxa de fluxo do gás, do tipo de agitador, da velocidade de agitação e das propriedades dos líquidos envolvidos no sistema (GANGADHARAN et al., 2011).

O fornecimento de oxigênio está ligado ao metabolimo celular, auxiliando na obtenção do produto desejado. Em células com metabolismo aeróbio, o oxigênio é utilizado como aceptor final de elétrons no término da cadeia respiratória e na reoxidação de coenzimas. Isso resulta na produção de adenosina trifosfato (ATP), fonte energética celular. Contudo, o oxigênio é pouco solúvel em água, precisando ajustar sua concentração de saturação em mg/L. Dessa forma, não é possível fornecer de uma só vez todo o oxigênio necessário em um bioprocesso. Esse deve ser continuamente liberado no bioreator durante o cultivo. O controle do suprimento e a demanda de oxigênio determinam a taxa ótima de transferência de massa ou taxa de absorção de oxigênio da fase gasosa para a líquida. A taxa de oxigênio dissolvido é dada pela diferença entre o oxigênio fornecido e o utilizado pelas células. O suprimento do oxigênio está relacionado com a velocidade de agitação, o grau de mistura, a vazão de ar, a temperatura e a geometria do biorreator. Esses fatores são relacionados com a transferência de massa gás-líquido. E a demanda de oxigênio está relacionada à fisiologia celular, que determina a quantidade necessária de oxigênio durante o bioprocesso (ZULKEFLEE e AZIZ, 2007).

Outro fator importante para produção bem sucedida, é a utilização de agentes indutores, que é de fundamental importância para a síntese enzimática, uma vez que a presença desses contribui para obtenção de atividades enzimáticas mais elevadas. A farinha de soja, resíduo da indústria de produção de óleo de soja, demonstra ser um excelente substrato indutor, estimulando o crescimento de uma variedade de micro-organismos e aumentando a atividade de diversas enzimas produzidas (LIMA et al., 2011), incluindo enzimas fibrinolíticas (SALES et al., 2013, MEDEIROS e SILVA et al., 2013).

Porém, após todas essas fases que incluem, encontrar as condições adequadas para o bioprocesso da produção enzimática, selecionar um

substrato indutor para produção da enzima fibrinolítica, otimizar a produção e aumentar a escala do produto almejado, para que este produto possa ser industrializado e posteriormente comercializado, é necessária a obtenção de um produto puro, principalmente em se tratando de um fármaco, como é o caso das enzimas fibrinolíticas. Diante dessa necessidade, diversos métodos de purificação vêm sendo investigados e, dentre esses, a fermentação extrativa é considerada uma alternativa promissora na redução de etapas dos processos de purificação.

1.5. Fermentação extrativa utilizando sistema de duas fases aquosas (SDFA)

Existe uma busca constante das indústrias por eficiência e baixo custo nos processos de obtenção de biomoléculas de alto valor agregado. A fermentação ou bioconversão extrativa tem sido apresentada como solução para superar a baixa produtividade dos processos biotecnológicos (DELOISA et al, 2009; NALINANON et al, 2009).

A bioconversão extrativa em sistema de duas fases aquosas (SDFA) é integrada pelas seguintes etapas: upstream, que antecede a transformação, seguida da etapa de transformação propriamente dita e a etapa de à jusante (downstream), momento que acontece o processo de recuperação do bioproduto desejado (RATANAPONGLEKA, 2010).

A fermentação extrativa é um processo que integra o cultivo microbiano à produção e recuperação do bioprotudo *in situ* de maneira simultânea (Figura 7), sendo utilizado para aumentar o rendimento dos processos fermentativos convencionais. O sistema de duas fases aquosas (SDFA) utilizado para uma fermentação extrativa é uma alternativa considerada bastante promissora quando comparado com os processos de fermentações convencionais, desde que seja um meio propício para o crescimento e estabilização das células microbianas e adequado para o favorecimento de produção da biomolécula, sem que esta sofra desnaturação ou algum tipo de desestabilização, aumentando assim a produtividade do processo. O SDFA é formado por dois polímeros ou um polímero e um sal em solução aquosa. Esta técnica é

constituída pela mistura de duas soluções acima de certa concentração crítica, indicada por um diagrama de fases em que a formação de duas fases aquosas imiscíveis é observada (SINHA et al., 2000; NG et al., 2013).

Em um comportamento ideal para um processo de fermentação extrativa, espera-se que as células e os componentes do meio se concentrem, preferencialmente, em uma das fases do sistema, enquanto que o produto (biomolécula de interesse) prefira a fase oposta. Esse comportamento facilita a extração do produto do meio de produção assim que este é formado, levando a uma purificação parcial do produto alvo, além de apresentar outras vantagens como eliminar a influência de inibidores que possam surgir eventualmente no processo de produção (SINHA et al., 2000; NG et al., 2013).

Os resultados do particionamento em SDFA dependem das propriedades da biomolécula produzida, tais como, peso molecular, ponto isoelétrico e hidrofobicidade, mas também das características físico-químicas de ambas as fases do sistema, que por sua vez são influenciados por fatores que compõem o sistema, tais como, massa molecular do polímero utilizado, concentração e o tipo de polímero e sal utilizados, além da temperatura e do pH estabelecidos para o sistema (PERICIN et al, 2009).

Uma estratégica maneira de manipular os resultados de um bioprocesso extrativo é por modificação química de um dos polímeros, anexando um ligante adequado para os receptores existentes no bioproduto ou fármaco de interesse que está sendo produzido (XU et al., 2001).

O impacto do custo total no processo de produção depende expressivamente do tipo de produto que se quer obter e da concentração de produto obtida ao final do processo. Dessa forma, o custo da venda de um determinado produto alvo tem correlação inversamente proprocional a sua concentração alcançada no fim do processo de produção. A recuperação *in situ* de biomoléculas tem sido proposta como solução para reduzir o número de etapas de purificação dos produtos biotecnológicos, sendo de fundamental importância para a viabilidade do processo, uma vez que reduz o custo para obtenção do produto final (NG et al., 2013).

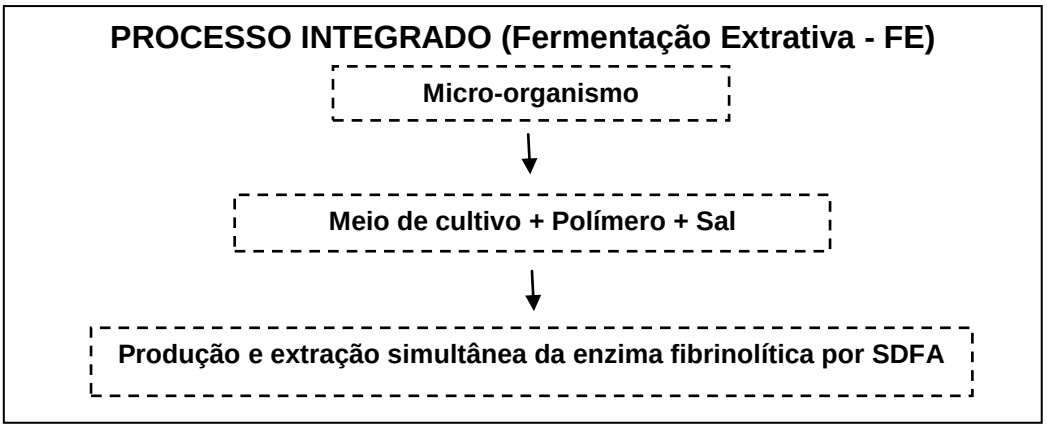

Figura 7. Representação simplificada do processo integrado da fermentação extrativa em sistema de duas fases aquosas (SDFA) para enzimas fibrinolíticas extracelulares

O processo de fermentação extrativa utilizando SDFA vem sendo reportado na literatura para extração de biomoléculas provenientes de espécies de *Bacillus* para recuperação de proteases alcalinas por *Bacillus licheniformis* (LEE e CHANG, 1990); α-amilase por *Bacillus amyloliquefaciens* (KIM e YOO, 1991); surfactina de *Bacillus subtilis* (DROUIN e COOPER, 1992); subtilina por *Bacillus subtilis* (KUBOI et al., 1994); enzimas que hidrolisam polissacarídeos produzidos por *Bacillus thuringiensis* H14 (BANIK e SANTHIAGU, 2002); e enzimas fibrinolíticas de *Bacillus subtilis* e *Bacillus* sp. UFPEDA 485 que são consideradas potentes fármacos promissores na medicina vascular (ASHIPALA et al., 2008; SALES et al., 2013).

A bioconversão extrativa em sistemas de duas fases aquosas está sendo cada vez mais cogitada, devido sua eficiência, especialmente para a separação de pequenas moléculas farmacêuticas, tais como, anticorpos, antibióticos, antígenos, proteínas recombinantes, aminoácidos, oligopeptídeos, ácido lático e principalmente enzimas para fins farmacêuticos (XU et al., 2001), incluindo enzimas fibrinolíticas (SALES et al., 2013).

Com iniciativas simples, de poucos esforços e baixo custo, a purificação de muitos produtos biofarmacêuticos pode ser conseguida em processos de bioconversão extrativa suave não desnaturante. Para aumentar a seletividade, vários bioligantes ou ligantes químicos com afinidade pelo polímero utilizado e alguns aditivos de superfície ativos podem ser inseridos nos sistemas de separação, ocasionando em resultados bem sucedidos (XU, 2001).

A aplicação de fármacos endovenosos, tais como, as enzimas fibrinolíticas microbianas, exige cepas não patogênicas ausentes de toxinas, além de uma alta purificação e estabilidade enzimática nas condições fisiológicas do sistema sanguíneo (pH 7,2 e temperatura 37°C), sendo também de extrema importância a obtenção de um fármaco que ocasione o mínimo de efeitos colaterais durante o período terapêutico.

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CAPÍTULO II

Optimization of production, biochemical characterization and evaluation of the therapeutic potential *in vitro* of a new fibrinolytic enzyme from *Bacillus amyloliquefaciens* UFPEDA 485

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Optimization of production, biochemical characterization and evaluation of the therapeutic potential *in vitro* of a new fibrinolytic enzyme from *Bacillus amyloliquefaciens* UFPEDA 485

[1]Fabiana América Silva Dantas de Souza; [1]Amanda Emmanuelle Sales; [5]Páblo Eugênio Costa e Silva; [1]Raquel Pedrosa Bezerra; [7]Germana Michelle de Medeiros e Silva; [6]Janete Magali de Araújo; [4]Galba Maria de Campos Takaki; [3]Tatiana Souza Porto; [2]José António Couto Teixeira; [1]Ana Lúcia Figueiredo Porto[a]

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

[2]Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus Gualtar, 4710-057, Braga, Portugal.

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

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

[5]Laboratory of Immunopathology Keizo Asami, Federal University of Pernambuco, 50670-901, Recife, PE, Brazil

[6]Department of antibiotics, Federal University of Pernambuco, 50670-901, Recife, PE, Brazil

[7]Department of Nanotechnology, Center of Strategic Technologies of the Northeast, 50740-540, Recife, PE, Brazil

* Corresponding author: analuporto@yahoo.com.br Phone and Fax: + 55 (81) 3320.6345

Abstract

Fibrinolytic enzymes have importance in the medicine and pharmaceutical industry because of their efficacy to degrade blood clots. In this work, forty-three microorganisms of the genus *Bacillus* were evaluated for its potential to produce fibrinolytic proteases. Thirty bacteria were confirmed as producers of fibrinolytic enzymes, being the best results obtained for the strain *Bacillus amyloliquefaciens* UFPEDA 485. The highest fibrinolytic activity of 813 $U.mL^{-1}$ and a degradation of blood clot *in vitro* of 62% were obtained in an optimized medium with 2 % of soybean flour and 1% glucose at 200 rpm after 48 h of cultivation, at pH 7.2 and 37°C. Optimal conditions for soybean flour and glucose concentration and agitation rate were obtained by applying a central composite design (CCD) star 2^3 and response surface methodology (RSM). Characterization of the enzyme extract was made from the best experimental condition with bigger fibrinolytic activity. Results concerning the effect of PMSF confirmed the enzyme as a serine-metallo protease once the fibrinolytic activity was inhibited by PMSF (fluoride methylphenylsulfonyl) 91.52% and EDTA (ethylenediaminetetraacetic acid). The optimum pH and temperature were 7.0 and 37^0C, respectively. Enzyme activity was inhibited by $FeSO_4$ (93.18%), and it was increased by $CaCl_2$ (110.22%), The obtained results confirm that the enzyme produced by *Bacillus amyloliquefaciens* UFPEDA 485, in particular its fibrinolytic activity at physiological conditions and long term stability, has suitable characteristics for human and veterinary applications, and promises to be a powerful drug for the treatment of vascular diseases.

Keywords: Screening; *Bacillus amyloliquefaciens*; Fibrinolytic enzyme; Optimization; Characterization.

1. Introduction

Proteases from microbial sources are hydrolytic enzymes that play an important role in cell metabolism and have attracted great interest from the pharmaceutical industry. Although several proteolytic enzymes are being produced, its production is not sufficient to meet the growing demand in the world market.[24]

Among the several proteases, enzymes with fibrinolytic activity have gained importance in the medical and pharmaceutical industry due to their effectiveness to degrade blood clots *in vitro* and *in vivo.*[13,9]

Fibrin, a protein component of blood clots is responsible for the appearing of thrombolytic disorders. The human body produces various kinds of enzymes that assist the formation of thrombus, but only one enzyme, plasmin (EC 3.4.21.7), may break and remove the clot, because it acts directly by dissolving the blood clot and maintaining blood flow at sites of vascular injury.[3]

Fibrinolytic agents that are commercially available for clinical use and are plasminogen activators such as urokinase and streptokinase (bacterial origin), have disadvantages as short half-life, high cost, need for high doses, collateral effects such as allergic reactions and hemorrhagic complications.[25] On the other hand, plasmin, and enzymes such as nattokinase, lumbrokinase (EC 3.4.17.13) and fibrolase are fibrinolytic enzymes which directly degrade fibrin thrombus dissolving blood clots quickly and completely. Thus, enzymes with such properties are required as alternatives for a superior therapy in the treatment of cardiovascular diseases and research for new fibrinolytic agents continues.[5]

Fibrinolytic enzymes were discovered in insects,[5] snake venom,[11] fruits such as *Campomanesia xanthocarpa*, popularly known as “guavirova”[17] brown algae,[16] fermented foods *Bacillus subtilis natto*[22] and are produced by various microorganisms, mainly bacteria of the genus *Bacillus*, widely studied due to their potential to produce potent fibrinolytic proteases.[1,21,6]

The obtention within a reasonable period of time of a microbial product of interest demands the use of methodologies than can accelerate the knowledge of the production process and the effect of the main process variables. Among

others, the central composite design (CCD) and response surface methodology (RSM) are statistical tools widely used in the optimization of fermentation processes involving enzyme production.[14]

Thus, considering the biotechnological potential of bioactive compounds production by microorganisms of the genus *Bacillus*, this work presents resulting on the screening of an efficient producer of fibrinolytic protease as well as a preliminary characterization of the obtained fibrinolytic protease, including the evaluation *in vitro* of its therapeutic potential.

2. Materials and Methods

2.1. Reagents

The chemicals reagents used were analytical grade and obtained from (Merck - Darmstadt, Germany) and (Sigma - St. Louis, MO).

2.2. Screening and culture conditions

Forty-three microorganisms were obtained from Cultures Collections of Department of Antibiotics, at the Federal University of Pernambuco and Catholic University of Pernambuco, Brazil. The stock culture was maintained in nutrient broth (peptone 1 % (w/v), meat extract 0.3 % (w/v) and 0.5 % NaCl (w/v)) in cryotubes (10 % v/v glycerol) at - 80 ◦C. For the inoculum, the colonies from the stock culture were reactivated and maintained in nutrient broth at 37 ^{0}C and 150 rpm. After 24 h growth in nutrient broth, the inocula was standardized by optical density (600 nm) and diluted when necessary.

For the screening experiments, the soybean medium (MS-2)[23] was used for the production of fibrinolytic proteases. The medium composition was: filtered soybean flour (2 % w/v), K_2HPO_4 (0.435 % w/v), NH_4Cl (0.1 % w/v), $MgSO_4.7H_2O$

(0.06 % w/v), glucose (1 % w/v) and 1 % of mineral solution (v/v) containing: $FeSO_4.7H_2O$ (100 mg); $MnCl_2.4H_2O$ (100 mg), and $ZnSO_4.H_2O$ (100 mg) in 100 mL of distilled water. Medium pH was 7.2. The production was done in shake flasks of 250 mL with a 100 mL working volumefor 48 hours at 150 rpm and 37 °C.

At the end of the production process, the culture medium was centrifuged at 10.000 xg for 20 min to obtain the enzyme extract and after to make analytical determinations.

2.3. Microbial identification

The microorganism was identified using 16S and rpoB genes and the sequencing was performed by STAB VIDA in Lisbon, Portugal. A sequence similarity search was performed in the National Center for Biotechnology (NCBI) database using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nih.gov/BLAST/). For 16S rRNA sequencing, total genomic DNA was extracted from the nutrient broth. The partial sequence of the 16S rRNA gene was amplified using a polymerase chain reaction (PCR) and bacterial universal primers specific to 16S rRNA gene. For further characterization of the microorganism the rpoB gene fragment which encodes the β subunit of RNA polymerase was investigated. The rpoB gene fragment was amplified from the genome DNA of *B. amyloliquefaciens* and *B. subtilis* by PCR using the following primers 5'- ATC GAA ACG CCT GAA GGT CCA AAC AT – 3' and 5' – ACA CCC TTG TTA CCG TGA CGA CC – 3'. The data base used was the Blast NCBI, and sequence comparison program was used Chromas Lite.

2.4. Selection of the best condition for production of fibrinolytic enzyme by Response Surface Methodology (RSM) using a Central Composite Design (CCD)

Central Composite Design (CCD) using two levels and three factors was composed of 17 runs with 3 repetitions at the central point, needed to calculate the pure error. The goodness of fit was evaluated by the adjusted coefficient of

determination (R^2_{adj}) and the analysis of variance (ANOVA), as well as comparative analysis by the plot of the predicted values with the observed experimental values. The independent variables were concentration of soybean flour (1.0; 2.0 and 3.0 % w/v); concentration of glucose (0.5; 1.0 and 1.5 % w/v) and agitation (100; 150 and 200 rpm) and the response variable was fibrinolytic activity ($U.mL^{-1}$). Statistical significance of the variables was determined at 5 % probability level ($p < 0.05$). The analysis of the results was carried with the program Statistic version 8.0. Response surface methodology (RSM) using CCD was employed to optimize the selected three variables by the second order polynomial equation (1):

$$Y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\beta_{ij} x_i x_j \qquad \textbf{Eq. (1),}$$

where Y is the measured response; Xi and Xj represent the independent variables; βo, βi, βii, βij, represent the independent term and (o, i, ii, ij) are the intercept coefficient, linear coefficient, quadratic coefficient, interaction coefficient, respectively.

2.5. Determination of total protein

Total protein concentration was determined by the Bradford method using as standard bovine serum albumin (BSA).[7]

2.6. *Assay of fibrinolytic activity by degradation of blood clots*

The used blood was extracted from the jugular of healthy young horses. The required volume of blood for clot formation was standardized and added without anticoagulant in test tubes having its walls previously wetted with saline

solution. After blood clotting, the tubes were placed in a water bath at 37°C for 1 hour to separate the serum from the clot of fibrin. The retracted clots were washed with saline solution up to the obtention of a translucent solution. Then the clots were transferred to test tubes with enzyme extract and kept at rest for 1 hour at 37°C. The sizes of the clots were standardized to 50% (w/v) in relation to the volume of enzyme extract. A saline solution was used as a negative control. The Percentage of Degradation of the Clot (PDC) was calculated according to equation (2), where $weight_0$ (zero) is the weight of the clot formed spontaneously before the treatment with the enzyme extract. The $weight_r$ is the weight of the residue remaining after treatment with the enzyme extract.[27]

$$PDC = \left(\frac{Weight_0 - Weight_r}{Weight_0} \right) \qquad \text{Eq. (2)}$$

2.7. *Determination of fibrinolytic activity at the fibrin plate*

The fibrin plate method[4] was used to evaluate the fibrinolytic activity in the screening process. The fibrin plate was composed by 2 mg/mL of fibrinogen solution (Fibrinogen from bovine plasma in Tris-HCl 150 mM containing NaCl 150 mM pH 7.75), 200 µL solution of Thrombin from bovine plasma (20 U/mL diluted in saline) was added to 4 ml of fibrinogen solution; 4 mL of agarose solution (2%) and 100 mL of $CaCl_2$ solution (1M). The reaction mixture was placed in plastic Petri dishes. After polymerization of the fibrin were made wells of 5 mm diameter, where 20 µL of enzymatic extract were placed. Then the plates were incubated at 37°C for 18 hours and the diameter of the halos measured. Assays for the standard curve were performed in triplicate and the values of the diameters of halos were obtained by average of the repetitions. One unit (U) of fibrinolytic activity corresponds to the correlation between the diameter of the degradation

halos in fibrin plate (mm) and the standard curve made using plasmin from human plasma. The activity was expressed in $U.mL^{-1}$.

2.8. *Fibrinolytic activity determination by spectrometry*

The fibrinolytic activity[26] of the enzyme extract obtained after the optimization experiments was was realized by reaction mixture containing 0.1 mL of 245 mM phosphate buffer (pH 7.0) and 0.4 mL of 0.72% fibrinogen solution was incubated at 37°C for 5 min; after that, 0.1 mL of a 20 $U.mL^{-1}$ thrombin solution was added and the reaction mixture was again incubated at 37°C for 10 min. After clot formation, 0.1 mL of diluted enzyme solution was added, and incubation continued at 37°C for 60 min, the reaction mixture being shaken every 20 min. The reaction was stopped by adding 0.7 mL of 0.2 M trichloroacetic acid (TCA). The reaction mixture was centrifuged at 15000 xg for 10 min, and the absorbance of the supernatant was measured at 275 nm by spectrophotometry. In this assay, one unit of fibrinolytic protease activity was defined as the amount of enzyme required to produce an increase in absorbance equal to 0.01 per minute, equivalent to liberation of tyrosine. All experiments were performed in triplicate.

2.9. *Determination of optimum pH and temperature of the enzyme*

The determination of the optimum pH and temperature of the fibrinolytic activity of the enzyme was done using fibrin as substrate as described in 2.7. For determination of the optimum temperature, the enzyme activity was analyzed at several temperatures between 4-80^{0}C under the standard assays conditions. For determination of the optimum pH, the reaction occurred at 37^{0}C and the enzyme activity was measured in the pH range (3.0-10.0). The buffers used were Glycine-HCl (pH 3.0), Sodium acetate (pH 4.0-5.0), Citrate phosphate (pH 6.0), Tris-HCl (pH 7.0-8.0), Glycine-NaOH (pH 9.0-10.0). The concentration of the buffers was standardized at 20 mM.

2.10. *Effect of pH and temperature on the stability of the enzyme*

Analysis of the effect of pH and temperature on enzyme stability was performed by incubation of the enzyme for 12h; aliquots were removed at intervals of 2h. Then, the remaining activities were determined (see 2.7). For measuring thermal stability analysis, the enzyme extract was incubated at temperatures (4, 25, 37, 40, 50, 60, 70 and 80ºC). To evaluate the effect of pH on enzyme stability, the enzyme extract was incubated in the same buffers used for the determination of the optimum pH.

2.11. *Effects of inhibitors and metal ions at the fibrinolytic activity*

The inhibitors and metal ions were dissolved in 150 mM Tris-HCl buffer (NaCl 150 mM pH 7.75); the concentration of the solutions was standardized at 5 mM. The enzyme extract was incubated for 1 h at 37^0C. Following incubation, the residual fibrinolytic activity of the protease was measured as described in 2.7. The following inhibitors were investigated PMSF (fluoride methylphenylsulfonyl - $C_7H_7FO_2S$), EDTA (ethylenediaminetetraacetic acid - $C_{10}H_{16}N_2O_8$), β-mercaptoethanol (2-hydroxy-1- ethanethiol - C_2H_6SO), Pepstatin A (4-amino-3-hidroxy-6-methyl-heptanoic - $C_{34}H_{68}N_5O_9$). The metal ions evaluated were $FeSO_4$, $CuSO_4$, $CaCl_2$, $MgSO_4$, $CoCl_2$, $ZnSO_4$, KCl and $MgCl_2$.

3. Results and Discussion

3.1. *Screening fibrinolytic enzyme producer*

Forty-three strains of *Bacillus* were evaluated, among which thirty (69.7%) showed fibrinolytic activity, as shown in Table 1. These results are similar to those reported in the literature and show the potential of the genus *Bacillus* as a producer of enzymes with fibrinolytic activity.

Table 1. Microorganisms of the genus *Bacillus* and their fibrinolytic activity after 48 h of cultivation in soybean medium (MS-2), pH 7.2, 150 rpm and 37°C

Microorganisms	***FA (U.mL^{-1})**	**Microorganisms**	***FA (U.mL^{-1})**
B. subtilis UCP 999	-	*B. circulans* UFPEDA 436	26.7 ± 0.83
B. licheniformis UCP 1008	34.2 ± 1.01	*Bacillus* sp. UFPEDA 437	2.25 ± 0.93
B. licheniformis UCP 1009	0.11 ± 0.02	*Bacillus* sp. UFPEDA 449	34.2 ± 1.61
B. licheniformis UCP 1010	-	*Bacillus* sp. UFPEDA 450	9.93 ± 0.54
B. licheniformis UCP 1013	6.06 ± 1.07	*Bacillus* sp. UFPEDA 451	-
B. licheniformis UCP 1016	16.3 ± 0.92	*B. cereus* UFPEDA 452	-
B. licheniformis UCP 1033	16.3 ± 0.82	*B. alvei* UFPEDA 461	9.93 ± 1.33
B. licheniformis UCP 1477	16.3 ± 0.82	*Bacillus sp.*UFPEDA 464	16.3 ± 0.73
B. licheniformis UCP 1482	9.93 ± 0.89	*Bacillus* sp. UFPEDA 465	3.69 ± 0.10
B. cereus UFPEDA 11	-	*Bacillus* sp. UFPEDA 466	-
Bacillus. sp UFPEDA 12	-	*Bacillus* sp. UFPEDA 469	26.7 ± 0.00
*Bacillus. sp.*UFPEDA 13	1.37 ± 0.74	*B. licheniformis* UFPEDA 470	-
B. mycoides UFPEDA 14	0.84 ± 0.08	*B. firmus* UFPEDA 471	-
B. subtilis UFPEDA 15	-	*B. pumilus* UFPEDA 472	20.9 ± 1.49
B. subtilis UFPEDA 16	-	*B. pumilus* UFPEDA 474	6.06 ± 1.46
B. subtilis UFPEDA 86	0.84 ± 0.09	*Bacillus* sp. UFPEDA 483	26.7 ± 0.08
B. megaterium UFPEDA 108	0.31 ± 0.09	*Bacillus* sp. UFPEDA 484	-
B. subtilis v. aterrimus UFPEDA 170	0.51 ± 0.08	***Bacillus amyloliquefaciens* UFPEDA 485**	**71.8** ± 0.08
Bacillus. sp UFPEDA 189	26.7 ± 1.51	*Bacillus* sp. UFPEDA 486	0.40 ± 0.08
Bacillus. sp UFPEDA 194	4.73 ± 0.74	*Bacillus* sp.UFPEDA 487	-
B. subtilis UFPEDA 260	0.51 ± 0.10	*Bacillus* sp.UFPEDA 488	0.15 ± 0.03
B. subtilis UFPEDA 404	20.9 ± 1.03		

*FA - Fibrinolytic activity by fibrin plate method and correlated with a standard curve of plasmin. Absence of fibrinolytic activity (-)

Other researchers[8] isolated microorganisms from the Chinese soybean cheese doufuru as well as Japanese natto and Chinese douche. Among the isolates, 16 microorganisms showed fibrinolytic activity being the highest value obtained for a *Bacillus subtilis* strain with a crude enzyme activity of 1833 IU/mL. In other research,[20] evaluated fibrinolytic proteases production by various bacteria, and, after 48 hours of culture, the *Bacillus* sp. strain AS-S20-I obtained the maximum fibrinolytic activity of 145.8 $U.mL^{-1}$ a value similar to the one obtained in this work by the strain *Bacillus amyloliquefaciens* UFPEDA 485 (71.78 $U.mL^{-1}$).

3.2. *Bacteria identification using 16S and rpoB genes*

Initially, the study for the identification was based on 16S rRNA gene sequence analysis. The results showed 99% similarity homologies with the species *Bacillus amyloliquefaciens* and *Bacillus subtilis*, thus, the analysis using the 16S rRNA gene sequence alone did not allow differentiating the species *Bacillus subtilis* and *Bacillus amyloliquefaciens*. Therefore, analysis was performed on the rpoB region which showed greater identity to the species *Bacillus amyloliquefaciens* than to the species *Bacillus subtilis*. The Blast from the consensus sequence obtained revealed about 98% homology with the region DNA-directed RNA polymerase β-subunit of the species *Bacillus amyloliquefaciens* and 89% homology to the same region of the *Bacillus subtilis* species.

For this study identification made by the analysis of the rpoB region sequencing offered advantages over the 16S because the rpoB gene has a higher degree of polymorphism compared to 16S, making it more accurate for species identification. The heterogeneity of the 16S rRNA gene hampers the quantification of bacterial species by PCR based assays. In contrast, the rpoB gene is common to all bacteria and occurs as a single copy in the genome.

Researchers obtained similar results in the process of identification of microorganism of the genus *Bacillus* by comparison of 16S and rpoB genes of the 13 *Bacillus species*. Found that, of the 13 species, the levels of similarity ranged from 90.3% (*B. hwajinpoensis* and *B. sporothermodurans*) to 99.8% (*B. anthracis* and *B. cereus*).[15]

In this work the results in conjunction of the 16S and rpoB region confirmed that the sample tested has a higher homology to the species *Bacillus amyloliquefaciens* and demonstrate that the polymorphism of the *Bacillus* rpoB gene can be used to identify *Bacillus* species, providing improvement over conventional methods of identification *Bacillus* species. The result was submitted to the GenBank database.

3.3. *Optimization of the conditions for production of the fibrinolytic enzyme from Bacillus amyloliquefaciens UFPEDA 485*

To achieve the optimization of the fibrinolytic activity in shake flasks by this central composite design (CCD) star 2^3, was previously realized other full factorial design 2^3, that studied other concentrations of soybean flour and glucose near optimal found in this work and by which was found the optimum temperature (37°C) and optimum pH 7.2 of production (data not shown), therefore, these factors were not investigated in the experiments realized in this work.

The optimization of the conditions for the production of the fibrinolytic enzyme from *Bacillus amyloliquefaciens* UFPEDA 485 was conducted according to a Central Composite Design (CCD) star 2^3 with two levels and three factors and the response variable Fibrinolytic Activity (FA) after 48 hours of cultivation of the *Bacillus amyloliquefaciens* UFPEDA 485 (Table 2).

The Increase of the concentration of the independent variables, soybean flour, glucose and the interaction between these two factors, had a linear effect positive and statistically significant for increased of the fibrinolytic activity. For the quadratic effect, the increase of the concentration of the independent variables, soybean flour, glucose and the interaction among the factors, was statistically significant and had a negative effect on the increased fibrinolytic activity. The results from the analysis of variances and effect estimates for the main factors are shown in Table 3. The variation around the mean of the residuals is fully explained by pure error, because there is no evidence of lack of fit, and a good model fit is obtained. The *p* value for lack of fit was < 0.00. The validity of the model is confirmed by the ANOVA analysis.

Table 2. Matrix of the Central Composite Design (CCD) star for the optimization of the conditions for production of the fibrinolytic enzyme from *Bacillus amyloliquefaciens* UFPEDA 485.

Runs	**Soybean flour (%)**	**Glucose (%)**	**Agitation (rpm)**	**Fibrinolytic activity ($U.mL^{-1}$)**
1	1.0 (-)	0.5 (-)	100 (-)	462 ± 0.014
2	1.0 (-)	1.5 (+)	200 (+)	682 ± 0.003
3	3.0 (+)	0.5 (-)	200 (+)	666 ± 0.013
4	3.0 (+)	1.5 (+)	100 (-)	492 ± 0.004
5 (C)	2.0 (0)	1.0 (0)	150 (0)	702 ± 0.006
6	1.0 (-)	0.5 (-)	200 (+)	710 ± 0.008
7	1.0 (-)	1.5 (+)	100 (-)	419 ± 0.005
8	3.0 (+)	0.5 (-)	100 (-)	413 ± 0.009
9	3.0 (+)	1.5 (+)	200 (+)	696 ± 0.011
10 (C)	2.0 (0)	1.0 (0)	150 (0)	701 ± 0.003
11	1.0 (-)	1.0 (0)	150 (0)	636 ± 0.004
12	3.0 (+)	1.0 (0)	150 (0)	613 ± 0.004
13	2.0 (0)	0.5 (-)	150 (0)	607 ± 0.026
14	2.0 (0)	1.5 (+)	150 (0)	604 ± 0.007
15	2.0 (0)	1.0 (0)	100 (-)	538 ± 0.006
16	**2.0 (0)**	**1.0 (0)**	**200 (+)**	**813 ± 0.014**
17 (C)	2.0 (0)	1.0 (0)	150 (0)	701 ± 0.004

Table 3. Analysis of variance (ANOVA) for the response fibrinolytic activity, over the independent variables soybean flour (%), glucose (%) and agitaton (rpm), according to Central Composite Design (CCD) star, with 95% confidence level.

	Fibrinolytic Activity	
Factor	**Estimates effect**	***p value***
(1) Soybean flour (%) (L)	178	0.00
Soybean flour (%) (Q)	- 51.3	0.00
(2) Glucose (%) (L)	506	0.00
Glucose (%) (Q)	- 282	0.00
(3) Agitation (rpm) (L)	2.93	0.00
1L by 2L	45.0	0.00
1L by 3L	- 0.14	0.00
2L by 3L	- 0.17	0.00
Regression coefficient ANOVA		
Pure Error = 0.18	$R^2 = 0.99$	$R^2_{adjusted} = 0,97$

Using the obtained values in the table 2, the corresponding multiple regression equation was calculated. The quadratic effect of the variable (X_3) was ignored in Equation (3) as its effect was not statistically significative.

From the regression analysis, the Equation (3) was obtained:

$$Y = - 151 + 178\ (X_1) - 51.3\ (X_1)^2 + 506\ (X_2) - 282\ (X_2)^2 + 2.93\ (X_3) + 45\ (X_1.X_2) - 0.14\ (X_1.X_3) - 0.17\ (X_2.X_3) \quad \text{Eq. (3)}$$

The optimum conditions found for the fibrinolytic activity (FA) were 1.88% for soybean flour and 0.988% for glucose, at 200 rpm. Under these conditions the value calculated was 808 $U.mL^{-1}$, very close to the value 813 $U.\ mL^{-1}$, observed by

applying Eq. 3 to the experimental conditions, using soybean flour (2%), glucose (1%) and agitation (200 rpm).This makes irrelevant the small adjustment of the optimum conditions of 1.88% to 2% of soybean flour and of 0.988% to 1% glucose.

Response surfaces were plotted using the model Equation (3), to visualize the interaction among the two most important variables (soybean flour and glucose) and to determine its optimum concentration for the production of fibrinolytic enzyme from *Bacillus amyloliquefaciens* UFPEDA 485. Response surface graph shows that the best result for the fibrinolytic activity occurred using soybean flour (2% w/v) glucose (1% w/w) and agitation at 200 rpm (Figure 1).

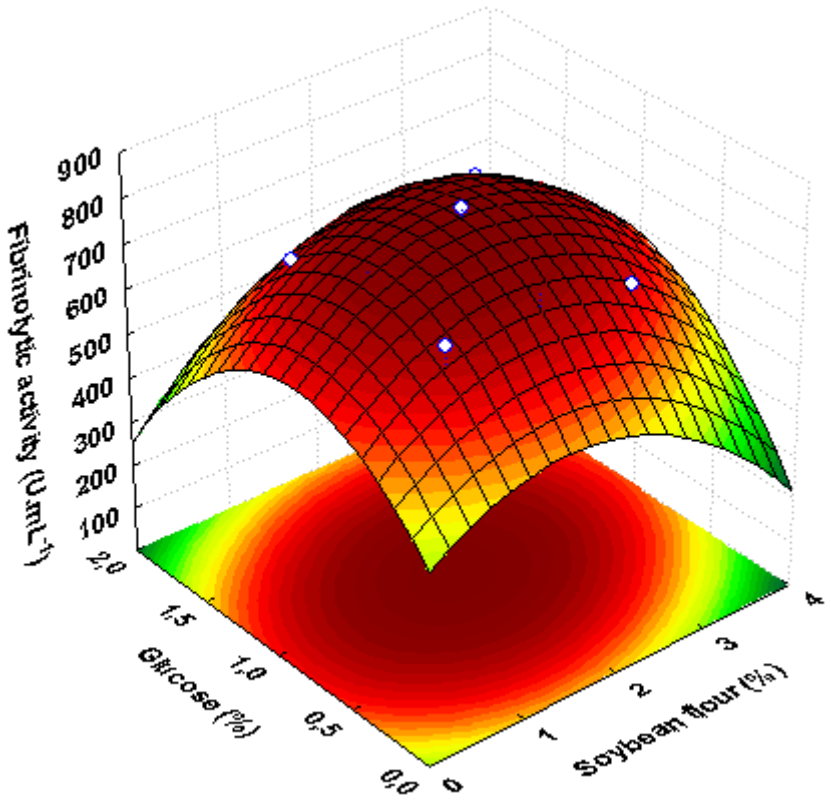

Figure 1 Response surface plot showing the effect of independent variables: soyben flour (%) and glucose (%) with agitation at 200 rpm, after 48 hours of cultivation on the response variable Fibrinolytic Activity (FA) of *Bacillus amyloliquefaciens* UFPEDA 485.

Previous researches have proved that glucose is a preferred carbon source for growth of *Bacillus* spp.. Other carbon sources such as fructose, maltose, lactose, sucrose and glycerol have shown minimum solubility, poor cellular uptake and unsuitable metabolic pathway reducing the production of fibrinolytic enzymes.[5] Researchers[5] reported an optimum concentration of glucose (1.25% w/v) for maximum fibrinolytic activity from *Bacillus sphaericus* MTCC 3672, result similar to this work, that obtained optimum concentration of glucose at (1% w/v).

Others researchers optimized the production of fibrinolytic enzyme by a central composite rotary design (CCRD), using soybean as a nitrogen source and glucose as carbon source. In this case the maximum activity, obtained in a bioreactor, reached in 12 hours, using glucose 0.5% and soybean meal 0.5%. Unlike this study, using a central composite design (star) 2^3 in shake flasks, and achieved the best result for fibrinolytic activity in 48 h, using soybean flour (2% w/v) and glucose (1% w/v).[10]

3.4. *Evaluation of fibrinolytic activity by degradation of blood clots*

The enzyme extract produced by *Bacillus amyloliquefaciens* UFPEDA 485 showed a high effectiveness in degrading blood clots *in vitro*. After 1 hour, the results showed a percentage degradation of the clot (PDC) of 62%. Negative control containing saline solution kept the clot intact (Figure 2). Other researchers studied the potential of the enzyme from *Bacillus* sp. SFN in degrading blood clots and showed that 20 U/mL of fibrinolytic enzyme could degrade completely the blood clot after 18 hours of incubation. The results obtained in this work suggest the high efficiency of the obtained enzyme in the degradation of blood clots as, in just one hour, a PDC of 62% was achieved.[19]

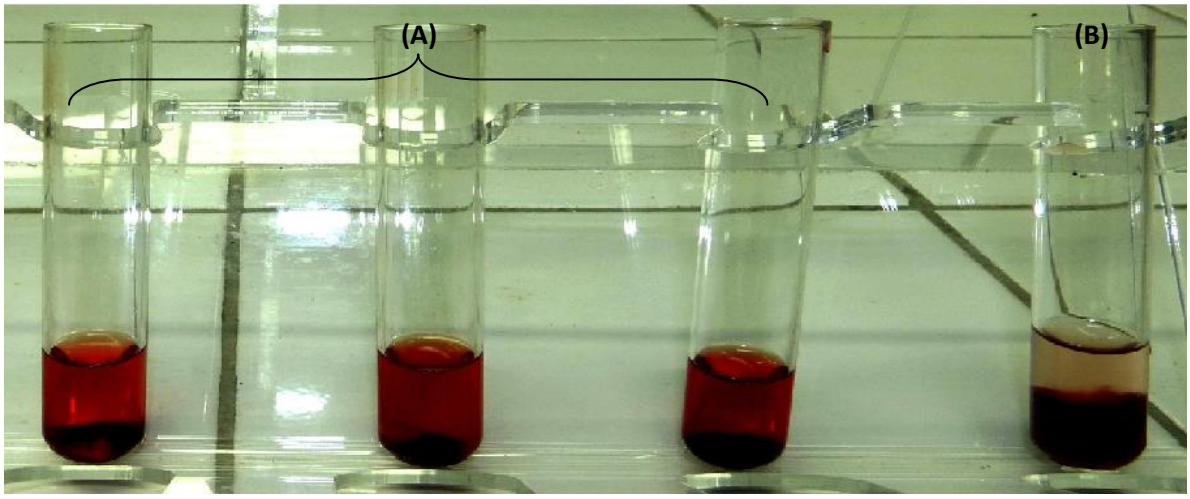

Figura 2. Degradation of blood clots by dispersion of red blood cells. **(A)** Blood clots after 1 hour in the enzyme extract from *Bacillus amyloliquefaciens* UFPEDA 485 (in triplicate), **(B)** Blood clot after 1 hour in physiological solution.

The efficiency of fibrinolytic enzymes in degrading blood clots *in vitro* has been studied. Researchers evaluated the effect of degradation clot in vitro using mouse blood.[16] Results showed that the enzyme was able to lyse the clot as effectively as commercial u-PA. Other researchers evaluated the ability of fibrinolytic protease from *Fusarium* sp. in degrading blood clots. The fibrinolytic effect was 36.5% for the degradation of blood clots of mouse at room temperature for 1 hour.[27] Compared to this work, the fibrinolytic enzyme from *Bacillus* sp.UFPEDA 485 showed to possess a potential almost 2 times higher for the degradation of blood clots within 1 hour. Other researchers investigated the ability of the enzyme from *Bacillus subtilis* natto 168, in degrading blood clots, also observing the degradation of blood clots after 1 hour. However, the percentage of degradation of the clot was not reported. [22]

3.5. *Effects of pH and temperature on the activity and stability of the enzyme*

The enzyme showed higher values of fibrinolytic activity at neutral pH, with an optimum at pH 7.0 (Figure 3A). After incubation for 12 hours, the enzyme retained 82.89% of its activity at pH 7.0 and 66.03% at pH 8.0, and remained

around 50% of its activity at pH 6.0 and 9.0 after 8 h of incubation (Figure 3B). Results corroborate with other researchers,[3] that studied a fibrinolytic enzyme produced by *Bacillus licheniformis* B4 - an optimum pH of 7.5 was observed, and the enzyme retained 100% of its activity at pH 7.0 after 30 min of incubation. Other researchers,[6] evaluated the fibrinolytic protease from *Bacillus cereus* NS-2 and, unlike this work, an optimum pH 9.0 was found and the fibrinolytic protease lost its activity in just 30 minutes. Thus, the fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* UFPEDA 485 is very stable in comparison with other fibrinolytic enzymes produced by other species of the genus *Bacillus*. Moreover, it has the best activity at physiological conditions.

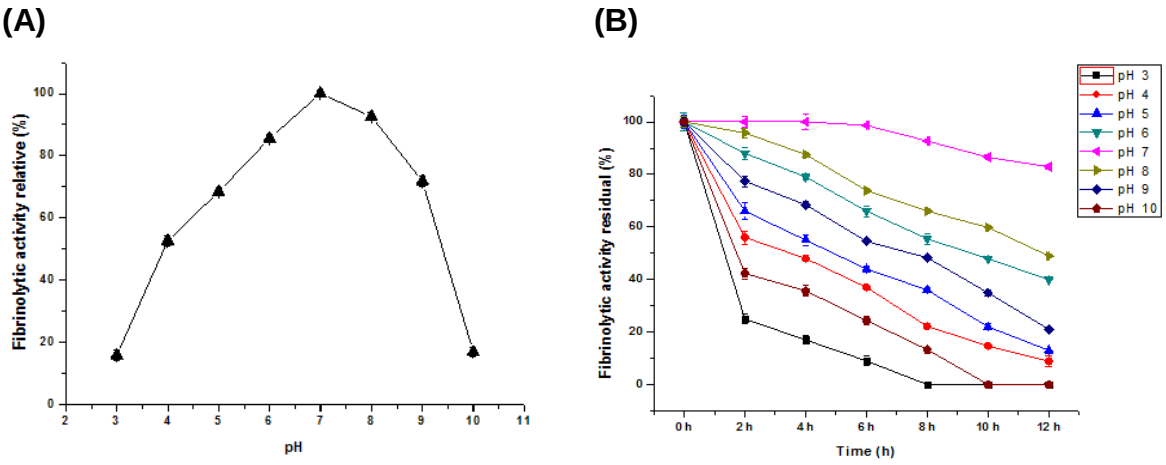

Figure 3. (A) Effect of pH on the fibrinolytic activity relative of the enzyme from *Bacillus amyloliquefaciens* UFPEDA 485 after 1h of incubation. **(B)** Effect of pH on the stability of enzyme measured at intervals of 2h per 12h of incubation of the enzyme and expressed as percentage of residual activity. Buffers used: Glycine-HCl (pH 3.0), Sodium acetate (pH 4.0-5.0), Citrate phosphate (pH 6.0), Tris-HCl (pH 7.0-8.0), Glycine-NaOH (pH 9.0-10.0). All buffer concentrations were 20 mM. Each value is the average of the results of three experiments, and the error bars show the standard deviations.

The optimum temperature was 37^0C (Figure 4A). Thermostability assays showed that, after 12h of incubation, the enzyme remained stable at 4, 25 and 37^0C, maintaining activities of 96.04, 95.88 and 98.26 %, respectively, and still

maintained 58.84% of its activity at 40^{0}C (Figure 4B). In other study,[3] was also found a fibrinolytic enzyme with an optimum temperature of 37^{0}C that retained its total activity after 1 hour of incubation when preserved at 20-40^{0}C; then the activity decreased with the increase of temperature. Differently, in other study,[6] was found an optimum temperature of 40ºC, and during the thermostability assays the enzyme lost 40-60% of its activity at each temperature of incubation. These results confirm the excellent thermostability of the fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* UFPEDA 485 when compared with the enzymes produced by *Bacillus* cited at the literature.

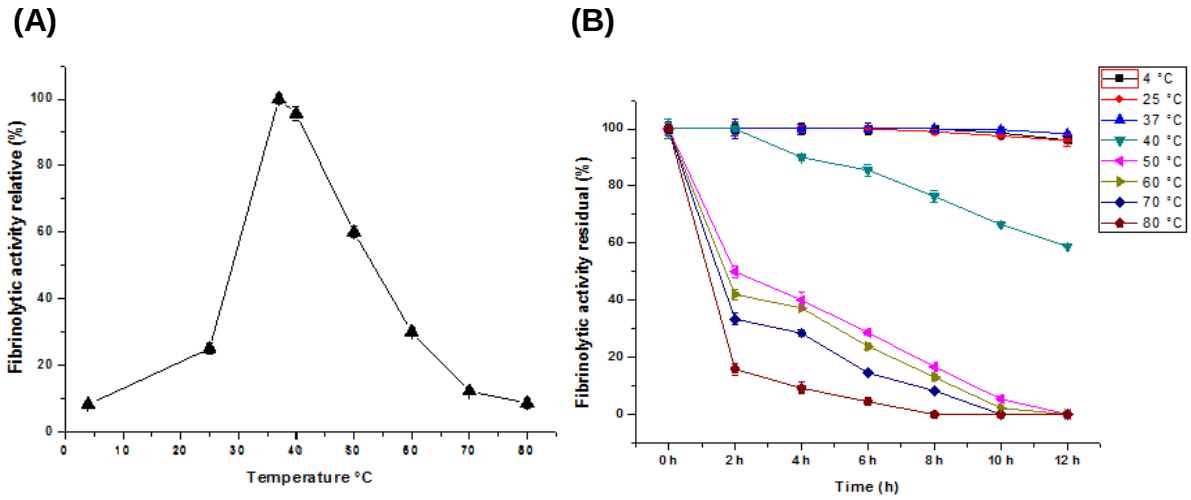

Figure 4 (A) Effect of temperature on the fibrinolytic activity relative of the enzyme from *Bacillus amyloliquefaciens* UFPEDA 485 after 1h of incubation; **(B)** Effect of temperature on the stability of enzyme measured at intervals of 2h per 12h of incubation of the enzyme and expressed as percentage of residual activity. Each value is the average of the results of three experiments, and the error bars show the standard deviations.

3.6. *Effects of inhibitors and metal ions on the fibrinolytic activity*

The fibrinolytic activity was almost completely inhibited after 60 min of incubation with PMSF (91.52%) and EDTA (89.64 %) in Table 4. The results show that the enzymatic extract from *Bacillus amyloliquefaciens* UFPEDA 485 contains

serine-metallo proteases, indicating that the hydroxyl (serine) group is located at or near the active site of one of the enzymes contained in the extract, hence being inhibited by PMSF (serine protease inhibitor); moreover, divalent metals seem to be required for the enzyme to maintain its activity, as an inhibition by chelating agents such as EDTA (metallo protease inhibitor) occurs. Enzyme activity was less inhibited by Pepstatin A (51.78%) an aspartic protease inhibitor, and β-mercaptoethanol (63.82%) a cysteine protease inhibitor.

Table 4. Effects of inhibitors on the fibrinolytic activity of the enzyme

Inhibitors and metal ions	Residual activity (%)
Control	100
PMSF	8.48 ± 0.01
EDTA	10.4 ± 0.02
Pepstatin A	48.2 ± 0.04
β-mercaptoethanol	36.2 ± 0.02

The values are presented as means ± SD (standard deviation).

Enzyme activity was significantly inhibited by $FeSO_4$ (93.18%) and the $CaCl_2$ (10%) slightly increased the fibrinolytic activity of the enzyme (Table 5). Both the activation as the inhibition the activity of an enzyme occurs due to allosterics effects. The connection of a chemical substance with the allosteric site of an enzyme may induce conformational changes in the spatial structure of the enzyme, changing the affinity by its substrate.

In another study[12] evaluated the influence of protease inhibitors and metal ions in fibrinolytic enzyme activity also reported an inhibition of 100% and 96.65%, in the presence of PMSF and EDTA, respectively. The enzyme was also classified as a serine-metallo protease. As wel as in this work, the enzyme when incubated with $CaCl_2$, increased its fibrinolytic activity to 118.15%

Table 5. Effects of metal ions on the fibrinolytic activity of the enzyme

Inhibitors and metal ions	Residual activity (%)
Control	100
$FeSO_4$	6.82 ± 0.04
$CuSO_4$	50.0 ± 0.05
$MgSO_4$	94.5 ± 0.03
$ZnSO_4$	52.6 ± 0.04
$CoCl_2$	48.3 ± 0,06
$MgCl_2$	96.8 ± 0,06
$CaCl_2$	110 ± 0,02
KCl	98.6 ± 0,01

The values are presented as means ± SD (standard deviation).

The influence of protease inhibitors was also evaluated in other study[18] unlike this work, when the enzyme was incubated with PMSF and EDTA, an inhibition of 99.8% and 8% was observed, respectively, making this enzyme to be considered only as a serine potease. However, for study of the effect of metal ions, as in this work, the $CaCl_2$ increased fibrinolytic activity to 111% and when incubated with $CoCl_4$ and $FeSO_4$ caused inhibition of 59% and 100%, respectively.

4. Conclusion

From the 43 strains evaluated, *Bacillus amyloliquefaciens* UFPEDA 485 was selected as the one producing the highest fibrinolytic activity.

Optimum conditions for production of the fibrinolytic enzyme from *Bacillus amyloliquefaciens* UFPEDA 485 were found.

The fibrinolytic protease from *Bacillus amyloliquefaciens* UFPEDA 485 has biochemical characteristics suitable for human and veterinary applications, namely the best activity at physiological conditions, besides having high thermal stability.

The results demonstrate the biotechnological potential of the *Bacillus amyloliquefaciens* UFPEDA 485 and its proteases to the pharmaceutical industry and the possible application on the treatment of vascular disorders.

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CAPÍTULO III

Process scale-up and biochemical characterization of an enzyme with anticoagulant and fibrinolytic potential from *Bacillus amyloliquefaciens* UFPEDA 485

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Process scale-up and biochemical characterization of an enzyme with anticoagulant and fibrinolytic potential from *Bacillus amyloliquefaciens* UFPEDA 485

[1] Fabiana América Silva Dantas de Souza; [1] Júlio Cézar dos Santos Nascimento; [1] André Manuel de Oliveira Mota; [3] Maikon Kelbert; [1]Raquel Pedrosa Bezerra; [1]Daniela de Araújo Viana Marques; [2] José Antônio Couto Teixeira; [1]Ana Lúcia Figueiredo Porto*

[1]Department of Morphology and Animal Physiology, Federal Rural University of Pernambuco, Rua Dom Manoel de Medeiros, s/n., Dois Irmãos, 52171-900, Recife, PE, Brazil.

[2]Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Largo do Paço, Campus Gualtar, 4710-057, Braga,

[3] University of Joinville Region, Street Paul Malschitzki, n.10, Bom Retiro, 89219-710, Joinville, Santa Catarina, Brazil.

* Corresponding author: analuporto@yahoo.com.br Phone and Fax: + 55 (81) 3320.6345

Abstract

Fibrinolytic proteases produced by *Bacillus* sp. have attracted an interest in the pharmaceutical industry due to its effectiveness in degrading and preventing the formation of blood clots. Fibrinolytic protease production by a *Bacillus amyloliquefaciens* strain was conducted in a 7L bioreactor. A full 2^2 factorial design was conducted to investigate the effects and interactions of independent variables aeration and agitation on the fibrinolytic and amidolytic activities of protease. The best conditions for protease production occurred at 12h, 800 rpm and 1.5 vvm, pH 7.2, at 37ºC. Under these conditions the enzyme achieved a fibrinolytic activity of 2169.11 $U.mL^{-1}$ and amidolytic activity of 1586.89 µmol. $min^{-1}.mL^{-1}$. Further characterization of the protease properties showed that the amidolytic activity was inhibited by PMSF and among the synthetic substrates evaluated the one with the highest affinity was *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA, indicating that the enzyme is a serine protease. The corresponding, K_m and V_{max} values were 0.68 mM and 357.14 mmol *p*NA min^{-1} mL^{-1}, respectively. The optimum pH for the hydrolysis of *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA was between 7 and 8 and for hydrolysis of fibrin pH 7.0. The optimum temperature for both fibrinolytic activity and amidolytic activity was 37 ºC. The fibrinolytic and amidolytic properties of the enzyme were preserved at 92.2% and 95.8% respectively after 9

months of storage. The results show the potential of the enzyme to be marketed and used as a new and potent drug in thrombolytic therapy.

Keywords: *Bacillus amyloliquefaciens*, fibrinolytic enzyme, anticoagulant, production scale-up, bioreactor

1. Introduction

Fibrinolytic proteases are hydrolytic enzymes that dissolve blood clots. The formation of blood clots is a natural phenomenon of protection of the human body to prevent excessive bleeding from injuries and wounds, but may sometimes block blood flow causing cardiovascular disorders such as stroke, myocardial infarction and others [19]. Thrombolytic therapy using fibrinolytic proteases has been shown as a potential solution to many vascular disorders.

The blood clots are composed of fibrin, a protein formed by the activation of fibrinogen by proteolytic action of thrombin (EC 3.4.21.5). Fibrin clot formation and fibrinolysis are normally well balanced in blood system. Generally the fibrin formed is hydrolyzed by plasmin (EC 3.4.21.7), but when fibrin is not dissolved because of some imbalance, thrombosis occurs [2].

Antithrombotic drugs, including anticoagulants and antiplatelets, are used to prevent and treat thrombosis. Drugs such as heparin and vitamin K antagonists (warfarin) are commonly used in anticoagulant therapy, but unfortunately these drugs have important limitations that drive continuous and intense efforts to develop new, efficient and safe anticoagulants, especially those targeting specific coagulation factors. Direct thrombin inhibitors such as argatroban, dabigatran, bivalirudin, and hirudin, as well as the factor (F) Xa inhibitor rivaroxaban, anticoagulants are currently marketed, but the nature of thrombosis involving complex clinical situations (patients with renal failure, cancer and thrombosis in pregnant women) continues to ask for new, safer and more effective anticoagulants with different pharmacological and pharmacokinetic properties [1] [12]

In recent years, various fibrinolytic enzymes derived from different sources have been discovered, *Daboia russelli* [2] [16], *Neanthes japonica* [26], *Cordyceps militaris* [4], *Campomanesia xanthocarpa* [11], *Yellow Mealworm* [7], *Euphorbia hirta* [17], *Artocarpus heterophyllus latex* [22], *Candida guilliermondii* [20]. The fibrinolytic protease from *Bacillus* sp. has attracted the interest of researchers and the pharmaceutical industry

because of its efficiency as thrombolytic agents in the process of fibrinolysis and plasmin activation [19]. Researchs have shown that *Bacillus* sp. produce a variety of fibrinolytic enzymes [6], [24], [23], [15].

The species of the genus *Bacillus* are attractive to the industry because they have high growth rates resulting in short cycles of cultivation and high production, and are able to secrete various proteins in the extracellular medium [21]. These features put the microorganisms of the genus *Bacillus* among the most important producers of industrial enzymes. Around 60% of commercially available enzymes are produced by *Bacillus* species [5].

Enzyme production on a large scale is mainly carried out by batch fermentation in stirred tank bioreactors and increase production of enzymes in the scale-up process is achieved by optimizing the relation between the microorganism and its environment. Agitation and aeration are impact factors in the fermentation process. The agitation influences mixing and heat and mass transfer being an important parameter for growth and performance of microbial cells. Aeration is also a key factor in aerobic processes due to its impact on oxygen supply. Overall, oxygen transfer depends on the gas flow rate, type of agitator, agitation speed and the properties of liquids [5].

The purpose of this study was to evaluate the best conditions of aeration and agitation for the production of the fibrinolytic enzyme from *Bacillus* sp. UFPEDA 485 in bioreactor, in order to maximize the enzyme activity produced and to minimize the production time, as well as to characterize the enzyme with amidolytic and fibrinolytic properties.

2. Materials and methods

2.1. Reagents

The chemicals reagents used were analytical grade and obtained from Merck (Darmstadt, Germany) and Sigma (St. Louis, MO).

2.2. Microorganism

The *Bacillus amyloliquefaciens* UFPEDA 485 strain was obtained from Culture Collection of Department of Antibiotics, at the Federal University of Pernambuco, Brazil. The microorganism was isolated

from the fermentation of sugar cane mills from Zona da Mata of Pernambuco, Brazil. The stock culture was maintained in nutrient broth (1 % peptone, meat extract 0.3 % and 0.5 % NaCl) in cryotubes (10 % v/v glycerol) at -80°C.

2.3. Culture condition

The soybean flour medium (MS-2) [18] was used for the production of fibrinolytic proteases. The medium composition was: filtered soybean flour (2% w/v), K_2HPO_4 (0.435 % w/v), NH_4Cl (0.1% w/v), $MgSO_4.7H_2O$ (0.06% w/v), glucose (1% w/v) and mineral solution (1% v/v) containing: $FeSO_4.7H_2O$ (100 mg); $MnCl_2.4H_2O$ (100 mg), and $ZnSO_4.H_2O$ (100 mg) in 100 mL of distilled water. For the selection of the best condition of production of the fibrinolytic enzyme was used 2^2 full factorial design. The experiments were performed in a 7L bioreactor (B. Braun Biotech International, Model Biostat®A, type A5, Melsungen, Germany) under controlled temperature (37^{0}C) and pH (7.2) by a micro DCU-twin controller. The liquid working volume was 5L and the culture pH was controlled by the addition of 6 N HCl or 6N NaOH.

For the inoculum, the colonies from the stock culture were reactivated in nutrient broth at 37°C and 200 rpm up to reach the end of the exponential growth phase; then, the cells were maintained on nutrient agar for 24 h and transferred to flasks with 250 mL and 100 mL working volume. In these flasks, cells were grown in soybean medium (MS-2) at 37°C and 200 rpm up to reach the end of the exponential growth phase, and the obtained cell culture was standardized by optical density and transferred into the bioreactor.

At the end of the production process, the culture medium was centrifuged at 10.000 xg for 20 min to obtain the enzyme extract and make analytical determinations.

2.4. Statistical analysis and experimental design

For statistical analysis, the variables were coded according to Eq. (1):

$$x_i = \frac{X_i - X_o}{\Delta X_i} \qquad \text{Eq. (1)}$$

where each independent variable is represented by x_i (coded value), X_i (real value), X_0 (real value at the center point), and ΔX_i (step change value).

A 2^2 full factorial design was used to analyze the influence of the two independent variables agitation (rpm) and aeration (vvm) on enzyme production. The effects of these two independent variables were determined having as response variable the fibrinolytic protease production (expressed as fibrinolytic and amidolytic activity). The experimental design was composed of 7 runs and 3 repetitions at the central point, needed to calculate the pure error (Table 1). Statistical significance of the variables was determined at 5 % probability level ($p < 0.05$).

The goodness of fit was evaluated by the coefficient of determination (R^2) and the analysis of variance (ANOVA); the first-order equation was determined by Fischer's test. The analysis of the results was carried with the program Statistic version 8.0.

2.5. Protein concentration

Total protein concentration was determined by Bradford method using as standard bovine serum albumin (BSA) [1].

2.6. Fibrinolytic activity

Fibrinolytic activity was determined according to Wang et al [23]. The reaction mixture containing 0.1 mL of 245 mM phosphate buffer (pH 7.0) and 0.4 mL of 0.72% fibrinogen solution was incubated at 37 °C for 5 min; after that, 0.1 mL of a 20 $U.mL^{-1}$ thrombin solution was added and the reaction mixture was again incubated at 37°C for 10 min. After clot formation, 0.1 mL of diluted enzyme extract was added, and incubation continued at 37°C for 60 min, the reaction mixture being shaken every 20 min. The reaction was stopped by adding 0.7 mL of 0.2 M trichloroacetic acid (TCA). The reaction mixture was centrifuged at 15000 xg for 10 min, and the absorbance of the supernatant was measured at 275 nm by spectrophotometry. In this assay, one unit of fibrinolytic protease activity was defined as the amount of enzyme required to produce an increase in absorbance equal to 0.01 per minute, equivalent to liberation of tyrosine. All experiments were performed in triplicate.

2.7. Evaluation anticoagulant effect

The anticoagulant effect of the enzyme extract was observed by monitoring of the no formation of a fibrin clot artificial. For the test was added in polypropylene tubes 0.6 mL of enzyme extract without dilution and diluted (1:2), (1:4) , (1:8) in 0.6 mL of a solution containing ; 0, 1 ml of phosphate buffer (245 mM, pH 7.0), 0.4 mL of fibrinogen (0.72%), 0.1 ml of thrombin solution (20 $U.mL^{-1}$). As a control, the enzyme extract was replaced by saline solution (0.15 M, pH 7.2). The assays were incubated at 37°C for 1 h. (Lu et al., 2010, Modified).

2.8. Amidolytic activity determination and substrate specificity

Amidolytic activity of the enzyme was performed according to Chang et al [2]. The following synthetic chromogenic substrates were analyzed: *N*-succinyl-Ala-Ala-Pro-Phe-pNA, *N*-benzoyl-Val-Gly-Arg-*p*NA, *N*-Succinyl-Ala-Ala-Val-*p*NA, Gly-Pro-*p*NA, Gly-Phe-*p*NA, D-Phe-Val-*p*NA, and D-Val-Leu-Lys-*p*NA. The reaction mixture, containing 63 µL of 1.7 mM synthetic substrate solution, 63 µL of 25 mM Tris-HCl buffer (10 mM $CaCl_2$, pH 7.8), and 25 µL of the enzyme extract, was incubated at 37 °C for 10 min, and then 63 µL of 0.2 M acetic acid was added to stop the reaction. The amount of liberated *p*-nitroanilide was measured using a microplate spectrophotometer (Biotek, model Synergy HT, Winooski – USA) at an absorbance of 405 nm and calculated using an extinction coefficient (ε) of 9950 cm^{-1} M^{-1}. One unit of amidolytic activity was expressed as µmol of *p*-nitroanilide released due to substrate hydrolysis per min per mL by the enzyme. Each value is the mean of 3 determinations.

2.9. Determination of kinetic parameters

The kinetic constants, K_m and V_{max}, of the enzyme were determined by the method of Lineweaver and Burk [14] with different concentrations (0.2-2.8 mM) of *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA as a substrate. Reactions were performed in 25 mM Tris-HCl buffer (10 mM $CaCl_2$, pH 7.8) at 37°C.

2.10. Determination of optimum pH and temperature for the enzyme activity

The determination of the optimum pH and temperature on the amidolytic activity and fibrinolytic activity of the enzyme were measured using *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA and fibrin as substrates, respectively. For determination of the optimum temperature, the enzyme activity was analyzed at several temperatures between 4-80 ºC under the standard assays conditions. For determination of the optimum pH, the reaction occurred at 37 ^{0}C and the enzyme activity was measured in the pH range (3.0-10.0). The buffers used were Glycine-HCl (pH 3.0), Sodium acetate (pH 4.0-5.0), Citrate phosphate (pH 6.0), Tris-HCl (pH 7.0-8.0), Glycine-NaOH (pH 9.0-10.0). The concentrations of the buffers were standardized 20 mM.

2.11. Effect of pH and temperature on the stability of the enzyme

Analysis of the effect of pH and temperature on enzyme stability was performed by incubation of the enzyme for 1h. Then, the activities remaining were determined. For measuring thermal stability analysis, the enzyme extract was incubated at temperatures (4, 25, 37, 40, 50, 60, 70 and 80ºC). To evaluate the effect of pH on enzyme stability, the reaction occurred at 37^{0}C and the enzyme activity was measured in the pH range (3.0-10.0). The buffers and their concentrations used were according to the item 2.10.

2.12. Effects of inhibitors and metal ions on the amidolytic activity of the fibrinolytic enzyme

The inhibitors and metal ions were dissolved in 25 mM Tris-HCl buffer (10 mM $CaCl_2$, pH 7.8); the concentration of the solutions was standardized at 5 mM. The enzyme extract was incubated for 1 h at 37ºC. Following incubation, the residual amidolytic activity of the protease was measured. The following inhibitors were investigated PMSF (fluoride methylphenylsulfonyl - $C_7H_7FO_2S$), EDTA (ethylenediaminetetraacetic acid - $C_{10}H_{16}N_2O_8$), β-mercaptoethanol (2-hydroxy-1- ethanethiol - C_2H_6SO), Pepstatin A (4-amino-3-hidroxy-6-methyl-heptanoic - $C_{34}H_{68}N_5O_9$). The metal ions evaluated were $FeSO_4$, $CuSO_4$, $CaCl_2$, $MgSO_4$, $CoCl_2$, $ZnSO_4$, KCl and $MgCl_2$.

2.13. Storage stability

The storage stability was analyzed at -20, 4 and 25°C for a total of 9 months for the fibrinolytic activity and amidolytic activity. Aliquots were withdrawn every 15 days for determination of the residual amidolytic activity and fibrinolytic activity.

3. Results and discussion

3.1. Conditions for production of enzyme by *Bacillus amyloliquefaciens* UFPEDA 485 in bioreactor

The enzyme production in the fermentations process depends critically on maintaining an oxygen transfer rate to satisfy the optimum oxygen demand of the microorganism for product formation. The most relevant factors concerning oxygen supply in a fermentor are the agitation and aeration rates. The (Fig. 1) shows the interaction between these variables and its effect on enzyme production. The results prove that both agitation and aeration are important factors for the production of fibrinolytic enzyme. The best value for fibrinolytic activity found was 2.169.11 $U.mL^{-1}$ obtained at the highest agitation and aeration rates (800 rpm and 1.5 vvm) in 12 h, at pH 7.2 and 37°C.

From the experiments in bioreactor, it was possible to find the best conditions for agitation and aeration. When compared to previous experiments conducted in flasks (data not shown), the highest fibrinolytic activity was 2.5 times higher and was obtained after 12 hours of fermentation (a fermentation time of 48 hours was required in the flasks experiments - data not shown). These results correspond to 10 fold increase enzyme productivity.

The same result was obtained from the effect of these two operating variables on amidolytic activity (Fig. 2). The best result for amidolytic activity was 1586.89 μmol min^{-1} mL^{-1} also found at 800 rpm and 1.5 vvm in 12h, which again proves the importance of agitation and aeration for the increase production of this enzyme.

The influence of the independent variables, agitation and aeration rate, on the amidolytic and fibrinolytic activities are described in Table 2. All independents variables and their interactions were statistically significant at 95%, and showed *p* values < 0.05. The analysis of variance revealed, $R^2_{adjusted} = 0.99$ for amidolytic activity, and $R^2_{adjusted} = 0.97$ for fibrinolytic activity.

Similar result was observed by Lee et al [13], when studying the production of a fibrinolytic enzyme from *Bacillus subtilis* BK-17 in bioreactor. The best result for fibrinolytic activity was 1,100 $U.mL^{-1}$, and was

achieved after 12h, at 800 rpm, aeration 1.3 vvm, initial pH 7.5 and temperature 37°C. Thus, the results reports in this work clearly demonstrate the biotechnological potential of *Bacillus amyloliquefaciens* UFPEDA 485 on the production of fibrinolytic enzyme, as a two fold increase in enzyme productivy is obtained when compared with the research of Lee et al [13] that used similar conditions of agitation and aeration.

In this work, the growth of the microorganism in the bioreactor under conditions established, showed not lag phase and the exponential phase lasted 12h (Fig. 3). The fibrinolytic and amidolytic activity increased simultaneously and accompanied cell growth. The maximum values for both fibrinolytic and amidolytic activities were achieved at the end of the exponential growth phase and there was no loss of enzyme activity when the fermentation process was extended.

Cho et al. [3] studied the optimum conditions for nattokinase production by submerged cultivation from *Bacillus subtilis*. Temperature and pH were controlled at 37°C and pH 7.0. Dissolved oxygen (DO) level was automatically controlled at 20% by changing agitation speed (500-900 rpm). In batch culture of *B. subtilis* in fermentor, the highest nattokinase activity was obtained at 10 h with 50 g/L of peptone supplementation. As in this work, the production of fibrinolytic enzyme obtained by Cho et al. [3] was also associated with the cell growth and the higher activity was also observed at the end of exponential growth phase. However, the enzyme did not remain stable during the cultivation process and a reduction in activity upon entering the stationary phase of cell growth was observed differently from what is observed in the process here presented.

3.2. Evaluation of the anticoagulant effect of the enzyme

For the test performed in triplicate, after 1h of incubation with enzyme extract it was not observed the formation of fibrin clots. In the control using saline solution, there was formation of the insoluble fibrin clot. The result indicated that the enzyme exhibited an efficient anticoagulant effect. As in this work, other researchers, also tested the anticoagulant effect of fibrinolytic enzyme from endophytic bacterium *Paenibacillus polymyxa* EJS-3, and there was not clot formation in the presence of the enzyme [3].

3.3. Substrate specificity and amidolytic activity of the fibrinolytic protease

The amidolytic activity of the fibrinolytic enzyme was investigated with several synthetic substrates (Table 3). The enzyme showed amidolytic activity and was able to hydrolyse all synthetic substrates tested, being *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA the substrate hydrolysed at the highest rate. *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA is a well-known substrate for subtilisin, chymotrypsin and cathepsin G (serine protease). The enzyme was less effective on the other synthetic substrates *N*-Benzoyl-Val-Gly-Arg-*p*NA; D-Val-Leu-Lys-*p*NA (for Plasmin and Streptokinase activated plasminogen); Gly-Phe-p-nitronilide; D-Phe-Val-*p*NA; *N*-succinyl-Ala-Ala-Val-*p*NA (for Elastase) and Gly-Pro-*p*NA.

The results presented corroborate with Chang et al [2] which used the same synthetic substrates and also showed higher affinity for *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA, showing amidolytic activity of 38.3 nmol.min^{-1} .ml^{-1}. The fibrinolytic enzyme from *Bacillus subtilis* fermented red bean, showed little activity for *N*-benzoyl-Val-Gly-Arg-*p*NA, D-Val-Leu-Lys-*p*NA and *N*-succinyl-Ala-Ala-Val-*p*NA and showed not amidolytic activity to other synthetic substrates tested. However, the enzyme produced in this work stands out of the enzymes currently found in the literature because it showed affinity for all synthetic substrates tested and achieved an amidolytic activity of 1.300.00 µmol.min^{-1}.mL^{-1} for the substrate with higher affinity (*N*-succinyl-Ala-Ala-Pro-Phe-*p*NA).

Choi et al. [4], investigated the amidolytic activity of a fibrinolytic enzyme from fruiting bodies of Korean *Cordyceps militaris* and, among various substrates, the enzyme also exhibited the highest specificity (34.9 µmol. min^{-1}) for *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA, a value that is much lower than the one obtained in this work.

Kim et al. [10] measured the amidolytic activity of a fibrinolytic enzyme from brown seaweed *Costaria costata* on four substrates. All tested substrates were hydrolyzed, but different of the results of this work, the enzyme exhibited a larger activity when the synthetic substrate H-D-Val-Leu-Lys-*p*NA was used.

3.4. Kinetic parameters of the enzyme with amidolytic activity

A Michaelis–Menten-type curve was obtained from the plot of the amidolytic activity of the enzyme using *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA as substrate. The K_m and V_{max} values obtained using a Lineweaver-Burk double-reciprocal plot were 0.68 mM and 357.14 mmole *p*NA min^{-1}.mL^{-1}, respectively. Investigating the fibrinolytic enzyme from a *Bacillus subtilis* and using the same substrate, Chang et al. [2] obtained a K_m 0.59 mM, a value similar to the obtained in this work and a different value for V_{max} (79.4 µmole *p*NA min^{-1}.mg^{-1}). Zambare et al. [25] evaluating the fibrinolytic enzyme from *Pseudomonas aeruginosa* MCM B-327, also used *N*-succinyl-Ala-

Ala-Pro-Phe-*p*NA and different this work reported a value for V_{max} of 36.90 $U.min^{-1}$ and a K_m of 8.81 mM, a value much higher than the one obtained in this work. These results point out the high specificity of the substrate for the enzyme produced by *Bacillus amyloliquefaciens* UFPEDA 485 here presented.

3.5. Effects of inhibitors and metal ions on the amidolytic activity of the fibrinolytic enzyme

The enzyme activity was almost completely inhibited (72.91%) after 60 min of incubation with PMSF (Table 4). These results indicate that hydroxyl (serine) group is located at or near the active site of the enzyme, and prove the results found in the study substrate specificity of the enzyme (affinity for *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA that is a well-known substrate for various serine proteases). The performance of the enzyme to hydrolyze *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA was slightly inhibited by EDTA (29.57%), β-mercaptoethanol (26.01%) and Pepstatin A (25.23%).

Enzyme activity was inhibited by the following ions: $FeSO_4$ (18.89 %), $CuSO_4$ (28.79 %), $MgSO_4$ (34.52 %), $ZnSO_4$ (39.01 %), $CoCl_2$ (35.14 %) and $MgCl_2$ (40.87 %); $CaCl_2$ and KCl slightly increased the amidolytic activity of the enzyme (Table 5). Both the activation as the inhibition the activity of an enzyme occurs due to allosterics effects. The connection of a chemical substance with the allosteric site of an enzyme may induce conformational changes in the spatial structure of the enzyme, changing the affinity by its substrate.

Kim et al. [10] investigated the effect of inhibitors and metal ions on the activity of a protease named CCP with antithrombotic action and in agreement with this work, the enzyme activity was significantly inhibited by PMSF and a slight increase in enzyme activity was observed when incubated with Ca^{++}. Another study that also agrees with this work was conducted by Chang et al. [2], using *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA as substrate for a enzyme from *Bacillus subtilis* The enzyme activity was almost completely inhibited after 30 min incubation with PMSF and, when in contact with calcium ion and potassium ion, the amidolytic activity appeared to increase slightly.

3.6. Effects of pH and temperature on the activity and stability of the enzyme

The enzyme from *Bacillus amyloliquefaciens* UFPEDA 485 had an optimum pH at 7 and 8 for the hydrolysis of *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA, while the highest activity occurred at pH 7 when fibrin was used as the substrate (Fig. 4a).

In what concerns amidolytic activity, the enzyme was very stable in the range of pH 5.0–9.0. The enzyme maintained 100 % activity after incubation at pH 6.0, 7.0 and 8.0 at 37°C for 60 min. The pH stability tests for fibrinolytic activity showed the enzyme was stable in the range of pH 6.0-8.0 and retained 100% activity at pH 7.0 at 37°C for 60 min (Fig. 4b).

The influence of temperature on the enzyme activity showed that the optimum temperature for both fibrinolytic activity and for amidolytic activity was 37°C (Fig. 5a). For the thermal stability tests, the enzyme was stable and retained 100 % activity at temperatures of up to 40°C, after 60 min of incubation. When incubated at 50°C for 60 min, the enzyme retained 64% and 52% of amidolytic activity and fibrinolytic activity, respectively (Fig. 5b).

Chang et al. [2], also analyzed the influence of pH and temperature on amidolytic and fibrinolytic activity, and unlike this work, the enzyme had an optimum pH at 9 for the hydrolysis of *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA, while the highest activity occurred at pH 11 when fibrin was used as substrate. For hydrolysis of *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA, the optimum temperature was 60°C. The enzyme produced in this work offers advantages over others enzymes because showed optimum pH and temperature at physiological conditions (pH 7.0 and temperature 37°C) for both hydrolysis of *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA and fibrin.

In another study, concerning pH and temperature stability, Mahajan et al. [15], using *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA as substrate, observed that the enzyme also was stable in a wide range of pH values (5.0-11.0), but unstable at temperatures above 37°C , as after 1h of incubation at 40°C and 50°C, the enzyme retained only 50% and 18% of its initial activity, respectively, and activity was completely lost at 60°C when incubated by 10 min. The enzyme produced in this work has a higher stability because retained 100% activity after 1h of incubation in the range pH 6.0-8.0, and also retained 100% activity at temperatures of up to 40°C, after 1h of incubation.

Overall, the enzyme produced by *Bacillus amyloliquefaciens* UFPEDA 485 presents relevant advantages, namely higher activity and stability at physiological pH and temperature, in relation to the other enzymes reported in the literature. These features are essential for its successful application.

3.7. Storage stability

The enzyme showed high stability when stored at -20°C temperature. The fibrinolytic and amidolytic activity of the enzyme are preserved to 92.2% and 95.8%, respectively, after 9 months of storage. When kept at a temperature of 4°C, the enzyme retained 86.2% of the amidolytic activity and 62.4% of fibrinolytic activity after 2 months of storage. After 30 days stored at 25°C, the enzyme retained 61.0% of the amidolytic activity and 52.5% of the fibrinolytic activity.

The stability of the fibrinolytic enzyme studied by Chang et al. [2], maintained 48.5%, 59.3% and 61.1% residual activity after being stored at 25, 4, and -20°C, respectively, for 28 days.

In comparison with the results presented in the literature, the enzyme from *Bacillus amyloliquefaciens* UFPEDA 485 proved to be highly stable when stored for long periods, demonstrating the potential of the enzyme to commercialization in the pharmaceutical industry.

4. Conclusion

The best condition of agitation and aeration for enzyme production in a bioreactor were defined allowing for a 10 fold increase in productivity when compared to previous experiments conducted in flasks (data not shown). The presented results also demonstrate the advantages of the enzyme produced by *Bacillus amyloliquefaciens* UFPEDA 485 as compared to other enzymes with amidolytic activity. The enzyme obtained was able to hydrolyse all synthetic substrates tested, showed an amidolytic activity higher than any other enzyme reported in the literature and was active at physiological conditions. Also, a higher storage stability than similar enzymes was observed.

The biotechnological potential of this strain of *Bacillus amyloliquefaciens* to produce a new enzyme of interest to the pharmaceutical industry because of its potential to be used as anticoagulant and fibrinolytic in the thrombolytic therapy and other cardiovascular disorders is demonstrated.

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Tables

Table 1 Levels and values of independent variables of the full 2^2 factorial design

Independent variables	Levels		
	Low (-1)	**Central (0)**	**High (+1)**
Agitation (rpm)	200	500	800
Aeration (vvm)	0.5	1.0	1.5

Table 2 Analysis of variance for the responses amidolytic activity and fibrinolytic activity, over the independent variables agitation and aeration, according to 2^2 full statistical design.

	Amidolytic activity		**Fibrinolytic activity**	
	Estimates effect	***p* Value**	**Estimates effect**	***p* Value**
Agitation	102.51	0.00	30.46	0.00
Aeration	43.61	0.00	8.86	0.01
1*2	6.71	0.02	4.41	0.04
Regression coefficient ANOVA	$R^2_{adjusted} = 0.99$		$R^2_{adjusted} = 0.97$	

All values are statistically significant at 95 % confidence level.

Table 3 Substrate specificity for amidolytic activity of the fibrinolytic enzyme

Synthetic substrate (1.7 mM)	**Substrate hydrolysis rate (μmol.min^{-1}.mL^{-1})**
N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide	1.300 ± 0.80
N-Benzoyl-Val-Gly-Arg-*p*-nitroanilide	344.4 ± 1.04
D-Val-Leu-Lys-*p*-nitroanilide	55.00 ± 2.20
Gly-Phe-*p*-nitronilide	20.08 ± 0.70
D-Phe-Val-*p*-nitroanilide	16.88 ± 1.82
N-succinyl-Ala-Ala-Val-*p*-nitroanilide	14.08 ± 2.22
Gly-Pro-*p*-nitroanilide	13.68 ± 3.06

Table 4 Effects of inhibitors on the amidolytic activity of the fibrinolytic enzyme

Chemical modification reagent, inhibitors	**Residual activity (%)**
Control	100 %
PMSF	27.09 ± 1.02
EDTA	70.43 ± 2.04
β-mercaptoethanol	73.99 ± 3.05
Pepstatin A	74.77 ± 2.01

Enzyme activity was determined using *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA as a substrate. The Values are presented as means ± SD (standard deviation).

Table 5 Effects of metal ions on the amidolytic activity of the fibrinolytic enzyme

Chemical modification reagent, metal ions	**Residual activity (%)**
Control	100 %
$FeSO_4$	81.11 ± 1.08
$CuSO_4$	71.21 ± 1.06
$MgSO_4$	65.48 ± 3.03
$ZnSO_4$	60.99 ± 1.07
$CoCl_2$	64.86 ± 2.03
$MgCl_2$	59.13 ± 1.01
$CaCl_2$	112.1 ± 0.85
KCl	108.5 ± 1.02

Enzyme activity was determined using *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA as a substrate. The Values are presented as means ± SD (standard deviation).

Figure Caption

Fig. 1 Scatterplot of the interaction between the independent variables agitation (rpm) and the aeration (vvm) for the response variables fibrinolytic activity of the protease from *Bacillus amyloliquefaciens* UFPEDA 485.

Fig. 2 Scatterplot of the interaction between the independent variables agitation (rpm) and the aeration (vvm) for the response variables amidolytic activity of the protease from *Bacillus amyloliquefaciens* UFPEDA 485.

Fig. 3 Cell growth (■), Fibrinolytic activity - FA (▲) and Amidolytic activity - AA (○) over time of culture of *Bacillus amyloliquefaciens* UFPEDA 485 in bioreactor under optimum conditions studied (800 rpm and 1.5 vvm).

Fig. 4 Fibrinolytic activity (▲) and Amidolytic activity (○) in **(A)** Effect of pH on the activity of enzyme from *Bacillus amyloliquefaciens* UFPEDA 485 and in **(B)** Effect of pH on the stability of enzyme measured after 1 h incubation and expressed as percentage of residual activity. Buffers used: Glycine-HCl (pH 3.0), Sodium acetate (pH 4.0-5.0), Citrate phosphate (pH 6,0), Tris-HCl (pH 7.0-8.0), Glycine-NaOH (pH 9.0-10.0). All buffer concentrations were 20 mM.. To determine the amidolytic and fibrinolytic activity was used as substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA and fibrin, respectively. The data are presented as means ± SD (n = 3) from three independent experiments.

Fig. 5 Fibrinolytic activity (▲) and Amidolytic activity (○) in **(A)** Effect of temperature on the activity enzyme from *Bacillus amyloliquefaciens* UFPEDA 485 and in **(B)** Effect of temperature on the stability of enzyme measured after 1 h incubation and expressed as percentage of residual activity. Effects of temperature were determined at (4, 25, 37, 40, 50, 60, 70 and 80 ^{0}C). To determine the amidolytic and fibrinolytic activity was used as substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA and fibrin, respectively. The data are presented as means ± SD (n = 3) from three independent experiments.

Figures

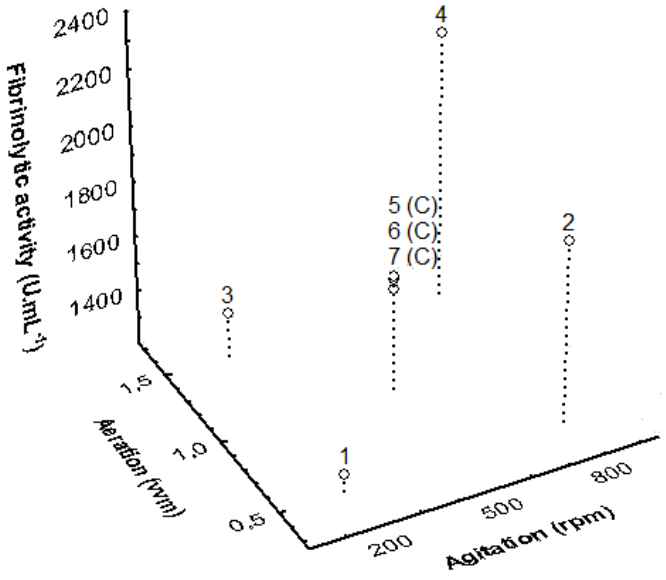


Fig. 1

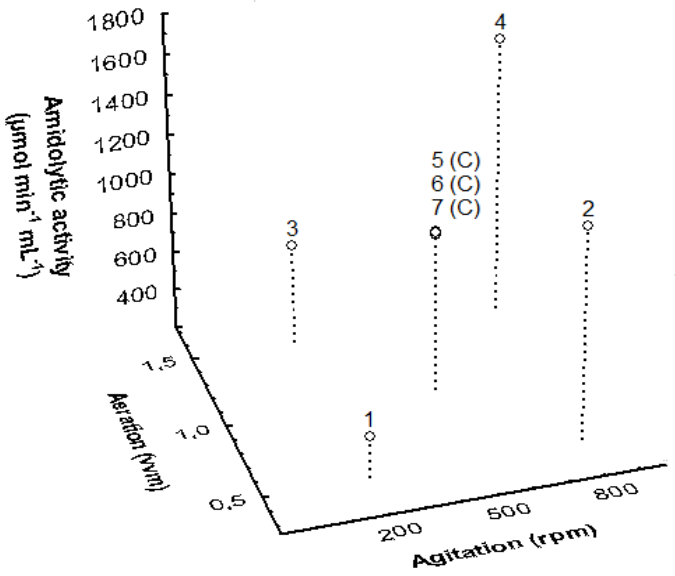

1800
1600
1400
1200
1000
800
600
400
Amidolytic activity
(µmol min-1 mL-1)
1.5
1.0
0.5
Aeration (v/v/m)
200
500
800
Agitation (rpm)
1
2
3
4
5 (C)
6 (C)
7 (C)

Fig. 2

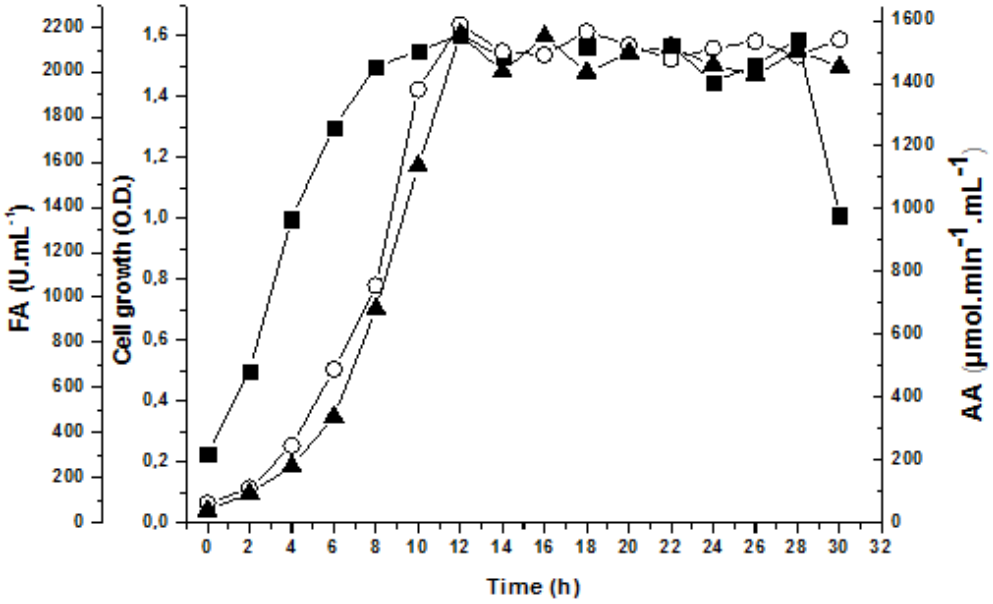
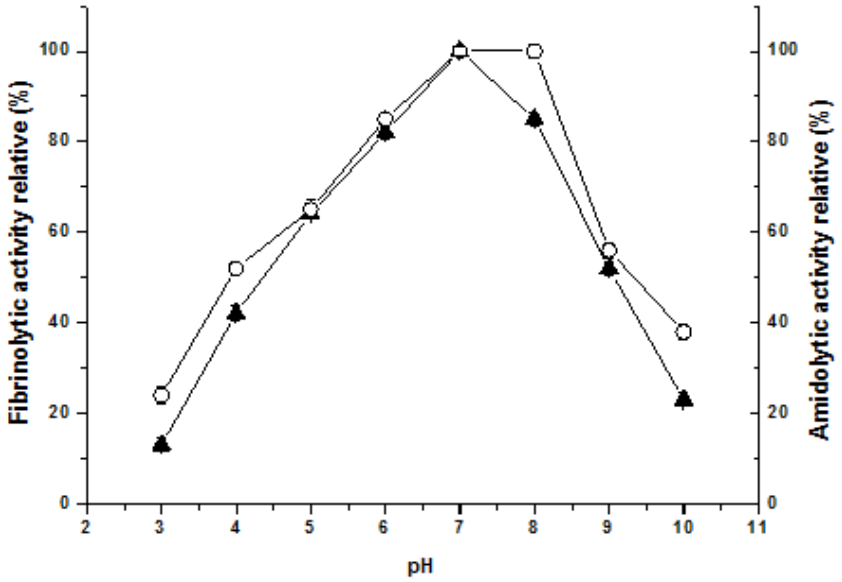
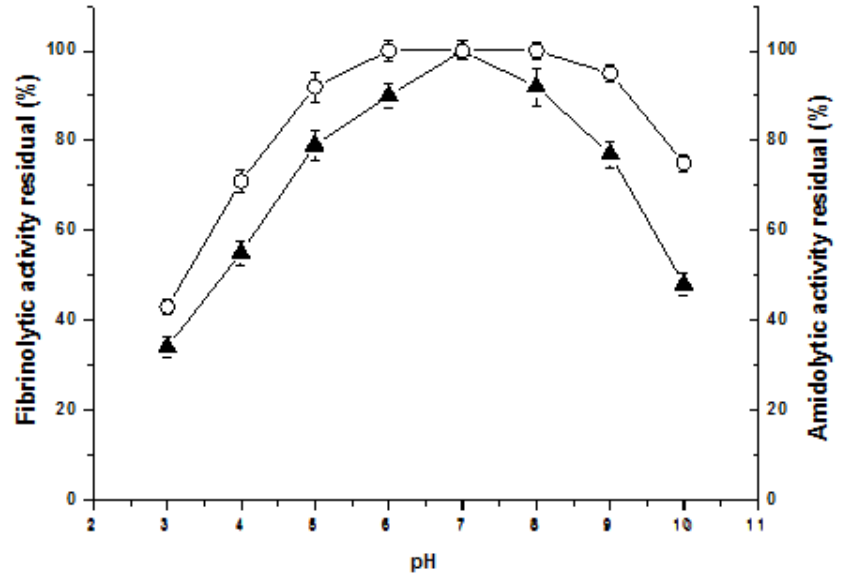


Fig. 3

(A)


Fibrinolytic activity relative (%)
Amidolytic activity relative (%)
pH
0
20
40
60
80
100
2
3
4
5
6
7
8
9
10
11

(B)


Fibrinolytic activity residual (%)
Amidolytic activity residual (%)
pH
0
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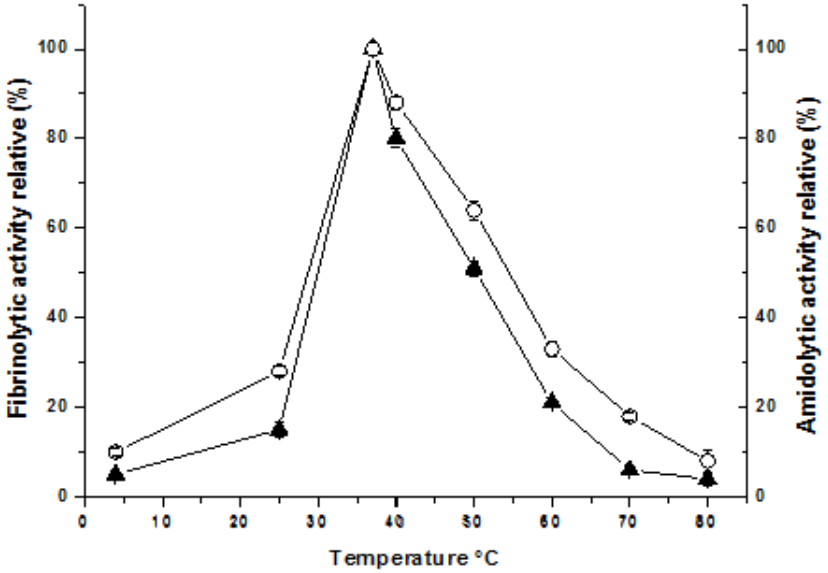
11

Fig. 4

(A)


Fibrinolytic activity relative (%)
Amidolytic activity relative (%)
Temperature °C
0
20
40
60
80
100
0
10
20
30
40
50
60
70
80

(B)

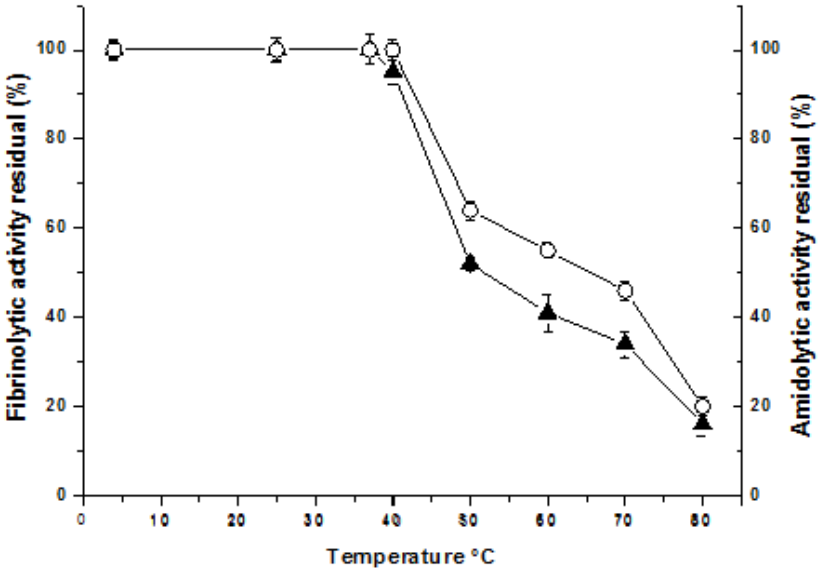
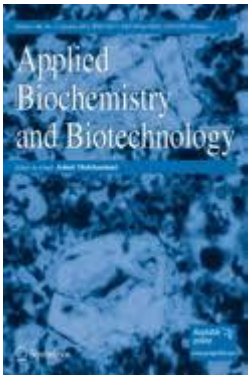

Fibrinolytic activity residual (%)
Amidolytic activity residual (%)
Temperature °C
0
20
40
60
80
100
0
10
20
30
40
50
60
70
80

Fig. 5

CAPÍTULO IV

Integrated Process Production and Extraction of the Fibrinolytic Protease from *Bacillus* sp. UFPEDA 485



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Integrated Process Production and Extraction of the Fibrinolytic Protease from *Bacillus* sp. UFPEDA 485

Amanda Emmanuelle Sales · Fabiana América Silva Dantas de Souza · José Antônio Teixeira · Tatiana Souza Porto · Ana Lúcia Figueiredo Porto

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Abstract Fibrinolytic proteases are enzymes that degrade fibrin; these enzymes are a promising alternative for thrombolytic therapy, and microorganisms produce them. The aim of this study was to evaluate the optimum conditions for the integrated production and purification of fibrinolytic protease from *Bacillus* sp. UFPEDA 485. Extractive fermentation was carried out in a culture medium containing soybean flour and by adding polyethylene glycol (PEG) and Na_2SO_4 according to a 2^3 experimental design. In all assays, the enzyme preferentially partitioned to the bottom phase ($K<1$), with an optimum activity of 835 U ml^{-1} in the bottom phase (salt-rich phase). The best conditions for extractive fermentation were obtained with 18 % PEG 8000 and 13 % Na_2SO_4. Characterization showed that it is a metalloprotease, as a strong inhibition—residual activity of 3.13 %—occurred in the presence of ethylenediaminetetraacetic acid. It was also observed that enzymatic activity was stimulated in the presence of ions: $CaCl_2$ (440 %), $MgCl_2$ (440 %), $FeSO_4$ (268 %), and KCl (268 %). The obtained results indicate that the use of a low-cost substrate and the integration of fermentation with an aqueous two-phase system extraction may be an interesting alternative for the production of fibrinolytic protease.

Keywords Fibrinolytic protease · *Bacillus* · PEG/sodium sulfate · ATPS · Extractive fermentation

A. E. Sales · F. A. S. D. de Souza · T. S. Porto · A. L. F. Porto (✉)
Department of Morphology and Animal Physiology, Federal Rural University of Pernambuco, 52171-900 Recife, PE, Brazil
e-mail: analuporto@yahoo.com.br

J. A. Teixeira
Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus of Gualtar, 4710-057 Braga, Portugal

T. S. Porto
Academic Unit of Garanhuns, Federal Rural University of Pernambuco, 55296-901 Garanhuns, PE, Brazil

Introduction

Proteases are hydrolytic enzymes which degrade proteins into amino acids and peptides. Such proteases are physiologically necessary for all living organisms and they are found in a wide variety of sources, including plants, animals, and microorganisms [1]. Fibrinolytic proteases are enzymes that have the potential to degrade fibrin, the major protein component of blood clots, which are formed from the activation of fibrinogen by thrombin [2]. Fibrinolytic proteases that are produced by bacteria of the *Bacillus* genus have attracted interest as thrombolytic agents due to their efficiency in the fibrinolytic process [3].

Cardiovascular diseases, such as acute myocardial infarction, ischemic heart disease, and high blood pressure, are the leading causes of death in the world. Among the different types of cardiovascular diseases, thrombosis is one of the most widely occurring diseases in modern life. Drugs using fibrinolytic enzymes are the most effective methods in the treatment of thrombosis [4].

Extractive fermentation and product recovery "in situ" have been suggested as a solution to overcome product inhibition and the low productivity of biotechnological processes [5, 6]. The concept of this "in situ" purification process involves the integration of an extractive step as the first stage of downstream processing to simultaneously synthesize and remove the product. This is not only to ensure primary recovery but also to increase the product formation rate by minimizing inhibition by the end product during fermentation [6].

A significant number of processes based on extractive fermentation using an aqueous two-phase system (ATPS) have been reported. Examples include the production of fibrinolytic protease [5], alkaline phosphatase [7], clavulanic acid [8], lipase [9], and products from cyanobacteria [10].

Aqueous two-phase systems are formed by adding two (or more) polymers or a water-soluble polymer and a specific salt in aqueous solution above certain critical concentrations and temperatures, resulting in two immiscible aqueous phases. Solutes of various sizes, such as inorganic ions, small organic molecules, biological macromolecules and inorganic colloidal particles, viruses, and cells, can be partitioned between the phases of ATPS [11].

There have been some prior attempts made by the industry to optimize procedures that ensure high yields with considerable purity [12]. In this regard, partitioning in ATPS provides a powerful method for separating and purifying biomolecules such as proteins. The ATPS also offers advantages such as short processing time, low power consumption, and a biocompatible environment for the biomolecule because of the large amount of water in the extraction systems. Thus, the ATPS has been recognized as an efficient and economical process for the recovery of biomolecules due to its relative low cost and ease of operation [12–14].

The objective of this study was to evaluate the optimum conditions for the integrated production and purification of fibrinolytic protease by *Bacillus* sp. UFPEDA 485 in extractive fermentation using a low-cost medium with polyethylene glycol (PEG)/Na_2SO_4, as phase-forming compounds, and the biochemical characterization of this enzyme.

Materials and Methods

Microorganism

The microorganism *Bacillus* sp. UFPEDA 485 was supplied by the Collection of Microorganisms, Department of Antibiotics at the Federal University of Pernambuco. This

microorganism was isolated from the fermentation of sugar cane mills native from the *Zona da Mata* of Pernambuco, Brazil. The microorganism was stored in nutrient broth (1 % peptone, 0.3 % meat extract, and 0.5 % NaCl) in cryotubes (10 % *v*/*v* glycerol) at −70 °C.

Culture Conditions

The microorganism was grown in nutrient broth, 1 % peptone, 0.3 % beef extract, and 0.5 % NaCl (pH 7.0). After 24 h growth in nutrient broth, 10-ml aliquots of this culture were transferred to conical flasks of 250 ml containing 90 g of extractive fermentation medium using ATPS.

For protease production (submerged fermentation and extractive fermentation), soybean medium (MS-2) described by Porto et al. [15] was used. The medium composition was soybean filtrate (2 % *w*/*v*), K_2HPO_4 (0.435 % *w*/*v*), and 1 ml of mineral solution containing $FeSO_4{\cdot}7H_2O$ (100 mg), $MnCl_2{\cdot}4H_2O$ (100 mg), and $ZnSO_4{\cdot}H_2O$ (100 mg) of distilled water q.s.p. 100 ml, NH_4Cl (0.1 % *w*/*v*), $MgSO_4{\cdot}7H_2O$ (0.06 % *w*/*v*), and glucose (1 % *w*/*v*).

Medium for Extractive Fermentation Using ATPS

The culture medium for extractive fermentation using ATPS was prepared by mixing PEG and sodium sulfate solutions according to the experimental design described in the succeeding section in this study and the culture medium MS-2. After adjusting the pH to 7.2, the system was autoclaved at 121 °C for 20 min.

Extractive fermentation lasted for 84 h and was performed at 150 rpm agitation and 37 °C under different conditions according to a factorial design. At the end of the process, the flasks were left to settle for 1 h and further centrifuged at 12,000×*g* for 15 min to separate both phases (PEG-rich or top and bottom or salt-rich). After that, the phases were subjected to the analytical determinations (protein content and fibrinolytic activity).

Factorial Design 2^3

The influence of phase-forming parameters in the fibrinolytic protease extractive fermentation process was performed according to a 2^3 factorial design shown in Table 1. The independent variables PEG molecular weight and PEG and salt (sodium sulfate) concentrations were selected according to Ashipala and He [5]. The response variables were the partition coefficient (K) and top (FA_t) and bottom (FA_b) phase activities. The experimental design consisted of 12 trials and 4 replicates in the central point, necessary to calculate the pure error.

Table 1 Levels of the independent variables of the full factorial design 2^3 for the extractive fermentation process of the fibrinolytic protease from *Bacillus* sp. UFPEDA 485

Variables	Levels		
	(−1)	(0)	(+1)
PEG molar mass (g mol^{-1})	4,000	6,000	8,000
PEG concentration (% *w*/*w*)	18	24	30
Sodium sulfate concentration (% *w*/*w*)	10.0	11.6	13.0

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 Statistica 8.0 (Statsoft Inc., Tulsa, OK, USA).

Determination of Total Protein

The protein content was determined by the Bradford method [16] using 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.

Determination of Fibrinolytic Activity on Fibrin Plates

Fibrinolytic activity was determined by the fibrin plate method [17]. The fibrin plate was composed of 2 mg ml^{-1} of fibrinogen solution (fibrinogen from bovine plasma in buffer 150 mM Tris–HCl and 150 mM NaCl, pH 7.75), and 200 μl of thrombin from bovine plasma solution (20 U ml^{-1} diluted in 150 mM sodium chloride solution) was added to 4 ml of fibrinogen solution, 4 ml of 2 % agarose, and 100 μl of 1 M $CaCl_2$ solution. The reaction mixture with the agarose was placed in plastic Petri dishes. After the polymerization of fibrin, 20 μl of enzymatic extract was added to 5-mm-diameter wells and then incubated at 37 ° C for 18 h. One unit of fibrinolytic activity corresponds to the correlation between the diameter of the degradation halos in the fibrin plate (in millimeters) and the standard curve made using plasmin from human plasma. The enzymatic activity was expressed as units per milliliter.

Calculating the pure error was difficult due differences in fibrinolytic activity values at the factorial design central points, so the determination of fibrinolytic activity was also carried out with the fibrin degradation assay. However, it is possible to establish an excellent correlation between the two methods.

Determination of Fibrinolytic Activity

Fibrinolytic activity was also measured using the fibrin degradation assay. First, 0.4 ml of 0.72 % fibrinogen was placed in a test tube with 0.1 ml of 245 mM phosphate buffer (pH 7.0) and incubated at 37 °C for 5 min. Then, 0.1 ml of a 20-U ml^{-1} thrombin solution was added. The solution was 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 0.2 M trichloroacetic acid was added and mixed. The reaction mixture was centrifuged at 15,000×*g* for 10 min. After that, 1 ml of the supernatant was collected and the absorbance at 275 nm was measured. Each experiment was performed in triplicate and the average value was then calculated after correction of the corresponding blank. In this assay, 1 U (fibrin degradation unit) of enzyme activity is defined as a 0.01/min increase in absorbance at 275 nm of the reaction solution [2].

Biochemical Characterization of Fibrinolytic Protease

After the integrated production and purification by the extractive fermentation process using ATPS, the sample that showed the best fibrinolytic activity was subjected to biochemical characterization to evaluate the optimum pH, optimum temperature, stability to pH and temperature, as well as the effect of enzyme inhibitors and metal ions.

Effect and Stability to pH and Temperature on Fibrinolytic Activity

To study the effect of pH and its stability, the fibrinolytic protease generated by extractive fermentation (salt ATPS phase) was mixed with different buffers: sodium acetate (pH 3.0 to 5.0), citrate phosphate (pH 5.0 to 7.0), Tris–HCl (pH 7.0 to 8.5), and glycine–NaOH (pH 8.5 to 11.0) and incubated at 37 °C for 60 min.

The temperature effect was determined by incubating the salt ATPS phase at temperatures ranging between 4 and 85 °C for 30 min. To determine the stability to temperature and pH, aliquots were withdrawn every 15 min for a period of 1 h. These aliquots were submitted to the determination of fibrinolytic activity by the fibrin plate method.

Effect of Metal Ions on Fibrinolytic Activity

Fibrinolytic activity samples from the bottom phase (extractive fermentation) were evaluated in the presence of ions. These ions are described as inhibitors or activators of fibrinolytic activity. The effect of ionic solutions was evaluated at a concentration of 5 mM. The bottom phase was exposed to the following ions: zinc sulfate [$(ZnSO_4)\cdot 7H_2O$], magnesium sulfate [$MgSO_4$], copper sulfate [$CuSO_4$], ferrous sulfate [$FeSO_4$], calcium chloride [$CaCl_2$], magnesium chloride [$(MgCl_2)\cdot 4H_2O$], potassium chloride [KCl], and cobalt chloride [$(CoCl_2)\cdot 2H_2O$] and incubated at 37 °C for 60 min. The ions were dissolved in Tris–HCl pH 7.75 with 0.15 M NaCl. Fibrinolytic activity was determined by the fibrin plate method.

Effect of Inhibitors in Fibrinolytic Activity

To evaluate the effect of inhibitors on enzyme activity, the bottom phase containing the fibrinolytic protease was exposed to the following inhibitors: PMSF (fluoride methylphenylsulfonyl—$C_7H_7FO_2S$), mercuric chloride ($HgCl_2$), 2-mercaptoethanol (2-hydroxy-1-ethanethiol—C_2H_6SO), and ethylenediaminetetraacetic acid (EDTA—acetic—$C_{10}H_{16}N_2O_8$) and incubated for 60 min at 37 °C. The inhibitors were dissolved in pH 7.75 Tris–HCl with 0.15 M NaCl, and the concentration of the solutions was standardized at 5 mM. The methodology for the determination of fibrinolytic activity was performed by the fibrin plate method.

Methodology for the Analysis of the Results

The distribution of the fibrinolytic protease between phases was expressed in terms of the partition coefficient (K), calculated as:

$$K = \frac{FA_t}{FA_b} \tag{1}$$

where FA_t refers to fibrinolytic activity in the top phase and FA_b refers to fibrinolytic activity in the bottom phase.

The specific fibrinolytic activity (SFA) was calculated by the ratio of fibrinolytic activity (FA) and the total protein (P) (in units per milligram):

$$SFA = \frac{FA_t}{P} \tag{2}$$

Results and Discussion

Extractive Fermentation of the Fibrinolytic Protease

Bacillus sp. UFPEDA 485 was the subject of this study on finding the best conditions for extractive fermentation using ATPS based on a complete experimental design. The matrix of the design variables and the results of the responses, the partition coefficient (K), and the activity on the top and bottom phases are shown in Table 2. In all the experiments, fibrinolytic activity ranged between 250 and 835 U ml^{-1}.

The fibrinolytic protease partitioned preferentially to the bottom phase in all experiments ($K<1$), while the cells were positioned at the interface. The system consisting of 18 % (*w*/*w*) PEG 8000 and 13.0 % (*w*/*w*) salt showed a partition coefficient of $K=0.3$.

The higher values in fibrinolytic activity (835 U ml^{-1}) were obtained in the bottom phase of the systems with the highest concentration of salt (13.0 % *w*/*w*). The highest activity values of fibrinolytic protease from *Bacillus* sp. UFPEDA 485 were observed at experimental runs 6 and 8, as shown in Fig. 2. High values of fibrinolytic activities were also obtained by Ashipala and He [5], but they found that, when using PEG 4000 (7 %) and sodium sulfate (4.8 %), the best obtained fibrinolytic activity was 1,224 U ml^{-1} with a partition coefficient of $K=0.44$. In the article of Ashipala and He [5], the authors only showed the fibrinolytic enzyme partition in ATPS, while in the current work, the simultaneous production and purification/partition in ATPS was described. In this paper, as the integrated production and purification of fibrinolytic protease was presented, the production medium, the cells, and ATPS together in one system may alter the cellular physiology, modify the viscosity of the medium due to the polymers inserted, and consequently, modify the production of the biomolecule and its partition. Moreover, each paper describes a different fibrinolytic enzyme, and the partition process is different, too.

Partition of penicillin acylase in ATPS composed of 10 % PEG 4000 and 7.5 % of sodium sulfate was evaluated by Gavasane and Gaikar [18], using similar conditions to this work,

Table 2 Matrix decoded and results of the full factorial design 2^3 for the responses: the partition coefficient (K) and activity in the ATPS top and bottom phases

Runs	MPEG (g mol^{-1})	CPEG (% *w*/*w*)	Csalt (% *w*/*w*)	FA_t (U ml^{-1})	FA_b (U ml^{-1})	K	V_r
1	4,000	18	10.0	250	605	0.4	1.0
2	8,000	18	10.0	410	685	0.5	1.0
3	4,000	30	10.0	360	675	0.5	1.8
4	8,000	30	10.0	280	485	0.5	1.0
5	4,000	18	13.0	350	740	0.4	1.0
6	8,000	18	13.0	300	835	0.3	0.8
7	4,000	30	13.0	345	430	0.8	1.2
8	8,000	30	13.0	415	770	0.5	1.0
9 (C)	6,000	24	11.6	390	495	0.7	1.0
10 (C)	6,000	24	11.6	360	455	0.7	1.0
11 (C)	6,000	24	11.6	375	380	0.9	1.0
12 (C)	6,000	24	11.6	295	415	0.7	1.0

FA_t fibrinolytic activity in the top phase, FA_b fibrinolytic activity in the bottom phase, K partition coefficient, V_r volume ratio of the phases, *(C)* central points

with the main difference that extraction was made separately from fermentation. The obtained partition coefficients of the enzyme and proteins were 0.01 and 0.07, respectively [19].

ATPS formed by PEG (2000, 4000, and 6000), polymers dextran T500 and dextran 40, magnesium sulfate salts, sodium sulfate, and trisodium citrate and were evaluated for the partition of alkaline phosphatase. The obtained partition coefficients (K=0.1 and K=0.06) for the PEG 6000/Na_2SO_4 and PEG 4000/Na_2SO_4 systems, respectively, indicated that the enzyme under study partitioned mostly to the bottom phase, as in this work [7].

The specific activity of the fibrinolytic protease obtained by conventional fermentation (data not shown) and extractive fermentation using ATPS was compared. The best result for enzyme-specific activity in conventional fermentation was of 1,054 U mg^{-1} of enzyme while using the same culture conditions, but in extractive fermentation (ATPS), the enzyme-specific activity was 9,240 U mg^{-1}. For this situation, the obtained enzyme recovery yield was 136 %. These results clearly demonstrate the advantages of using extractive fermentation as it was possible to obtain a ninefold concentration of the target enzyme as a result of process selectivity and reduced product inhibition.

Higher concentrations of enzyme were also obtained in a study with extractive fermentation using aqueous two-phase systems (PEG 4000, 6000, and 9000 with potassium phosphate) [19]. In the production of the alkaline protease of *Bacillus thuringiensis*, a 2.8 times larger activity was obtained with the 13.6 % (*w*/*w*) PEG 4000 and 13.7 % (*w*/*w*) potassium phosphate ATPS relative to conventional fermentation [19].

Statistical analysis showed that the variables concentration of PEG (CPEG), the interaction between PEG molar mass (MPEG) and concentration of sodium sulfate (Csalt), as well as the interaction between all variables were statistically significant for the fibrinolytic activity response in the bottom phase (FA_b) (Fig. 1), since the values were higher than the level of statistical significance, as shown in Table 3. However, the variable (CPEG) PEG concentration showed a negative effect on fibrinolytic activity in the bottom salt-rich phase. This indicates that the decrease in PEG concentration contributes to the increase in fibrinolytic activity in the bottom phase (Fig. 1).

The interaction between the variables MPEG and Csalt was statistically significant and revealed that an increase in their levels has improved the fibrinolytic activity values in the bottom phase (FA_b) (Fig. 2). So, the best condition for the response (FA_b) was obtained with the system MPEG 8000 18.0 % (*w*/*w*) and Csalt 13.0 % (*w*/*w*).

These results show that the enzyme can be entrapped by metal ions of the lower salt-rich phase due to ionic interaction between the metal ions and the anionic amino acids of the enzyme molecule [19]. Studies have been conducted to evaluate the interaction between the metal ions from the lower phase of the system and the biomolecule of interest aiming at partition of the biomolecule to the lower phase through the existing ionic interactions [20].

Similar results were obtained for purification in ATPS of a lipase produced by *Bacillus subtilis*, with the PEG molar mass being the most significant effect. The partition coefficient of the enzyme was reduced as a consequence of the increased hydrophobicity of the top phase due to the increase in chain length of PEG that had less hydroxyl groups for the same concentration of the polymer [21].

Partition Coefficient of the Fibrinolytic Protease

Table 3 demonstrates that no variable or interaction between them had a statistically significant effect for the response partition coefficient (K) because the values of the estimated effects are below the level of statistical significance (p=0.05). In all experiments,

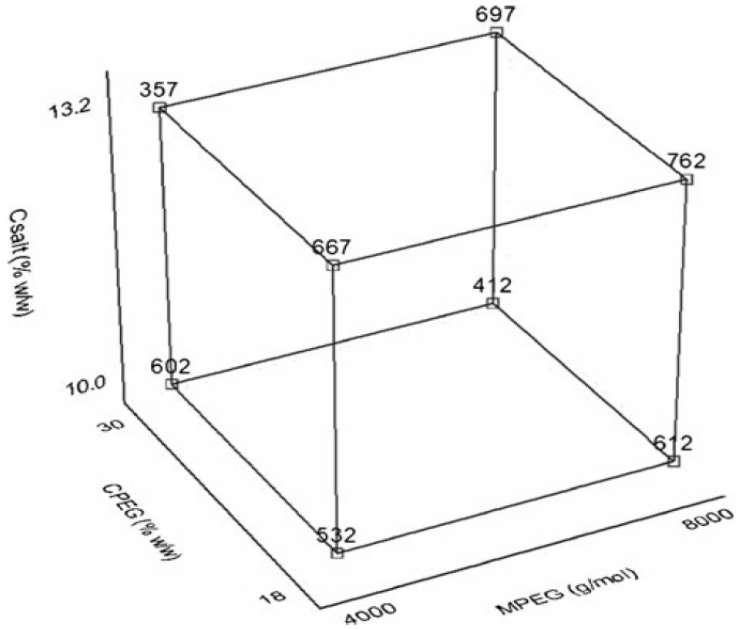

Fig. 1 Cubic plot of the interaction between the variables salt concentration (*Csalt*), PEG concentration (*CPEG*), and PEG molar mass (*MPEG*) for fibrinolytic activity response in the bottom phase of extractive fermentation

values of the partitioning coefficient (K) were <1 and ranged from 0.3 to 0.9, indicating that the fibrinolytic protease preferentially partitioned in the bottom phase (Table 3).

In contrast to these results, the PEG molar mass had no influence on the partition coefficient as the hydrophobic character of PEG increases with the chain length. In a study of phenylalanine ammonia–lyase purification, the system formed by PEG 1000 at a concentration of 13 % and sodium sulfate at 14 % was regarded as the best condition for partition. The partition of the enzyme decreased with increasing PEG molar mass (from 1,000 to 3,350) due to the excluded volume effect [22].

The presence of salts can affect partitioning by weakening or strengthening the interactions or the interaction between ionized groups with the opposite net charge of proteins. The effectiveness of the salt is mainly determined by the nature of the anion. Multicharged anions

Table 3 Calculated effect of the responses in the factorial design 2^3 for the integrated production and purification of the fibrinolytic protease by *Bacillus* sp. UFPEDA 485

Effects	Fibrinolytic activity		
	K	FA_t	FA_b
1. MPEG	−3.73	0.84	2.31
2. CPEG	1.74	0.76	−3.59*
3. Csalt	−2.72	0.93	2.31
1×2	−1.27	−1.01	−0.18
1×3	2.22	−0.51	3.87*
2×3	−0.38	1.09	−1.74
1×2×3	−0.23	3.04	3.66*

*$p<0.05$, data were statistically significant at the 95 % confidence level

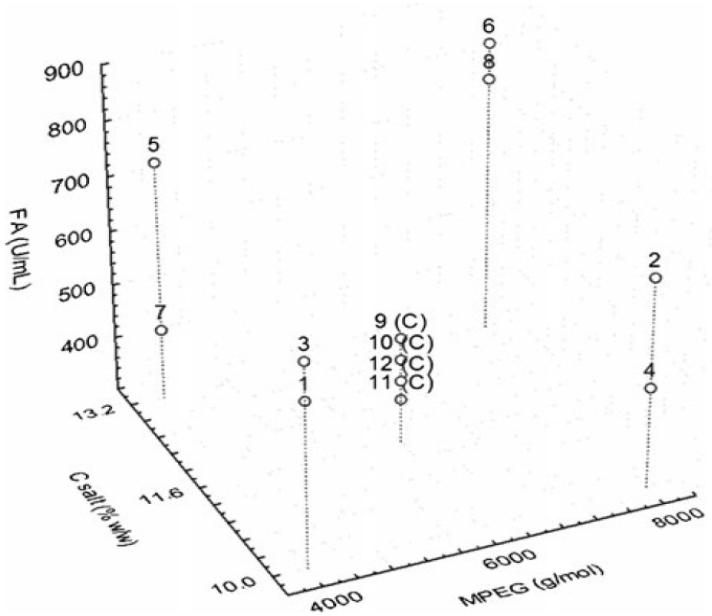

Fig. 2 Simultaneous effects of salt concentration (*Csalt*) and PEG molar mass (*MPEG*) for fibrinolytic activity in the bottom phase of extractive fermentation

are the most effective in the order: $SO_4^{2-}>HPO_4^{2-}>CH_3COO^->Cl^-$. The order of cations is usually given as $NH_4^+>K^+>Na^+>Li^+>Mg^{2+}>Ca^{2+}$ [23].

The potential difference between the phases will influence the partitioning of macromolecules and charged particles, especially those carrying a large number of electric charges on their surface. The electric potential created can be adjusted by changing the salt's composition and concentration; therefore, it can be used to control the partition coefficient [24].

Protease Inhibitors on Fibrinolytic Activity

The enzyme was subjected to the action of inhibitors and metal ions after pre-purification by extractive fermentation. Enzyme activity was significantly inhibited by the metalloprotease inhibitor, EDTA, showing a residual activity of 3.1 %. It has been also slightly inhibited by β-mercaptoethanol (98.9 %) but was not inhibited when subjected to PMSF, the inhibitor of serine proteases. These results allowed characterizing fibrinolytic protease as a metalloprotease. Similar results were obtained by Simkhada et al. [25] and Park et al. [26]. The effect of the different inhibitors can be seen in Table 4.

Table 4 Effect of inhibitors on the fibrinolytic activity

Inhibitors	Residual activity (%)
Control	100
EDTA	3.1
β-Mercaptoethanol	98.8
PMSF	100

Metal Ions on Fibrinolytic Activity

Enzyme activity was not affected by the ions $ZnSO_4$, $MgSO_4$, $CuSO_4$, and $CoCl_2$. On the other hand, enzymatic activity was stimulated in the presence of the following ions: $CaCl_2$ (440 %), $MgCl_2$ (440 %), $FeSO_4$ (268 %), and KCl (268 %) and inhibited only by the HgCl ion (94.20 %). The effect of metal ions can be seen in Table 5.

The $MgCl_2$, $FeSO_4$, and KCl ions also stimulated the activity of fibrinolytic protease produced by the *Paenibacillus polymyxa* EJS-3 bacterium whose residual activity was 111, 110, and 103 %, respectively [27]. The $CaCl_2$ ion also increased the fibrinolytic activity of the protease produced by *Streptomyces* sp. CS684; however, enzyme activity was inhibited by $ZnSO_4$ (35 %), $CuSO_4$ (1.6 %), and $CoCl_2$ (41.8 %) [25]. When incubated with the ions $CaCl_2$ and $MgCl_2$, the fibrinolytic protease activity produced by *Ganoderma lucidum* Vk12 [28] was also stimulated, as in this work.

Optimum Temperature and Stability of the Fibrinolytic Protease

The optimum temperature of the fibrinolytic protease was 37 °C, as can be seen in Fig. 3. At room temperature (25 °C), the enzyme showed 88 % activity; at 45 °C, the activity was 84 %, while it decreased to 76 % when subjected to 55 and 65 °C. These results show that the enzyme activity of the crude extract of conventional fermentation was reduced to 60 % at 55 °C and completely lost the activity at 65 °C (data not shown). These results show that, after extractive fermentation, the fibrinolytic protease became more stable at high temperatures.

Fibrinolytic activity was maintained at 80 % after 60 min of exposure to optimum temperature. These results differed from those obtained for the biochemical characterization of the fibrinolytic protease produced by *Bacillus* sp. nov. SK006, whose optimum temperature was 40 °C, had reduced activity at 50 °C, and was completely inactivated at 65 °C for 10 min of exposure [2]. Similarly, the optimum temperature of nattokinase produced by *B. subtilis* TKU007 was 40 °C. The enzyme retained 70 % activity at 60 °C and it was reduced to 52 % at 70 °C [2].

Optimum pH and Stability of the Fibrinolytic Protease

Results on the effect of pH on enzyme stability can be observed in Fig. 4. The optimum pH of the fibrinolytic protease obtained by extractive fermentation was detected between pH 7.0 and 8.5 in the presence of 0.05 M Tris–HCl and NaOH–glycine buffer, respectively. The

Table 5 Effect of the metal ions on the fibrinolytic activity

Metal ions	Residual activity (%)
Control	100.0
$MgSO_4$	100.0
$CuSO_4$	100.0
$CoCl_2$	100.0
$CaCl_2$	440.7
$MgCl_2$	440.7
$FeSO_4$	268.8
KCl	268.8
HgCl	94.2

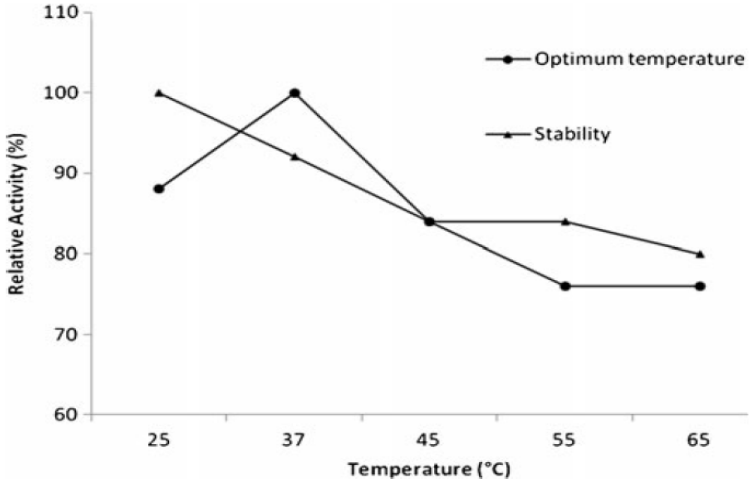

Fig. 3 Optimum temperature and stability of the fibrinolytic protease produced by *Bacillus* sp. UFPEDA 485 in the bottom phase of extractive fermentation

enzyme retained 95.2 % of its activity at pH 7.0 and 8.5. It has also maintained 92.8 % of its activity at pH 11.0, 85 % at pH 5.0, and 52 % at pH 3.0. The results show that, after pre-purification, the enzyme is stable over a broad range of pH values and a considerable lost in activity only occurs at acidic pH (3.0). The enzyme was characterized as a neutral to alkaline protease since it has maintained its activity when tested in the neutral–alkaline range (pH 7.0–8.5).

Similar results were obtained in the characterization of the fibrinolytic protease produced by *B. subtilis* ICTF-1 after purification, where the enzyme remained active in the pH range between 7.0 and 11.0, decreasing at pH 5.0 [4]. Fibrinolytic protease produced by *B. subtilis* also retained its activity between 5.0 and 11.0 [4]. In this work, the optimum pH of the enzyme was detected at pH 9.0, and at pH 7.0, the enzyme showed only 68 % activity [4].

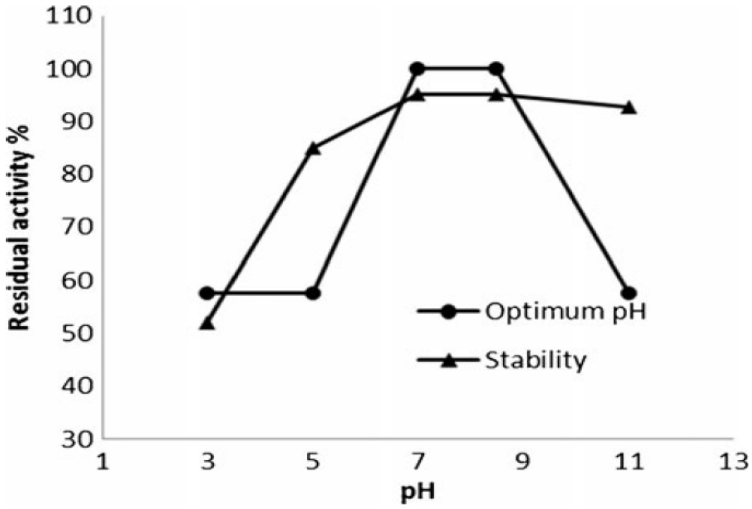

Fig. 4 Optimum pH and stability of the fibrinolytic protease produced by *Bacillus* sp. UFPEDA 485 in the bottom phase of extractive fermentation

Conclusions

It can be concluded that the integration of fermentation and purification made possible the purification of the fibrinolytic protease produced by *Bacillus* sp. UFPEDA 485. Furthermore, the use of ATPS in extractive fermentation can direct the development of a low-cost process by replacing the initial stages of conventional separation processes.

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CONSIDERAÇÕES FINAIS

- Das 43 bactérias do gênero *Bacillus*, 30 bacérias apresentaram atividade fibrinolítica, e o *Bacillus amilolyquefaciens* UFPEDA 485 foi considerado o melhor produtor por apresentar a maior atividade fibrinolítica.
- Foi possível otimizar em frascos agitados as condições de produção da enzima fibrinolítica produzida pelo *Bacillus amyloliquefaciens* UFPEDA 485, com o auxílio de ferramentas estatísticas.
- Os resultados demonstram o potencial biotecnológico do *Bacillus amyloliquefaciens* UFPEDA 485 de poduzir uma nova enzima com propriedades fibrinolítica e anticoagulante de interesse para indústria farmacêutica para aplicação no tratamento de distúrbios vasculares.
- A protease fibrinolítica do *Bacillus amyloliquefaciens* UFPEDA 485 se destaca por apresentar propriedades fisico-químicas adequadas ao sistema sanguíneo, além de possuir alta estabilidade de estocagem quando comparada com outras enzimas citadas na literatura.
- Foi selecionada a melhor condição de agitação e aeração para a produção da enzima em biorreator pela constatação do aumento de 10 vezes mais na produtividade, quando comparado com os experimentos desenvolvidos em frascos agitados.
- O processo de produção e purificação integrado por fermentação extrativa em sistema de duas fases aquosas permitiu a purificação parcial da enzima fibrinolítica do *Bacillus amyloliquefaciens* UFPEDA 485, podendo substituir as fases iniciais dos processos de separação convencionais, reduzindo assim o custo para obtenção final da enzima.