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Laboratório de Imunopatologia Keizo Asami

Páblo Eugênio da Costa e Silva

PRODUÇÃO DE ENZIMA FIBRINOLÍTICA A PARTIR DA MICROALGA *Chorella vulgaris* UTILIZANDO RESÍDUOS INDUSTRIAIS

Recife, 2013

Páblo Eugênio da Costa e Silva

Produção de Enzima Fibrinolítica a partir da Microalga *Chlorella vulgaris* Utilizando Resíduos Industriais

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Dedico

aos meus pais, irmãos, amigos, avó Inês Pereira (In Memoriam), as minhas entidades e a todo meu povo que sempre me guiaram

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“A inveja é a falta de capacidade”

Vó Zefa

Resumo

As doenças cardiovasculares são as principais causas de morte em todo o mundo e podem ser causadas pelo o acúmulo de fibrina nos vasos sanguíneos. Vários micro-organismos produtores de enzimas fibrinolíticas já são descritos na literatura, dentre eles bactérias e fungos. O objetivo do trabalho foi produzir enzimas fibrinolíticas a partir da microalga *Chlorella vulgaris* utilizando glicerol e milhocina, resíduo agroindustrial do processamento do milho, como fonte de carbono e nitrogênio, respectivamente. No cultivo utilizando 0,5 % de milhocina em meio Bold' Basal (BBM) foi observado uma produção de 745 U mL^{-1} de enzima fibrinolítica pela microalga. Após a seleção do meio de cultura, foi realizado um delineamento estatístico utilizando Metodologia de Superfície de Resposta (RSM). A concentração celular foi otimizada, e o valor estimado pela equação foi de 1,52 g L^{-1}, utilizando 0,9 % de glicerina e 1,2 % de milhocina. A produtividade celular, produção de protease e enzima fibrinolítica não conseguiram ser otimizadas, no entanto alcançaram níveis de 232 mg L^{-1} dia^{-1}, 416 U mL^{-1} e 704 U mL^{-1}, respectivamente. O presente trabalho mostrou que *C. vulgaris* cultivada em meio de cultura utilizando glicerol e o resíduo agroindustrial milhocina, pode ser viável para obter altos níveis de concentração de enzima fibrinolitica, bem como ser utilizada como um recurso no combate a doenças cardiovasculares.

Palavras-chave: *Chlorella vulgaris*, Enzima Fibrinolítica, Milhocina, Resíduo agroindustrial

Abstract

Cardiovascular diseases are the leading causes of death worldwide and may be caused by the accumulation of fibrin in the blood vessels. Various micro-organisms producers of fibrinolytic enzymes are already described in the literature, including bacteria and fungi. The objective was to produce fibrinolytic enzymes from the microalgae *Chlorella vulgaris* using glycerol and corn steep liquor, agroindustrial residue from corn processing, as a source of carbon and nitrogen, respectively. In the culture conditions that using 0.5 % corn steep liquor in medium Bold 'Basal (BBM) liquid was observed 745 U mL^{-1} of fibrinolytic enzyme from microalgae. After selection of culture medium, it was done a statistical design using Response Surface Methodology (RSM). Cell concentration was optimized and predicted value by the equation was 1.52 g L^{-1} using 0.9 % glycerol and 1.2 % of corn steep liquor. Cell productivity, protease production and fibrinolytic enzyme was not optimized, however, reached values of 232 mg L^{-1} day^{-1}, 416 U mL^{-1} and 704 U mL^{-1}, respectively. The present study showed that *C. vulgaris* grown in culture medium, using glycerol and corn steep liquor, may be feasible to obtain high levels of fibrinolytic enzyme concentration, as well as being used as a resource against cardiovascular disease.

Keywords: *Chlorella vulgaris*, Fibrinolytic Enzyme, Corn Steep Liquor, Agroindustrial Waste

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LISTA DE ABREVIATURAS E SIGLAS

OMS - Organização Mundial da Saúde

RSM – Metodologia de Superfície de Resposta

FDA – Food and Drug Administration

TNK – Tenecteplase

rt-PA – Alteplase

PLG – Plasminogênio

PLa – Plasmina

u-PA – Uroquinase Ativadora do Plasminogênio

t-PA – Ativador do Plasminogênio Tecidual

TAFI – Inibidor da Fibrinólise Ativado pela Trombina

CVD – Cardiovascular Disease

PEG – Polyethilene Glycol

CHO – Chinese Hamster Ovary

CIP – Codium intricatum protease

CLP – Codium latum protease

CDP – Codium divaricatum protease

pH - Potencial Hidrogeniônico

BBM – Bold's Basal Medium

C_{gly} – Glycerol Concetration

C_{CSL} – Corn Steep Liquor Concentration

X_m – Maximum Cell Concentration

Px – Cell Productivity

P_{act} – Activity Protease

F_{act} – Activity Fibrinolytic

TCA – Trichloroacetic Acid

CCD – Central Composite Design

SUMÁRIO

1. Introdução

Nos últimos anos, as doenças cardiovasculares são as principais causas de morte em muitos países. De acordo com a Organização Mundial de Saúde (OMS) cerca de 17,3 milhões de pessoas morreram vítimas de doenças cardiovasculares em 2008, e existe uma estimativa de que o número de mortes chegue a 23,6 milhões no ano de 2030. Cerca de 80% das mortes registradas foram notificadas em países de renda média a baixa (WHO, 2011).

Doenças cardiovasculares são desordens que acometem o coração e os vasos sanguíneos, e são as principais causas de morte em todo o mundo (SIMKHADA et al., 2010), podendo estar relacionadas ao desequilíbrio na formação dos coágulos sanguíneos.

A formação do coágulo sanguíneo, porém, é considerado um fenômeno de proteção pelo corpo humano, bloqueando o fluxo sanguíneo para fora do corpo após feridas e lesões vasculares, mas algumas vezes, em situações descontroladas, podem bloquear o fluxo de sangue e acarretar em enfarto do miocárdio e acidente vascular cerebral. A fibrina é uma malha insolúvel que forma o principal componente do coágulo sanguíneo. A plasmina é uma serino protease que é ativada por ativadores do plasminogênio e tem a função de digerir proteoliticamente a rede de fibrina. O equilíbrio na formação da rede de fibrina e na dissolução do coágulo é necessário, afim de manter corretamente o fluxo de sangue através da formação de um coágulo no local da lesão e remoção da fibrina em locais indesejados (BAJZAR et al.,1996).

Uma série de anexos (ativadores do plasminogênio) são utilizados no tratamento de distúrbios relacionados ao coágulo sanguíneo. Dentre eles podemos destacar a uroquinase (GUREWICH et al., 1984), ativador do plasminogênio tissular (COLLEN & LIJNEN, 2004), estreptoquinase (VERSTRAETE et al., 1985) e estafiloquinase (COLEN, 1998), que são amplamente sugeridos para remoção de coágulos. Mas os problemas com estes anexos envolvem hemorragias internas (GURWITZ et al., 1998) e o seu elevado custo (BLANN et al., 2005).

Proteases microbianas representam uns dos três maiores grupos de enzimas industriais possuindo uma ampla aplicação em produtos industriais e

domésticos, incluindo detergentes, alimentos, couro, seda e indústria de produtos farmacêuticos (MUKHERJEE et al., 2008; RAI et al, 2009; 2010). As vantagens da produção de enzimas por via microbiana é seu baixo custo e alta eficácia (DEEPAK et al., 2010). Uma das principais aplicações farmacêuticas das protease microbianas (protease fibrinolítica) é no tratamento da trombose, que é considerada uma das doenças cardiovasculares mais comuns da vida moderna (MINE et al., 2005).

Enzimas fibrinolíticas estão sendo extraídas a partir de diversos vegetais, animais e micro-organismos. Enzimas fibrinolíticas a partir de fontes microbianas têm sido relatadas em várias espécies de *Bacillus*, *Pseudomonas*, *Staphylococcus*, *Alteromonas*, bactérias *Coryneform*, *Penicillium*, *Aspergillus*, *Fusarium*, *Trichotecium*, *Actinomyces*, *Esherichia coli* e *Streptomyces*.

O cultivo de microalgas destinados a obtenção de enzimas fibrinolíticas ainda não foi relatado, mas pode ser uma alternativa na bioprospecção de novas enzimas. Segundo Lourenço (2006) as aplicações mais simples de microalgas compreendem seu uso na alimentação de animais e do homem. Outras aplicações correntes são: a extração de substâncias de importância farmacêutica, a produção de substâncias úteis como corantes de alimentos e produtos industrializados, a produção de cosméticos, o uso como indicadores ambientais e outros.

A biotecnologia de microalgas fez grandes avanços nas últimas três décadas e diversas microalgas como *Botryococcus*, *Chlorella*, *Dunaliella*, *Haematococcus* e *Spirulina*, são cultivada para produção de proteínas, astaxantina, β-caroteno, glicerol, combustíveis líquidos, formulações farmacêuticas e muitos produtos químicos (APT & BEHRENS, 1999; SPOLAORE *et al.* 2006).

A procura de compostos bioativos de interesse farmacológico em microalgas e cianobactérias têm recebido grande atenção, principalmente no tocante à identificação de diversas substâncias sintetizadas por estes micro-organismos. A imensa biodiversidade e consequente variabilidade na composição bioquímica em extratos dos micro-organismos fotossintetizantes tem levado à descoberta de uma

variedade de atividade biológica. Vários desses compostos podem ter aplicação em medicina humana e animal ou na agricultura.

Portanto, aplicação biotecnológica de microalgas na produção de enzimas fibrinolíticas pode ser uma alternativa bastante viável e econômica, uma vez que os cultivos de microalgas não são dispendiosos, pois requerem apenas sais, fonte de carbono (CO_2) proveniente do ar atmosférico e luminosidade artificial ou adivinda do sol, tornando uma fonte de baixo custo para produção de bioprodutos e biomassa.

2. Revisão de Literatura

2.1 Proteases

A clivagem proteolítica de peptídeos é uma das mais frequentes e importantes modificações de proteínas. Historicamente, a proteólise enzimática foi associada com a digestão de proteínas e chamou a atenção de fisiologistas e bioquímicos, interessados neste processo fisiológico em animais, inclusive o homem. Assim, as proteases digestivas de secreções gástricas e pancreáticas são as enzimas mais bem caracterizadas, gerando o conhecimento atual sobre as estruturas e funções das enzimas proteolíticas em geral. Investigações da cinética, especificidade de inibição, associadas com análises detalhadas de suas estruturas por cristalografia de raios X e de suas sequências de aminoácidos, levaram à identificação dos componentes e da geometria dos sítios ativos de proteases, permitindo a dedução de seus mecanismos de ação. Como resultado, tornou-se evidente que as proteases podem ser classificadas em famílias e que membros de uma mesma família apresentam estruturas e mecanismos de ação similares (NEURATH, 1990).

Proteases ou proteinases são enzimas proteolíticas que catalisam a hidrólise de proteínas. Com base na sua estrutura ou propriedade do sítio ativo, há vários tipos de proteases, tais como, de serina, carboxil, metalo, ácidas, neutras e alcalinas. As proteases são enzimas industrialmente importantes e

constituem um quarto da produção de enzima global (KALAIARASI & SUNITHA, 2009). As proteases são industrialmente importante devido a suas amplas aplicações na processamento de couro, indústria de detergentes, indústrias de alimentos, indústria farmacêutica, têxtil, etc (DENG et al., 2010; JELLOULI et al., 2009).

Sabe-se que já existe uma grande variedade de proteases comercialmente utilizadas que variam de aditivos detergentes a efetivos terapêuticos. As proteases terapêuticas tem sido muito bem avaliadas (CRAIK et al., 2011).

A utilização de engenharia de proteases para aplicações terapêuticas tem sido um objetivo na indústria farmacêutica. A atividade catalítica das proteases faz delas um terapêutico potencial único, permitindo dosagens menores, potencial para inativar numerosas proteínas alvo e maior eficácia. Em combinação, estes efeitos podem conduzir a doses mais baixas e menos frequentes e o mais importante, custos mais baixos. Várias terapias com proteases estão atualmente sob investigação clínica, algumas das quais são esperadas para chegar ao mercado ao longo dos anos. A Food and Drug Administration (FDA) já aprovou uma variedade de proteases naturais para o uso em tratamentos tais como desbridamento de feridas (colagenase), trombólise (uroquinase), hemofilia (fator VIIa), sepsia (proteína C ativada) e espasmos musculares (toxina botulínica A e B) (CRAIK et al., 2011; DESAI & HENTZ, 2010).

Agentes trombolíticos, anticoagulantes, antiagregantes e trombolíticos diretos (fibrinolíticos), são utilizados para o tratamento de doenças trombóticas (MACKMAN, 2008). Atualmente, as enzimas fibrinolíticas por via microbiana tem conquistado grande interesse em buscas de novas biomoléculas.

2.2 Proteases fibrinolíticas

A fibrina é o principal componente dos coágulos sanguíneos, e é formada a partir da clivagem dos fibrinopeptídeos do fibrinogênio por ação da trombina. A formação da rede de fibrina insolúvel é então iniciada, e após a fibrinólise a

fibrina é hidrolisada em produtos de fibrina degradados pela plasmina (WEISEL, 2005).

Enzimas fibrinolíticas, capazes de hidrolisar o coágulo de fibrina, vem sendo descobertas por uma infinidade de micro-organismos incluido bactérias e fungos. Dentre elas é possível citar algumas produzidas por bactérias do gênero *Bacillus*, como a Nattokinase (FUJITA et al., 2003), CK (KIM et al, 1996), Subtilisina DFE (PENG et al., 2003), outras produzidas por bactérias do gênero *Streptomyces* como FP84 (SIMKHADA et al., 2010) e SOT (UESUGI et al., 2011), além das produzidas por fungos, Fu-P (WU et al., 2009) e KSK-3 (SHIRASAKA et al., 2012).

Matsubara et al. (1998, 1999, 2000) verificou que macroalgas do gênero *Codium* sp. também eram produtoras de enzimas fibrinolíticas. Abrindo novos horizontes para a bioprospecção de enzimas fibrinolíticas a partir de organismos fotossintetizantes.

Portanto, a busca por enzimas fibrinolíticas de baixo custo e com elevada especificidade vem sendo continuamente investigada (LEE et al., 2005).

2.3 Mecanismo Hemostático (hemostasia)

Hemostasia é o processo que mantém a integridade fechada do sistema circulatório de alta pressão, após dano vascular. Uma lesão na parede de um vaso gera o extravasamento do sangue e rapidamente se iniciam eventos na parede do vaso para selar a violação. Plaquetas circulantes são recrutadas para o local da lesão, onde se tornam o componente principal do desenvolvimento do trombo, a coagulação do sangue é iniciada pelo fator tecidual que culmina na geração da trombina (EC 3.4.21.5) e fibrina, como descrito na Figura 1 (FURIE, 2008).

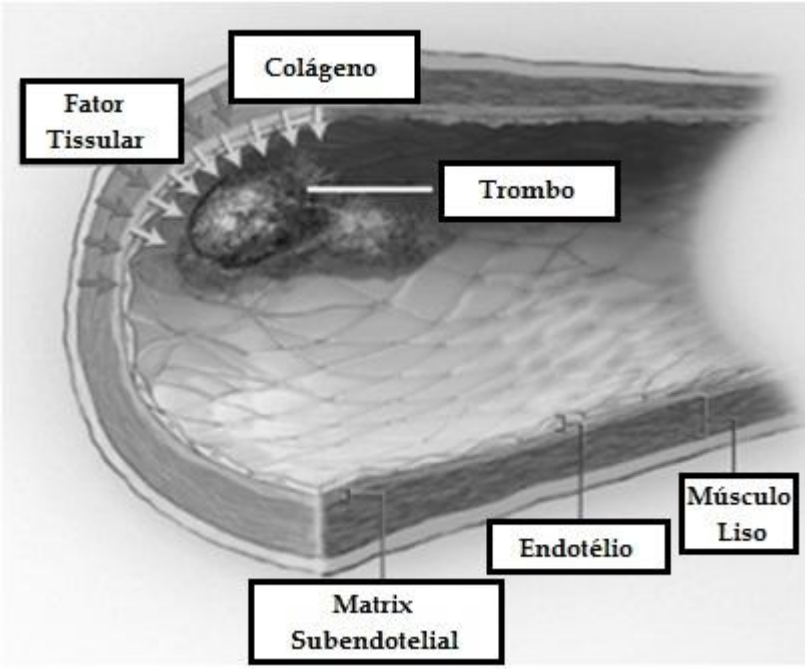

Fig. 1 – Ilustração da formação de um trombo (FURIE, 2008).

2.3.1 Fibrinogênio

O fibrinogênio (Fig. 2) é uma proteína trinodular de 450 Å e 340 kDa presente em altas concentrações (2-4 mg/mL) no plasma. O fibrinogênio consiste de três diferentes pares de cadeias polipeptídicas de dissulfeto ligadas: Aα, Bβ e γ. Estas seis cadeias polipeptídicas estão com sua porção N-terminal dispostas ao domínio central E da molécula. A porção terminal da cadeia Bβ e γ estendem-se para fora opostamente ao domínio D. A porção C-terminal da cadeia Aα são globulares e situadas perto do domínio central E do fibrinogênio onde elas interagem intra-molecularmente (MEDVED et al., 1983).

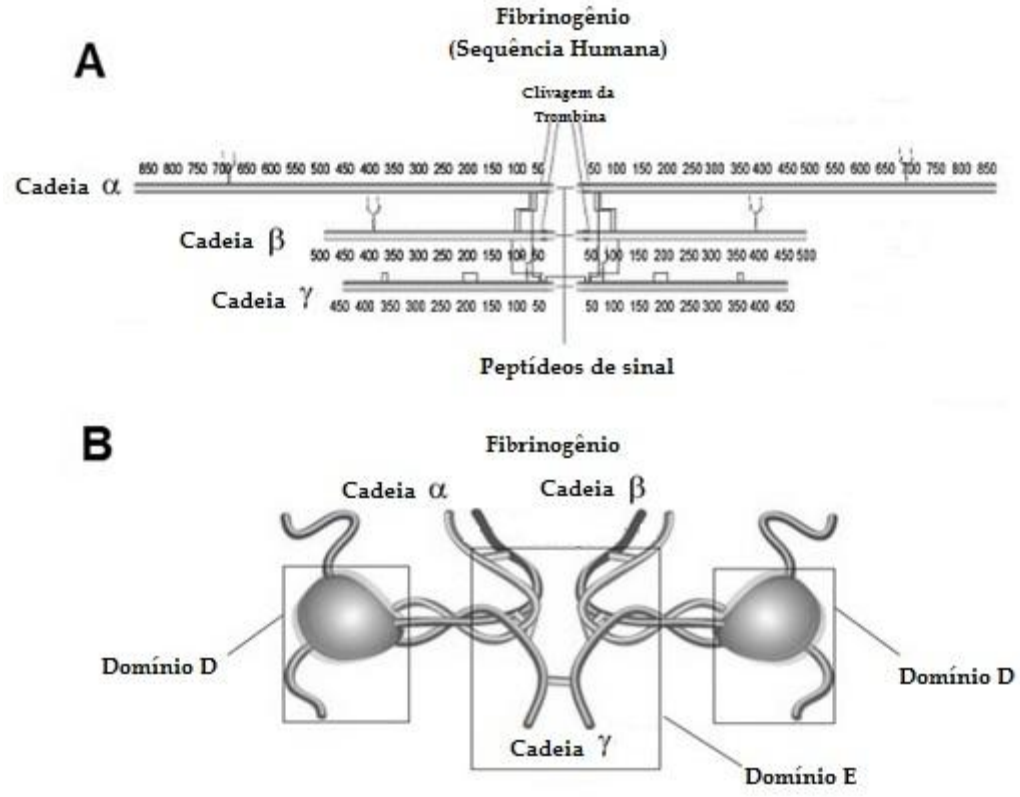

Fig. 2 – Estrutura plana (A) e tridimensional (B) do fibrinogênio (SIGMA-ALDRICH, 2013).

2.3.2 A formação de fibrina

A fibrina, causadora da coagulação sanguínea é ativada pela trombina, pela degradação do fibrinogênio e pode ser lisada pela plasmina (JEONG et al., 2001).

Normalmente a formação de fibrina e fibrinólise são mantidas em equilíbrio; no entanto, em uma situação de desequilíbrio surgida quando a fibrina não pode ser hidrolisada, pode-se surgir doenças cardiovasculares, tais como trombose devido a acumulação de fibrina nos vasos sanguíneos (FUJITA et al., 1995).

A formação de um coágulo de fibrina é mediada por um grupo de proteases plasmáticas firmemente reguladas e por cofatores. Embora este sistema seja essencial para minimizar a perda de sangue a partir de um vaso sanguíneo lesionado (hemostasia), também pode contribuir patologicamente com a formação

de fibrina e ativação plaquetária, que podem obstruir vasos (trombose) (GAILANI & RENNÉ, 2007).

A polimerização da fibrina é iniciada pela clivagem enzimática dos fibrinopeptídeos, convertendo o fibrinogênio em monômeros de fibrina (Fig 3). Em seguida várias reações não enzimáticas produzem uma sequência ordenada de passos para montagem da macromolécula. Várias proteínas do plasma se ligam a rede de fibrina resultante. O coágulo é então estabilizado através de ligações covalentes ou de ligações cruzadas de aminoácidos específicos por uma transglutaminase, Fator VIIIa (WEISEL, 2005).

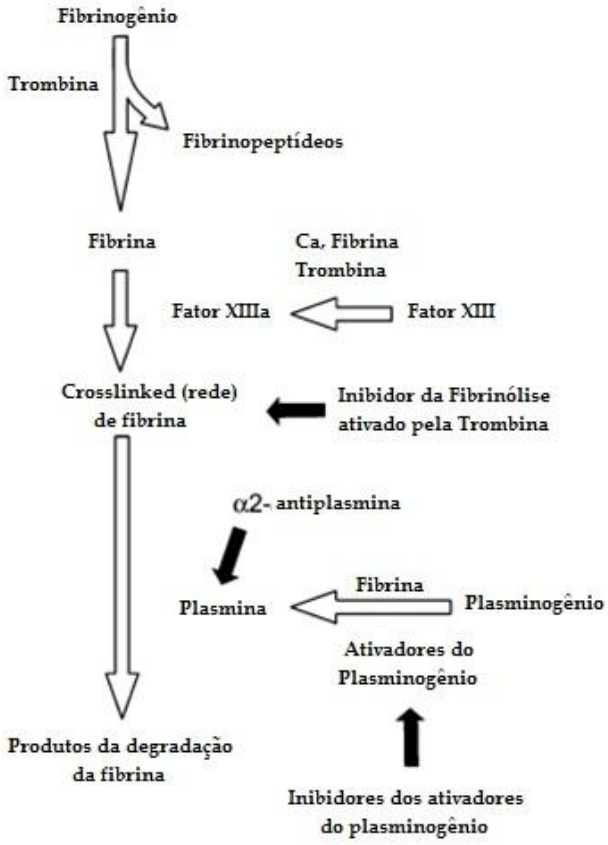

Fig. 3 – Esquema da polimerização da fibrina e fibrinólise. O coágulo é formado pela conversão do fibrinogênio em fibrina pela clivagem dos fibrinopeptídeos pela ação da trombina, seguido pela estabilização da rede com ligações isopeptídicas pelo Fator XIIIa transglutaminase. O coágulo é dissolvido por proteólise pela enzima plasmina, a qual é ativada na superfície de fibrina por ativadores do plasminogênio. Este processo é controlado por várias reações inibitórias (setas pretas) (WEISEL, 2005).

2.3.3 Fibrinólise

A fibrinólise tem contribuído para redução da mortalidade de pacientes hospitalizados com infarto agudo do miocárdio. Desde o aparecimento dos primeiros fibrinolíticos como uroquinase e estreptoquinase, até os recentemente comercializados como tenecteplase (TNK) ou alteplase (rt-PA), a efetividade tem melhorado e efeitos colaterias reduzidos, essencialmente em hemorragias (CURIEL-BALSERA et al., 2011).

Segundo Cesarman-Maus & Hajjar (2005) a fibrinólise pode ser definida como a degradação da fibrina mediada pela plasmina. Este sistema tem por base a ativação de uma pró-enzima, o plasminogênio (PLG), que depois de ativado se transforma em plasmina (PLa), uma proteína com capacidade de degradar a fibrina.

A ativação da coagulação gera trombina, o que resulta na formação do trombo por conversão do fibrinogênio em fibrina e por ativação das plaquetas. Plasmina (EC 3.4.21.7) é a mais importante protease fibrinolítica (Fig. 4). Plasminogênio (PLG), é um zimogênio que circula no plasma, que pode ser convertido em plasmina pelo ativador do plasminogênio tecidual (t-PA) bem como pela uroquinase (u-PA). Através de mecanismos de retroalimentação positiva, a plasmina cliva tanto o t-PA quanto o u-PA, transformando-os de uma cadeia simples para duas cadeias ativas de polipeptídios. A fibrina, um importante substrato da plasmina, regula sua própria degradação pela ligação do PLG com o t-PA na sua superfície, assim, localizando e aumentando a geração de plasmina. Embora o t-PA seja um ativador fraco do plasminogênio na ausência de fibrina, a sua eficiência catalítica para ativação do PLG é reforçada por pelo menos duas ordens de grandeza na presença de fibrina. A afinidade de t-PA e PLG são baixas na ausência de fibrina, mas aumentam significativamente na sua presença (CESARMAN-MAUS & HAJJAR, 2005).

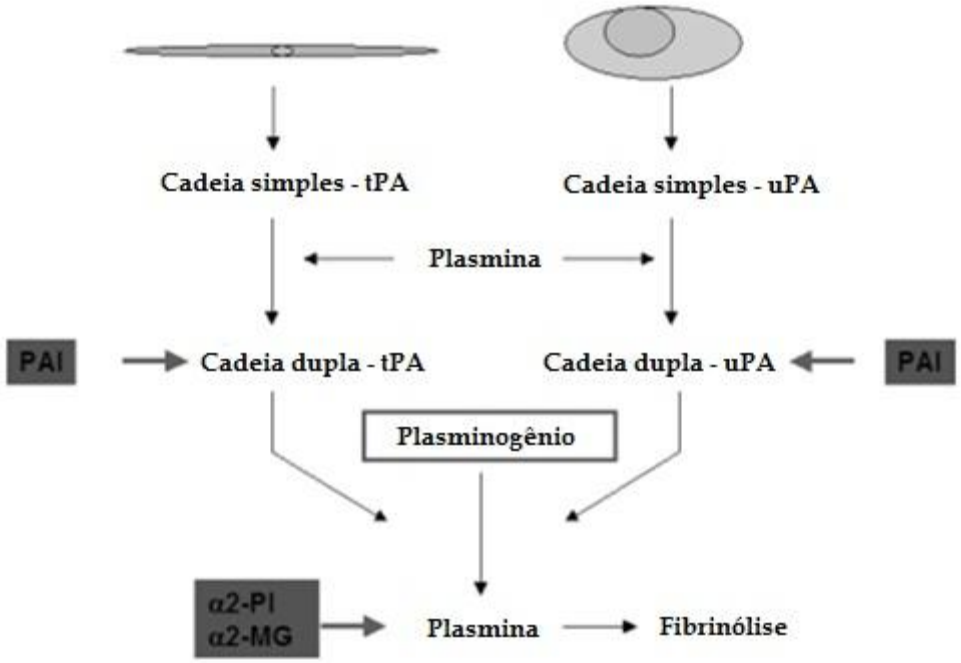

Fig. 4 – Visão geral do sistema fibrinolítico. O plasminogênio é convertido em serino protease ativa, plasmina, principalmente através da ação de duas cadeias de ativadores do plasminogênio tissular (tc-tPA) ou duas cadeias de uroquinase (tc-uPA). Estes ativadores são secretados como cadeias simples (sc-tPA e sc-uPA) a partir de células endoteliais e a partir de epitélio renal, monócitos, macrófagos ou células endoteliais respectivamente. Ambos tPA e uPA podem ser inibidos pelo inibidor de ativadores do plasminogênio-1 (PAI), enquanto que a plasmina é inibida pelo seu maior inibidor, inibidor α2-plasmina (α2-PI) e em menor grau pela α2-macroglobulina (α2-MG). Uma vez que a plasmina é gerada, converte as cadeias simples de tPA e uPA em cadeias duplas. Em seguida, são rapidamente inibidos, a menos que se mantenham ligados a fibrina ou a receptores da superfície celular. Os inibidores estão indicados por caixas vermelhas (CESARMAN-MAUS & HAJJAR, 2005).

Uma vez formada, a plasmina cliva a fibrina, gerando produtos de degradação solúveis e expondo resíduos carboxi-terminais de lisina (Lys) (Fig. 5). Domínios de proteínas específicos do t-PA e do PLG contém sítios de ligação à lisina, que medeiam mais ligações a fibrina, conduzindo a maior produção de plasmina e remoção de fibrina. As ligações podem ser bloqueadas por lisina análogas, tais como o ácido ε-aminocapróico e o ácido tranexâmico, bem como pela recentemente caracterizada, inibidor da fibrinólise ativado pela trombina (TAFI). Quando ativado pela trombina, o TAFI remove resíduos carboxi-terminais de lisina, atenuando a geração de plasmina, estabilizadora da fibrina nos trombos, e estabelecendo uma conexão regular entre coágulo e trombo. A dissolução da

fibrina é também regulada por inibidores da ativação do PLG, tais como inibidor do ativador do plasminogênio-1 (PAI-1), e por inibidores de plasmina em si, tal como inibidor da α_2-plasmina (α_2-PI). Além disso, a plasmina ligada à fibrina é protegida pela α_2-PI, devido a ocupação dos seus sítios de ligação da lisina. Por outro lado, o TAFI, diminui esta proteção através da supressão do resíduo de lisina ligado a plasmina em fibrina (CESARMAN-MAUS & HAJJAR, 2005).

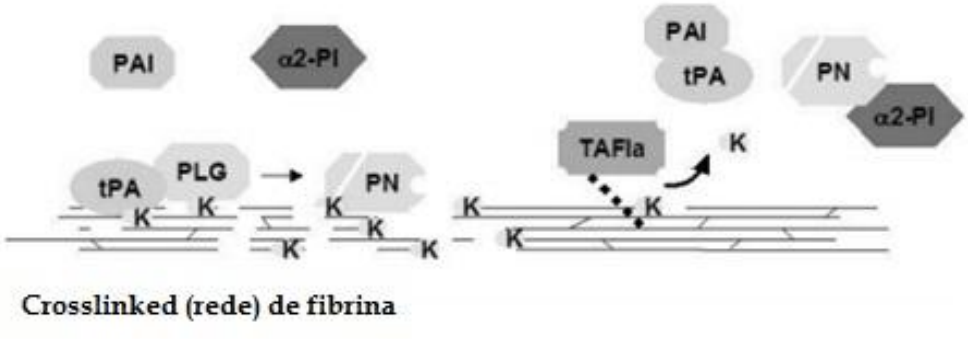

Fig 5 - Fibrina e receptor reforçado de geração de plasmina. (A) Ativador do plasminogênio tissular (tPA) e plasminogênio (PLG), ligamentos de fibrina através de resíduos de lisina (K). Este conjunto trimolecular aumenta bastante a geração de plasmina (PN), o que resulta em maior exposição de lisinas carboxi-terminais e em última análise, na degradação de fibrina. Fibrina associada a plasmina e tPA são protegidas contra seus principais inibidores, o inibidor α2-plasmina (α2-PI) e inibidor do plasminogênio-1 (PAI), respectivamente. Inibidor da fibrinólise ativado pela trombina (TAFIa), uma carboxipeptidase do plasma, cliva os resíduos de fibrina e atenua a dissolução de fibrina através da diminuição dos sítios de ligação à fibrina (K) para as enzimas fibrinolíticas. A uroquinase (uPA) atua independentemente da fibrina (CESARMAN-MAUS & HAJJAR, 2005).

A fibrinólise, no entanto, compreende a parte do processo hemostático cuja função é dissolver o coágulo de fibrina formado a partir de uma injúria vascular (BAJAJ et al., 2013).

2.4 – Microalgas

As microalgas fazem parte de um grupo muito heterogêneo de organismos que são predominantemente aquáticos e geralmente microscópicos unicelulares, podendo formar colônias. Sua coloração variada é característica oportunizada pela presença de pigmentos e mecanismo fotoautotrófico (LOURENÇO, 2007). O

termo microalgas não tem valor taxonômico, uma vez que engloba micro-organismos algais com clorofila e outros pigmentos fotossintéticos capazes de realizar a fotossíntese. Várias microalgas são utilizadas para obtenção de diversos produtos e biomassa, dentre os quais podemos citar a *Arthrospira* (*Spirulina*), *Dunaliella* e *Chlorella*. No entanto, ainda hoje a *Chlorella* ocupa o primeiro lugar no uso comercial (RICHMOND, 2004).

2.5 – *Chlorella vulgaris*

A microalga *Chlorella vulgaris* pertence ao Filo Chlorophyta, Classe Trebouxiophyceae, Ordem Chlorellales e Família Chlorellaceae. São cosmopolitas, possuindo forma esférica e coloração verde devido a presença de pigmentos fotossintéticos (clorofila a e b) em seus cloroplastos (Figura 6). Através da fotossíntese, estas microalgas se multiplicam rapidamente mediante reprodução assexuada.

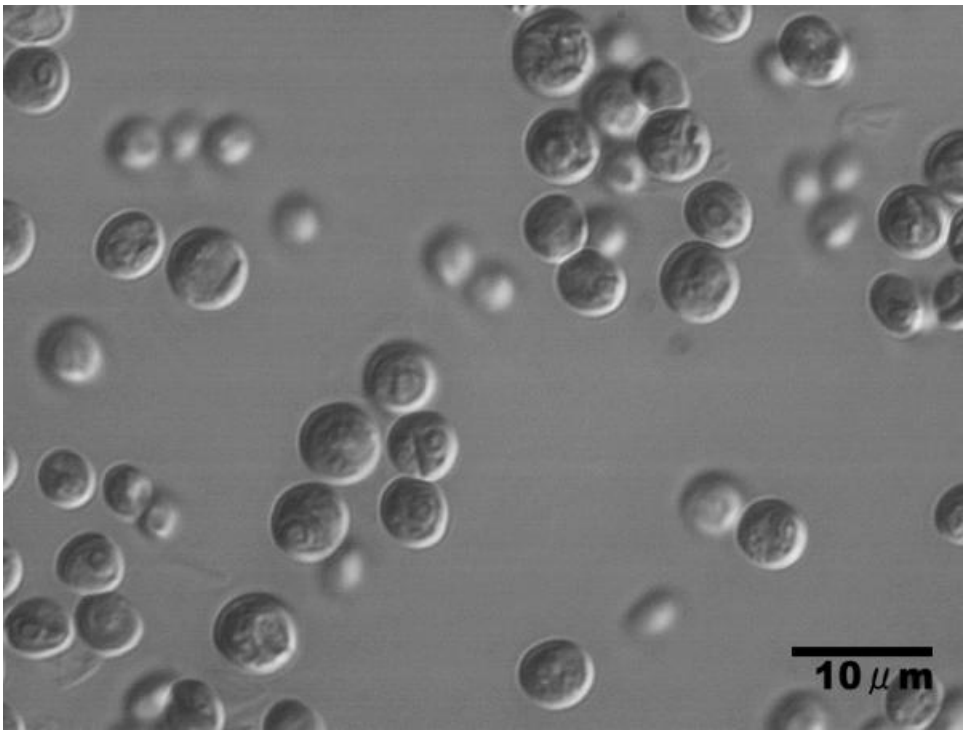

Fig. 6 – Microscopia da microalga *Chlorella vulgaris*. (Fonte: ALGAL RESOURCE DATABASE: http://www.shigen.nig.ac.jp/algae/strainDetailAction.do?stockNo=NIES-642)

Por décadas estes micro-organismos têm sido estudados visando a produção de biomassa para diferentes aplicações. A primeira cultura de microalga foi estabelecida por Beijerinck, em 1980, constituindo-se na espécie *Chlorella vulgaris*. Em escala comercial, a produção de microalgas iniciou-se no Japão, na década de 60, com espécies do gênero *Chlorella* sp., visando a utilização como suplemento alimentar. A partir de então, o cultivo de microalgas passou a ser gradativo no mundo inteiro, destinando-se às mais variadas aplicações, como produção de proteínas, lipídios, clorofila, carotenóides, enzimas, agentes antioxidantes, vitaminas, antibióticos (LOURENÇO, 2007).

A biomassa da *Chlorella vulgaris* apresenta algumas particularidades como elevado teor protéico e lipídico, contendo aminoácidos e ácidos graxos essenciais a alimentação humana e animal. Por possuir estas características, são recomendadas como complemento alimentar (ECIMAT, 2013).

2.6 Cultivo de microalgas e utilização de resíduos agroindustriais

A produção comercial de microalgas pode ser simples ou de forma complexa utilizando equipamentos sofisticados dependendo da sua aplicação. Atualmente numerosas aplicações comerciais são reconhecidas para microalgas, dentre elas a utilização de biomoléculas de alto valor agregado, tais como pigmentos, ácidos graxos essenciais, proteínas e vitaminas, é bastante promissora (LOURENÇO, 2007).

Alguns cultivos utilizando fotobiorreatores estão sendo utilizados para aumento da densidade celular e produtividade das microalgas. Os cultivos são realizados em sistemas fechados, como os flat-planes, serpentinas, espirais ou cilindros, construídos com tubos plásticos, vidro ou policarbonato. Nos fotobiorreatores, é possível controlar as condições de cultivo (quantidade de nutrientes, temperatura, iluminação, pH e outros). Isto resulta numa alta densidade celular e elavada produtividade, viabilizando a produção comercial de uma série de composto de alto valor agregado (TREDICI, 2004).

Estudos recentes revelaram que *Chlorella vulgaris* pode ser cultivadas em condições autotrófica, mixotrófica ou heterotrófica. Os cultivos mixotróficos podem gerar um aumento na produção de biomassa quando comparados com cultivos autotróficos e heterotróficos (HEREDIA-ARROYO et al., 2011).

Vários estudos tem relatado o cultivo mixotrófico de *Chlorella vulgaris* para acumulação de biomassa e lipídios, e diversas fontes de carbono e efluentes vem sendo utilizadas sob condições mixotróficas, tais como glicose, sacarose, acetato e glicerol, bem como extrato de galinheiros de frangos e águas residuais da indústria do tapete (HEREDIA-ARROYO et al., 2011; FENG et al., 2005; BHATNAGAR et al., 2011).

Pouco se sabe sobre o uso de resíduos agroindustriais no cultivo de microalgas, no entanto, dentre os poucos estudos e resíduos utilizados é possível citar o uso de milhocina (MAHBOOB et al., 2012) e soro de queijo (ABREU et al., 2012) no cultivo de *C. vulgaris*.

A utilização de resíduos agroindustriais no cultivo de microalgas podem gerar uma alternativa de diminuir os custos de produção de biomassa e biomoléculas a partir de substratos caros, como glicose, peptona, extrato de carne, extrato de levedura, e outros. Esses resíduos geralmente são lançados no meio ambiente acarretando sérios riscos de contaminação, e causando sérios danos a saúde animal e humana, e a agricultura.

2.7 Glicerol

Glicerol é o nome comum do composto orgânico 1,2,3-propanotriol, descoberto por Carl W. Scheele em 1779. Os seus sinônimos são glicerina, trihidroxipropano, glicil álcool, gliceril e 1,2,3-trihidroxipropano. O glicerol na sua forma apresenta-se como um líquido viscoso, incolor, inodoro e higroscópico, com sabor doce, solúvel em água e álcool, insolúvel em éter e em clorofórmio (ARRUDA et al., 2006).

Devido às suas características físicas e químicas e ao fato de ser inócuo, o glicerol é uma substância de valor agregado a qual é muito empregada na indústria farmacêutica e cosmética sendo utilizada na composição de cápsulas, supositórios, anestésicos, xaropes e emolientes para cremes e pomadas. Por ser não tóxico, não irritante, sem cheiro e sabor, o glicerol tem sido aplicado como emoliente e umectante em pastas de dente, cremes de pele, loções pós-barba, desodorantes, batons e maquiagens. Pode ainda ser usado como umectante e para conservar bebidas e alimentos tais como refrigerantes, balas, bolos, pastas de queijo e carne, ração animal seca o glicerol apresenta diferentes aplicações na indústria de cosméticos, farmacêutica, detergentes, na fabricação de resinas e aditivos e na indústria de alimentos (ARRUDA et al., 2006).

Na natureza, o glicerol existe em vegetais (soja, mamona, babaçu, girassol, palma, algodão, coco, dendê, pinhão manso) e animais em formas combinadas de glicerina com ácidos graxos. O glicerol é também um composto considerado fundamental dentro do sistema metabólico de micro-organismos, onde atua como precursor de numerosos compostos e como regulador de vários mecanismos bioquímicos intracelulares (LAGES et al.,1999).

Em microrganismos eucarióticos, o glicerol constitui o principal composto formado para regular as variações de atividade de água em ambientes altamente osmofílicos (WANG et al., 2001).

Segundo Berh (2008), uma tonelada de biodiesel produz cerca de 110 kg de glicerol bruto ou cerca de 100 kg de glicerina pura. Devido ao aumento na produção de glicerol pela fabricação do biodiesel, deve ser encontrado um destino adequado para esta produção a fim de evitar o acúmulo deste material, por isso neste trabalho sugere-se o uso do glicerol no meio de cultivo como alternativa de reaproveitamento de resíduos.

2.8 Milhocina

A milhocina é um subproduto da produção de amido de milho. Industrialmente, o milho seco é macerado em uma solução de ácido sulfúrico a quente; a fração solúvel sofre uma suave fermentação láctica natural que ocorre

devido à presença de bactérias na solução, e é, então, submetida a uma operação de evaporação, na qual a milhocina é concentrada. A milhocina é usada principalmente como suplemento alimentício para ruminantes, fonte de nutrientes para aves, na confecção de iscas atrativas para as moscas das frutas e fonte de nutrientes para o processo de fermentação industrial (FILIPOVIC; RISTIC; SAKC*,* 2002).

A composição da milhocina é variável, dependendo da origem da matéria-prima e de seu processamento. No entanto, basicamente a milhocina é composta por uma mistura de proteína solúvel, aminoácidos, carboidratos, ácidos orgânicos (por exemplo, o ácido láctico), vitaminas e sais minerais. Hull et al. (1996) encontrou uma série de pequenos polipeptídeos presentes na milhocina.

Uma composição típica é mostrada na Tabela 1. No entanto, a composição pode ainda variar devido às diferenças entre as plantações de milho de região para região (AKHTAR et al., 1998).

Tabela 1 - Composição típica da milhocina (AKHTAR et al., 1998)

Subastâncias	**Porcentagem**
Milhocina em base seca	50,7
pH	3,9
Proteína	40,8 (base seca)
Ácido Lático	16,0 (base seca)
Açúcares redutores	12,8 (base seca)
Compostos variados	30,4 (base seca)

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4. Objetivos

4.1 Objetivo Geral

Produzir enzimas fibrinolíticas a partir da microalga *C. vulgaris* utilizando glicerol e o resíduo agroindustrial milhocina.

4.2 Objetivos Específicos

1. Avaliar a produção de enzimas fibrinolíticas do extrato da microalga *C. vulgaris*;
2. Avaliar o efeito da milhocina no aumento da produção de proteases e enzimas fibrinolíticas;
3. Avaliar o efeito do uso do glicerol no aumento da concentração celular e produtividade;
4. Otimizar o aumento da concentração celular, produtividade celular, produção de protease e enzima fibrinolítica, com o uso de glicerol e milhocina, utilizando Metodologia de Superfície de Resposta (RSM) como ferramenta para delineamento estatístico.

5. Artigo 1

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An overview about fibrinolytic enzymes from microorganism and algae: Production and characterization

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Abstract

Cardiovascular diseases are the leading causes of death through the world. A common cause of cardiovascular diseases is an abnormal clot formation, called “thrombus”. Although several commercial thrombolytic agents - plasminogen activators (PA), including tecidual plasminogen activators (tPA), recombinant t-PA (r-tPA), urokinase (u-PA), streptokinase and anisoylated plasminogen streptokinase-activator complex are an accepted therapeutic approach for thrombotic occlusive disease, these agents are expensive and have a number of drawbacks, such a short half-life after intravenous administration, uncontrollable acceleration of fibrinolysis and haemorrhage. Widespread systemic activation of fibrinolysis leads to potentially life-threatening side effects. To overcome these risks, a safer thrombolytic agent is need for treating thrombolytic processes. Safe and specific thrombolytic agents produced on industrial scale may substantially

contribute to reducing the costs of thrombolytic therapy. This review will deal primarily with the fibrinolytic enzymes production by microorganisms, culture conditions and their biochemical characteristics.

1. Introduction

An estimated 17.3 million people died from Cardiovascular disease (CVD) in 2008, representing 30% of all global deaths. Of these deaths, an estimated 7.3 million were due to coronary heart disease and 6.2 million were due to stroke. According to the World Health Organization (2012), in 2030, almost 25 million people will die from cardiovascular diseases, mainly from heart disease and stroke.

A common cause of cardiovascular diseases is abnormal fibrin accumulation in the blood vessels or a fibrin clot adhering to the unbroken vessel walls of the endoepithelium. An abnormal clot formation is called a “thrombus”. Intravascular thrombosis is caused by the disorderly formation of a clot of blood in a blood vessel and it is one of the main causes of a variety of CVDs. Thrombus can stop blood circulation in vessels (arteries or veins), and may cause a hypoxiation syndrome such as acute myocardial infarction, high blood pressure, ischemic heart and stroke (Mine et al., 2005).

Over the years, thrombolytic therapies via injecting or orally administrating thrombolytic agents to lyse thrombi in blood vessels have been extensively investigated (Goldhaber and Bounameaux, 2001; Tough 2005). Pharmacologic dissolution of an established thrombus is now an accepted therapeutic approach for thrombotic occlusive disease. Intravenous infusion of commercial thrombolytic agents approved by Food and Drug Adminstration - plasminogen activators (PA), including recombinant tPA (r-tPA), u-PA, urokinase, streptokinase, and anisoylated plasminogen streptokinase-activator complex is effective in restoring blood flow in occluded arteries and veins (Liu et al., 2005). However, these agents are expensive and have a number of drawbacks, such as rapid degradation, uncontrollable acceleration of fibrinolysis and hemorrhage. Widespread systemic activation of fibrinolysis leads to potentially life-threatening side effects. To

overcome these risks, a safer thrombolytic agent is need for treating thrombolytic processes.

Thrombolytic therapy occurs by the use of tissue plasminogen activator (t-PA), a serine protease that activates plasminogen in plasmin, resulting in the dissolution of fibrin clot. Major plasminogen activators in use are streptokinase (SK), urokinase (u-PA), and tissue type plasminogen activator (t-PA) (Bernik and Kwaan, 1969), buy they do not have a direct fibrinolytic activity and their therapeutic action is via the activation of blood plasminogen to the clot dissolving plasmin (Banerjee et al., 2004). Unlike tPA and uPA, which are proteases, streptokinase possesses no enzymatic activity of its own (Castellino, 1981). Streptokinase is not an enzyme *per se* but rather a potent activator that interacts with plasminogen to form a stoichiometric 1:1 complex. This interaction results in the activation of plasminogen to plasmin, which is the active fibrinolytic component of the circulatory system. Streptokinase–plasminogen activator complex is a high-specificity protease that proteolytically activates other plasminogen molecules to plasmin (Bajaj and Castellino, 1977; Castellino, 1981). Thus, the plasminogen activating action of streptokinase is fundamentally different from the proteolytic activation brought about by tPA and uPA.

SK is secreted to the growth medium by many beta-hemolytic *Streptococcus* strains and both t-PA and uPA occur naturally in the blood. SK activates plasma plasminogen to plasmin (Malke and Ferretti,1984) causing fibrinolysis (Khil et al., 2003; Sun et al.,2004) while that both t-PA and uPA are trypsin-like serine proteases that activate plasminogen directly (Coleman et al., 2005). Plasmin is a proteolytic enzyme that is capable of breaking cross-links between fibrin molecules, which provide the structural integrity of blood clots. The other type is plasmin-like proteins, which directly degrade the fibrin in blood clots, thereby dissolving the thrombi rapidly and completely.

Streptokinase (SK) is a life-saving clot-dissolving drug routinely prescribed universally in the management of heart attack (Banerjee et al., 2004). Immunogenicity of streptokinase and its relatively short half-life in circulation limit therapeutic potential of this protein. Streptokinases structurally modified have been produced in several ways including genetic mutation, recombinant DNA technology and chemical or enzymatic modification of the native streptokinase.

Recombinant streptokinase more resistance to proteolysis and with reduced immunogenicity have been produced. Recombinant streptokinase produced in the yeast *Pichia pastoris* is glycosylated, and this appears to enhance its resistance to proteolysis (Pratap et al., 2000). Following a similar path, attempts have been made to extend the half-life of native nonglycosylated streptokinase by complexing it with polymers such as polyethylene glycol (PEG) (Koide et al., 1982; Rajagopalan et al., 1985).

Plasmin-resistant, long-life variants of protein-engineered streptokinase have been produced in a protease-deficient recombinant *Bacillus subtilis* WB600 (Wu et al., 1998). It appears that the streptokinase domains responsible for activity, stability and immunogenicity have considerable overlap. Therefore, a future therapeutically optimal streptokinase will not be necessarily the most active nor the longest lived. A mutant streptokinase that lacked the C-terminal 42 amino acids was found to be less immunogenic than the native molecule (Torrens et al., 1999). One chemical modification has involved complexing streptokinase with PEG (Koide et al., 1982; Rajagopalan et al., 1985; Pautov et al., 1990), primarily for reducing immunogenicity.

β-hemolytic *Streptococci* of groups A, C and G are producers of streptokinase. Although the majority of group A strains of beta hemolytic *Streptococci* produces moderate amounts of fibrinolysin (Comission on acute respiratory diseases, 1947), the group C are preferred for producing because they lack erythrogenic toxin. The group C strain *Streptococcus equisimilis* H46A (ATCC 12449) has been widely utilized for streptokinase production because it yielded the most active (Bernejje 2004), not produce erythrogenic toxins and is less fastidious in its growth requirements than the majority of group A strains (Christensen, 1945).

Due to the pathogenicity and production of several toxins that complicate the downstream purification by *Streptococcus* sp. (Pimienta et al., 2007), the gene encoding SK has been cloned and expressed in several heterologous hosts such as *B. subtilis* WB600 (Wong et al., 1994; Wu et al., 1998), *E. coli* (Yazdani and Mukherjee, 2002) and yeast *Pichia pastoris* (Hagenson et al., 1989; Estrada et al., 1992; Pratap et al., 2000), *Lactococcus lactis* (Sriraman and Jayaraman, 2006) and *Streptomyces lividans* (Kim et al., 2010). Streptokinase is a effective as recombinant tPA in treating acute myocardial infarction (Sherry and Marder, 1991),

and it is certainly more cost-effective; however, its use is not risk free. Streptokinase in circulation is proteolytically degraded by plasmin.

Recombinant t-PA can be produced by mammalian cells lines, insect cells, *Saccharomyces cerevisiae*, *Aspergillus nidulans* and *Escherichia coli*, but the levels of t-PA produced are low for clinical use. Recombinant t-PA in bacteria such as *B. subtilis* has been recognized as a good host for the expression of fibrinolytic enzymes because of its nonpathogenicity and capability of secreting functional extracellular proteins to the culture medium (Wong, 1995). On the other hand, when expressed in *E. coli* is problematic due to its accumulation in an unfolded state as inclusion bodies, from which recovery of biologically active PA by refolding is low in efficiency. The mammalian cell expression system is high in cost and low in yield and requires stringent control procedures to detect infectious agents. Since yeast can be grown rapidly on simple media to high cell density, secreting expression of foreign proteins by yeast has advantages over other expression systems (Wang et al., 2000).

The main commercial sources of t-PA are namely alteplase and activase. These are produced in chinese hamster ovary (CHO) cells, although the nonglycosylated, truncated t-PA (reteplase, retavase), which can be produced in *E. coli*, is also available for therapeutic use (Wiebe et al., 2001). Urokinase-type plasminogen activator (uPA), a strong fibrinolytic agent, can also be produced by vascular endothelial cells, SMC (Smooth Muscle Cells), monocytes and macrophages, fibroblasts, epithelial cells, and also by malignant tumor cells of different origin. However, therapy expensive prices and undesirable side effects, such as the risk for internal hemorrhage within the intestinal tract when orally administered, has led researchers to search for cheaper and safer resources.

Fibrinolytic enzyme therapy, such as the intravenous administration of urokinase, is expensive, and patients may suffer from undesirable side effects such as resistance to reperfusion, occurrence of acute coronary reocclusion and bleeding complications (Bode, Runge, & Smalling, 1996). Consequently, several lines of investigation are currently being pursued to enhance the efficacy and specificity of fibrinolytic therapy. Recently, fibrinolytic enzymes have been discovered from both food and non-food sources. These enzymes have proved to be effective and they have been proposed as one potent fibrinolytic enzyme.

Protease production by microbial sources has the advantage of being low cost and high efficacy (Deepak et al., 2010). The production cost is a limiting factor for large-scale fibrinolytic protease production from microrganisms and can be reduced significantly by using nutrients low cost or even no cost, as the use of agro-industrial waste, and consequently facilitate industrial production. Thus, intense researches has been conducted with the goal of finding new enzymes with thrombolytic effect, as well as optimizing production aiming the production of a potent drug with low side effects and low cost.

Microbial proteases represent one of the three largest groups of industrial enzymes, they have numerous and wide application in industrial and household products, including detergents, food, leather, silk and pharmaceutical industry (Mukherjee et al., 2008; Rai et al, 2009, 2010). One of the main applications of pharmaceutical microbial protease (protease fibrinolytic) is in the treatment of thrombosis, considered one of the most frequent cardiovascular diseases in modern life (Mine et al. 2005). The purpose of this review is to present the main producers of fibrinolytic enzyme, culture conditions and characterization of these enzymes that are described in the literature.

2. Sources producing fibrinolytic enzymes

In the literature, different sources of fibrinolytic enzymes are reported such as isolated from venoms, bacteria, fungi and more recently, macroalgae. Streptokinase (SK) was the first fibrinolytic proteins isolated and produced and to a lesser extent, staphylokinase, are the best investigated of microbial origin. Fibrinolytic proteins have been isolated from other species including fungi (Bärwald et al., 1974).

Several natural and recombinants sources have been cited in the literature. Among them, natural thrombolytic agents more effective that have been identified and characterized are from snake venoms, *Bacillus* sp., *Pseudomonas* sp., *Staphylococcus*, *Alteromonas*, *Corineforme*, *Penicillium*, *Aspergillus*, *Fusarium*, *Tricotecium*, *Actinomycetes*, *Streptomyces*, earthworms, insects, marine green alga and recombinant enzymes with *Escherichia coli* marking a new early

treatment of CVDs. *Streptococcus equisimilis*, *Bacillus*, *E. coli, P. pastoris* and *Streptococcus* sp. also are some hosts utilized to production of SK recombinant.

The *Streptococcus equisimilis* H46A *skc* gene encoding streptokinase has been cloned and expressed in several heterologous hosts due to the pathogenicity of its natural host. Since the recovery of extracellular proteins is generally easier than that of cytoplasmic proteins, the expression and subsequent secretion of SK have been studied in several heterologous hosts like *Escherichia coli*, *Bacillus subtilis* and *Pichia pastoris.* High-level expression of *skc* in *E. coli* has been reported, but the formation of inclusion bodies consisting of highly aggregated SK molecules makes its recovery in an active form difficult (Boersma et al., 2003; Estrada et al., 1992).

Fibrinolytic agents isolated from snake venom and obtained from *Bacillus* sp. are currently the most studied natural source because they have a strong fibrinolytic activity. Table 1 shows the fibrinolytic activity of different *Bacillus* species described in the literature.

Table 1 – Fibrinolytic activity obtained from different *Bacillus* sp. described in the literature.

Source fibrinolytic	Fibrinolytic Activity	References
B. subtilis 168 (S1)	252 U/mg	CHANG et al., 2012
B. subtilis 168 (S2)	355 U/mg	CHANG et al., 2012
B. subtilis 168 (P1)	160 U/mg	CHANG et al., 2012
B. subtilis 168 (P2)	6.284 U/mg	CHANG et al., 2012
B. subtilis ICTF-1	280,280 U/mg	MAHAJAN et al., 2012
B. polymaxa NRC-A	15,042 U/mg	MAHMOUD et al., 2011
Bacillus sp. strain AS-S20-I	2,408 ± 70 U/mg	MUKHERJEE and RAI, 2011
B. spharecus	4,258 U/mg	BALARAMAN and PRABAKARAN, 2007
B. licheniformis KJ-31	242,8 U/mg	HWANG et al., 2007

U/mg = milligram protein; mm = area lytic

Thrombolytic therapy using streptokinase, urokinase and tissue plasminogen activator (t-PA) has been widely used in clinical practice as fibrinolytic agents. However, the biological activity of these substances is of short duration in the circulation and can lead to significant risks of hemorrhagic

complications due to strong activation of the fibrinolytic activity in some situations (Thomas et al, 1996; Goodchild and Boylan, 1992; Hanaway et al., 1972). It is from these obstacles that researcher's currently seeking new sources fibrinolytic enzyme, with lower costs and fewer side effects. Microbial sources have currently been the subject of further investigations.

In eastern countries, *Bacillus* sp. which produce fibrinolytic enzymes have been isolated mainly from fermented foods like Korean Jang-Chungkook (Kim et al., 1996), Chinese douche (Wang et al., 2006), Japanese natto (Sumi et al., 1987), Japanese shiokara (Sumi et al., 1995), Korea Doen-Jang (Kim and Choi, 2000), Asian fermented shrimp paste (Wong and Mine, 2004), and Indonesian tempeh (Kim et al., 2006). The daily intake of these foods is recommended to prevent thrombosis and related disorders. In Table 2 are described some fibrinolytic enzymes obtained from microrganisms isolated from food.

Table 2 – Differents *Bacillus* sp. isolated from traditional food.

Microorganisms	Food	Name of enzyme	References
B. subtilis	Natto-red bean	S1, S2, P1, P2	Chang et al., 2012
B. licheniformis KJ-31	Jeot-gal, Korea	bpKJ-31	Hwang et al., 2007
Bacillus sp. DJ-2	Doen-jang, Korea	bpDJ-2	Choi et al., 2005
B. subtilis QK02	Fermented soybean	QK-1 and QK-2	Ko et al., 2004
Bacillus firmus NA-1	Natto	–	Seo and Lee, 2004
B. amyloliquefaciens	Douchi, China	Subtilisin DFE	Peng et al. 2003
Bacillus sp. KDO-13	Soybean paste, Korea	–	Lee et al., 2001
B. subtilis IMR-NK1	Natto, Taiwan	–	Chang et al., 2000
Bacillus sp. DJ-4	Doen-jang, Korea	Subtilisin DJ-4	Kim and Choi, 2000
Bacillus sp. KA38	Jeot-gal, Korea	Jeot-gal enzyme	Kim et al., 1997
Bacillus sp. CK	Chungkook-jang, Korea	CK	Kim et al., 1996
B. natto	Natto, Japan	Nattokinase, NK	Fujita et al., 1993
B. subtilis	Fermented soybean	Nattokinase	Kamata et al., 1989

Nowadays, fibrinolytic enzyme produced by food micro-organisms has become a research hotspot for its various advantages including: low cost, high activity, safe and non-toxic. Nattokinase from *Bacillus amyloliquefaciens* DC-4 in Douchi, subtilisin DJ-4 of *Bacillus* sp. DJ-4 from Korean food Doen-Jang (Kim et al., 2000), fibrinolytic enzyme of *Bacillus* sp. CK11-4 from Chungkook-Jang (Kim et al., 1996), fibrinolytic enzyme of *Bacillus* sp. KA38 from fermented fish (Kim et al 2010), fibrinolytic enzyme of *Bacillus subtilis* from fermented red beans (Chang et al., 2010) and protease of *Fusarium* sp. BLB from Tempeh (Sugimoto et al., 2007) are some example of fibrinolytic enzyme obtained from food microbe. These fibrinolytic enzymes show promise in the prevention and treatment of thrombosis for their advantages of safety and low price when used as functional food additives

and potential drugs. The production of fibrinolytic enzymes by this way has been performed biotechnologically, but the performance scale industrial is still absent.

These enzymes produced by micro-organisms are usually extracellular which contributes to reducing production cost. On the other hand, high level of intracellular SK has also been obtained during continuous fermentation of recombinant *Pichia pastoris* but protein recovery requires cell lysis (Wong 1989). *Pseudomonas* sp. TKU015 isolated from soil (Wang et al., 2009); *Pseudomonas* sp. from human urine (Dubey et al., 2011); *Alteromonas piscida* (Demina et al., 1990); *Streptomyces* sp. CS684 from soil Korean (Simkhada et al., 2010) also produce fibrinolytic enzyme. Although different bacteria genus produces fibrinolytic enzymes, the genus *Bacillus* is what produces enzyme with high fibrinolytic activity. *Streptomyces* sp. is second bacteria genera most frequently reported in the literature for the production of fibrinolytic enzymes. The highest fibrinolytic activity produced by *Streptomyces* sp. XZNUM 0004 was 2,751 U/mg (Ju et al., 2012), whereas *Bacillus subtilis* the highest fibrinolytic activity reported was 280,280 U/mg (Mahajan et al., 2012) (Table 1 and 3).

Table 3 – Fibrinolytic activity obtained from differences *Streptomyces* sp. described in the literature.

Microorganism	Name of enzyme	Fibrinolytic activity	References
Streptomyces sp. XZNUM 00004	SFE1	2,750 U/mg	Ju et al., 2012
Streptomyces sp. CS624	FES624	1,060 U/mg	Mander et al., 2011
Streptomyces omiyaensis	SOT	136.2 ± 7.4 U/mg	Uesugi et al., 2011
Streptomyces sp. CS684	FP84	19 U/mg	Simkhada et al., 2010

U/mg = milligram protein

Fungi such as *Aspergillus ochraceus*, *Cochliobolus lunatus*, *Fusarium oxysporum*, *Fusarium* sp., *Penicillium chrysogenum*, *Pleurotus ostreatus*, *Rhizopus chinensis*, *Tricholoma saponaceum*, *Ganoderma lucidum*, *Cordyceps sinensis*, *Flammulina Velutipes* and *Schizophyllum commune* also produce

proteases with fibrinolytic activity (Peng et al. 2005). Less common fungi such as *Armillaria mellea* (Lee et al. 2005) and *Cordyceps militaris* (Cui et al. 2008; Kim et al. 2006) have been recognized as potent fibrinolytic enzyme producers among fungi (Table 4). In general, the fibrinolytic activities of fungi exhibit lower values when compared with those of bacteria.

Table 4 – Fibrinolytic activity obtained from fungi described in the literature.

Micro-organism	Name of enzyme	Fibrinolytic activity	References
Aspergillus oryzae KSK-3	KSK-3	1,005 U/mg	Shirasaka et al., 2012
Bionectria sp. LY 4.1	-	66.7 mm^2	Rovati et al., 2010
Bionectria sp. LY 4.2	-	21.9 mm^2	Rovati et al., 2010
Bionectria sp. LY 4.4	-	58.3 mm^2	Rovati et al., 2010
Fusarium sp. CPCC 480097	Fu-P	76,111 U/mg	Wu et al., 2009
Fusarium sp. BLB	-	665 U/mg	Ueda et al., 2007

U/mg = milligram protein; mm^2 = area lytic

In recent years, analyzes with green algae of the genus *Codium* and brown alga *Sargassum fulvellum* has been the target of research looking for new sources with fibrinolytic activity. Algal enzymes are also promising source in the degradation of the fibrin clot and plasminogen activators (Matsubara et al., 2002; Wu et al., 2009). Sumi et al. (1992) have reported that seaweed *Codiales codium* possessed the ability to degrade the fibrin clot, from fibrinolytic activity (60 mm^2), as well as the plasminogen activation (85 mm^2).

Matsubara et al. (1998) related that among five different *Codium* algal species, only *Codium cylindritum* showed no fibrinolytic activity (Table 5). *C. intricatum* produced two enzymes named CIP-I and CIP-II with high fibrinolytic activity and fibrinogenolytic activity with preference to the Aα chain over Bβ or γ chains.

Table 5 – Fibrinolytic activity from different *Codium* sp.

Algae	**Degradation fibrin area (mm^2)**
Codium fragile	79
Codium divaricatum	113
Codium pugniformis	176
Codium cylindricum	Nd
Codium intricatum	346

nd = not detected

Codium latum produces a protease known as *C. latum* Protease (CLP) that hydrolyzed the fibrin clot with fibrinolytic activity of 314 mm^2, using the purified CLP enzyme (Matsubara et al., 1999), and presented activity higher than the values of some powerful snake venoms (Datta et al., 1995).

According Matsubara et al. (2000) a fibrinolytic enzyme was isolated from a marine green algae, *Codium divaricatum*, and designated *C. divaricatum* Protease (CDP) is a protease that effectively hydrolysed fibrinogen Aα chain, while it had very low hydrolyzing efficiency for Bβ and γ chains. The high specificity for Aα chain was very similar to the α-fibrinogenase in snake venom described by Ouyang and Teng (1976), Ouyang et al. (1977). Matsubara et al. (2002) also shows that the three enzymes (CIP, CLP and CDP) may also activate plasminogen, as described in Table 6.

Table 6 – Fibrinolytic activity of three enzymes in the presence or absence of plasminogen (Matsubara, et al., 2002)

	Lytic area (mm^2)	
	Plasminogen	
	+	-
Codium intricatum protease	50	38
Codium latum protease	491	177
Codium divaricatum protease	79	38

+ presence; - absence

3. Cultivation of microorganisms

Microorganisms are capable of growing on a wide range of substrates and can produce a remarkable spectrum of products. The relatively recent advent of in vitro genetic manipulation has extended the range of products that may be produced by microorganisms and has provided new methods for increasing the yields of existing ones. The commercial exploitation of the biochemical diversity of microorganisms has resulted in the development of the fermentation industry and the techniques of genetic manipulation have given this well-established industry the opportunity to develop new processes and to improve existing ones (Hewitt et al., 2007)

Streptococcus sp., *Staphylococcus* sp. and *Bacillus* sp. are heterotrophic microorganisms and needs nutrients organic as energy source. *Streptococci* commonly require complex and rich media supplemented with various nutritional factors for growth. The medium contained peptone, phosphate salts, glucose, biotin, riboflavin, thiamine, tryptophan, glutamine and nucleotides (adenine and uracil) that are high cost nutrient for the scale up. In addition, commercial production of streptokinase requires special attention to biosafety considerations because the protein is potentially immunogenic to process workers. In addition, care is necessary if streptokinase is being produced using natural strains of *Streptococci* because all streptokinase producing *Streptococci* are potentially pathogenic (Banerjee et al., 2004).

Bacillus sp. strain AS-S20-I has been cultivated in different carbon (glucose, fructose, galactose, maltose, sucrose, lactose, carboxymethyl cellulose, casein, and starch) and nitrogen sources (yeast extract, beef extract, tryptone, ammonium sulphate, potassium nitrate, ammonium nitrate, and sodium nitrate) because they are nutrients that have the greatest impact on the production of proteases. Using response surface methodology and submerged fermentation was obtained 749.0 x 10^3 U L^{-1} in optimizing the production of fibrinolytic proteases with 3.0% (w/v) casein and 0.12% (w/v) ammonium sulphate at pH 10.9 and 45°C. The fibrinolytic enzyme degraded the fibrin specific activity 2,408 ± 70.0 U mg^{-1} (Mukherjee and Rai, 2011).

Scaling up of fibrinolytic enzyme production by *Bacillus sphaericus* was evaluated by Balaraman and Prabakaran (2009). 60 L of yeast nutrient salt medium (NYSM) consisting of a mixture of glucose (5 g), peptone (5 g), NaCl (5 g), beef extract (3 g), yeast extract (0.5 g), mineral solutions ($MgCl_2$, $CaCl_2$, $MnCl_2$) was used to produce 4,258 U/mg of fibrinolytic activity.

Optimal fermentation conditions for fibrinolytic enzyme production of subtilisin DFE by *B. amyloliquefaciens* DC-4 was compositions of fermentation medium constituted 2% fibrin, 2% dextrin, 0.5% peptone, 0.15% yeast extract, 0.40% K_2HPO_4, 0.04% NaH_2PO_4, 0.30% $CaCO_3$, pH 7.0; 32 ºC; 210 rpm and fermentation time of 72 h (Peng and Zhang, 2002). The addition of yeast extract and especially fibrin makes the culture medium very expensive, which prevents large-scale cultivation. Therefore, with the increase in yield and productivity and simultaneous cost reduction, the industrial fibrinolytic production by microorganisms can be regarded as possible and economically attractive.

Chitte and Dey (2002) showed that the fibrinolytic production from *Streptococcus megasporus* SD5 can be stressed when use sodium citrate, sodium acetate, glycerol–arginine, fructose, mannose and arabinose, dextran, rhamnose and/or lactose as carbon source and peptone and/or yeast extract as nitrogen source. Casein and casitone were also suitable for enzyme production, while potassium nitrate, elastin, methionine and phenylalanine might not be the suitable substrates as *S. megasporus* SD5.

Mushrooms (fungi) grow in culture medium made of organic substances for saprophytic utilization. Moreover, cultivation of mushroom fruiting body is space occupied and labor-intensive. In the last decade, technology for mushroom submerged path culture has been established in the laboratory and at an industrial level. Choi and Sa (2000) isolated a metalloprotease with fibrinolytic activity from cultured mycelium of *Ganoderma lucidum*. Fukushima et al. (1991) reported that when soy sauce oil was the carbon source for *Asperyillus oryzae*, protease secretion was increased significantly during submerged cultivation.

Aspergillus oryzae KSK-3 produced a high fibrinolytic enzyme with activity of 1,0 U mg^{-1} when cultivated during 7 days in liquid medium contain 1.0 % glucose, 0.3 % yeast extract, 0.5 % polypeptone and 0.3% malt extract, at 28 °C, 170 rpm (Shirasaka et al., 2012). Fibrinolytic activity of 76,1 U mg^{-1} was obtained

from *Fusarium* sp. CPCC 480097 cultivated in liquid medium containing 1.5 % Indian meal powder, 1.0% flour powder, 1.0% corn powder, 1.0 % ground pea powder, 0.3% $CaCO_3$, 0.1% $MgSO_4$, 0.1% NaCl and 0.1% KH_2PO_4, at 28°C, 220 rpm after 144 h (Wu et al., 2009), despite the long cultivation time when compared with other fungi, it is necessary to reduce the cost of culture media by making it more advantageous for industrial scale.

Fibrinolytic activity from *Codium* algae has been studied by Matsubara et al. (1998, 1999, 2000). *C. fragile, C. divaricatum, C. pugniformis, C. cylindricum* and *C. intricatum* were collected at the coast of Hiroshima and Kochi Prefecture, and *C. latum* was collected off the coast of Kagoshima Prefecture, all in Japan. *C. intricatum* had the highest fibrinolytic activity, with 346 mm^2 of lytic area. Studies of fibrinolytic activity with algae are still very rare, however, may be a promising source of new discoveries.

The collection of macroalgae is generally carried out in wild environment, and most of them are also grown on farms using aquaculture techniques. Eastern countries are currently one of the largest producers in the world algae and algae cultivation is performed on ropes suspended in the ocean (FAO, 2013), making it difficult to control the growth conditions. However, the cultivation of microalgae compared with the macroalgae is much more feasible, as it enables better control of the cultivation conditions due to easy handling.

Microalgae are sunlight-driven cell factories that convert carbon dioxide and water into biomass containing lipids, proteins and carbohydrates. Microalgae have become an emerging source for the production of various product. Various production systems are used to cultivate microalgae on a large-scale: open systems such as raceway ponds, and closed systems like flat panel and tubular photobioreactors. The maximum yield and production in closed systems is higher than in open raceway ponds (Norsker et al., 2011). It is expected that algae productivities between 40 and 80 tonnes of dry matter per hectare per year can be realised in closed systems (Wijffels et al., 2010).

Fibrinolytic enzyme production by genus *Bacillus* is generally carried out at a temperature of 30 to 37 °C at pH ranging from 7.0-8.1 and stirring in shake rotary 180-210 rpm; and to *Streptomyces* genus is generally carried out at a temperature of 28 °C and agitation of 160-180 rpm. Fungi of the genus *Fusarium*

and *Aspergillus* are usually grown at a temperature similar to that of *Streptomyces*, 28 °C. Macroalgae are usually collected in the environment wild and then extracted the fibrinolytic enzymes.

4. Biochemical characteristics of fibrinolytic enzymes

Biochemical properties of fibrinolytic enzymes such as molecular weight, optimal pH and temperature, stability and substrate specificity must be determined so that the enzyme has a high efficiency and a characteristic closer to the human fibrinolytic enzymes. Table 7 shows properties of the some microbial fibrinolytic enzymes. Regardless of the source microbial the fibrinolytic enzymes presented molecular weight between 18-35 kDa. The molecular weights of the enzymes of bacteria are generally higher than those of fungi. Moreover, fibrinolytic enzymes from fungi are glycosylated, which protect it from degradation of other proteases, preventing its degradation (Pratap et al., 2000). Enzymes Subtilisin DFE, CK, Subtilisin QK-2 of bacterial origin have similar molecular weight enzyme Fu-P of *Fusarium* sp. SCLC 480097 (Table 7).

Table 7 – Properties of microbial fibrinolytic enzymes

Microbial / Enzyme	M.W. (kDa)	Opt. pH	Opt. temp. (°C)	pH stability	Temp. stability (°C)	Comments	Reference
Streptomyces sp. CS684 / FP84	35	7.5	45	6-9	<40	Serine metalloprotease	Simkhada et al. (2010)
Fusarium sp. BLB	27	9.5	50	2.5-11.5	<50	Serine protease belong to neither chymotrypsin, trypsin, or subtilisin	Ueda et al. (2007)
B. subtilis DC-33 / Subtilisin FS33	30	8.0	55	NI	NI	Subtilisin-like serine protease	Wang et al. (2006)
Armillaria mellea / AMMP	21	6.0	33	NI	NI	Chymotripsin-like metalloprotease	Lee et al. (2005)
Rhizopus chinensis 12	18	10.5	45	6.8-8.8	37 to 24 h	Metalloprotease	Liu et al. (2005)
B. subtilis / Subtilisin QK-2	28	8.5	55	3-12	40 to 30 min	Subtilisin-family serine protease	Ko et al. (2004)
B. amyloliquefaciens DC-4 / Subtilisin DFE	28	9.0	48	6-10	50 for 60 min	Subtilisin-family serine protease	Peng et al. (2003)
Bacillus sp. DJ-4 / Subtilisin DJ-4	29	NI	40	4-11	RT to 48 h	Plasmin-like serino protease	Kim and Chio (2000)
Streptomyces spp. / SW-1	30	8.0	NI	4-9	4-37	Serine metalloprotease	Wang et al. (1999)
Bacillus sp. / KA38	41	7.0	40	NI	NI	Metalloprotease	Kim et al. (1997)
Bacillus sp. CK 11-4 / CK	28.2	10	70	7-10.5	50 for 60 min	Thermophilic alkaline serine protease	Kim et al. (1996)

NI = no information; M.W. = molecular weight; Temp. = optimum temperature; Opt. pH = optimum pH; RT = room temperature; h = hours; min = minutes

Some fibrinolytic agents used commercially in thrombolytic therapy have very different molecular weights, which means that this feature is not a factor limiting fibrinolytic activity, compared with the temperature, pH and others. Streptokinase possesses a molecular weight of 47 kDa, Alteplase t-PA of 70 kDa, Reteplase r-PA of 39 kDa and Tenecteplase TNK-tPA of 70 kDa.

Nattokinase can withstand temperatures of up to 50 °C and remains active after five folds of freezing and thawing (Fujita et al., 1993). It is stable between pH 6 and 12 (optimal pH 10.3–10.8) but becomes inactive in an acidic medium. Nattokinase has been shown to be absorbed across the rat intestinal tract (Fujita et al., 1995).

Serine proteases with fibrinolytic activity from *Bacillus* sp. are generally active at neutral and alkaline pH, and have optimal pH between 8.0 and 10, with molecular weights between 27.7 and 44 kDa and isoelectric points at about 8.0 (Kim and Choi 2000, Ko et al. 2004).

The optimal pH for these mushroom fibrin(ogen)olytic enzymes is 5-10; the optimal temperature is 20-60°C. An overview of mushroom fibrinolytic enzymes may be classified as serine protease (i.e., inhibited by serine protease inhibitors) and metalloprotease (i.e., inhibited by metalloprotease inhibitors) according to protease inhibitor specificity.

Most enzymes showed optimum pH above 8.0 (Table 7), however FP84 and KA38 had optimum pH 7 and 7.5 respectively, which would be more appropriate since blood pH is about 7.2. Enzymes with pH close to that of blood may have its catalytic activity favored, since the pH is one of the biochemical characteristics essential for enzymatic activity.

The stability of the pH and temperature factors are very important for the catalytic mechanism of fibrinolytic enzymes. In Table 7 it is possible to see that both the enzymes of the bacteria as fungi have pH stability within the pH range of blood. Already temperature stability is quite varied and lasts no longer than 60 minutes, except for the enzyme produced by the fungus *Rhizopus chinensis* which has very durable temperature stability at 37 °C for 24 hours, and a good stability on pH.

The enzyme produced by *Streptomyces* sp. CS684 and named FP84 has a number of characteristics that favor its use on an industrial scale. In addition to

having a pH of 7.5 and stability within the range of the blood, the enzyme has a stable average temperature close to body temperature (36-37 °C), even with a temperature optimum at 45 °C, which makes it a fibrinolytic enzyme very attractive.

Mahajan et al. (2012) evaluating the fibrinolytic enzyme purified produced by *Bacillus subtilis* ICTF-1 in the presence of various metallic ions found that Ca^{2+} acts as an enzyme activator increasing their activity by 129%, in contrast ions Zn^{2+}, Fe^{3+} and Hg^{2+} inhibited the enzyme activity by 69%, 96% and 98% respectively, and the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) inhibited more than 99% of the catalytic activity of the enzyme at a final concentration of 5 mM. The activity in the absence of metal ions and inhibitors was taken as 100%. Mahmoud et al. (2011) evaluated the effect of inhibiting the catalytic activity of the enzyme produced by a *Bacillus polymaxa* NRC found that the enzyme was also inhibited by PMSF in 76% and 95% at a concentration of 5 mM and 10 mM respectively. Similar results were obtained in the fibrinolytic enzyme produced by *Bacillus amyloliquefaciens*, the enzyme was completely inhibited by PMSF (1 mM) (Peng et al. 2003).

The fibrinolytic protease (FP84) purified from *Streptomyces* sp. CS684 slightly increased residual activity after using 2 mM $CaCl_2$, 110 ± 2.5%. On the other hand, the activity was completely inhibited by $FeSO_4$ (2 mM) (Simkhada et al., 2010). Mander et al. (2011) found that proteolytic enzyme with fibrinolytic activity produced by *Streptomyces* sp. CS624 was resistant PMSF (4 mM) with relative activity 99 ± 3.46%, while the activity was clearly inhibited by the serine protease inhibitor Pefabloc SC (2 mM), with a relative activity of 81 ± 3.28%, which suggests that the enzyme is from the family of serine proteases. The activity was also significantly inhibited by metalloprotease inhibitors, thylenediamine tetraacetic acid (EDTA), ethylene glycol-bis (β-aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA) with relative activity of 12 ± 2.49% and 23 ± 1.64% respectively, both at 1 mM and completely inhibited by $FeSO_4$ (5 mM) suggesting that this enzyme also belongs the family of metalloproteases.

Wu et al. (2009) evaluated a new fibrinolytic enzyme (Fu-P) produced by the fungus *Fusarium* sp. CPCC 480097 found that the enzyme was strongly inhibited by PMSF and EDTA in a concentration of 4.5 mM, obtaining relative

activity of 6.4 ± 2.2 and 4.7 ± 2.4%, respectively. The enzyme was completely inhibited by Zn^{2+} ions and Cu^{2+}, both in a concentration of 5 mM. The enzyme also increased its activity with Zn^{2+} and Ca^{2+} (1 mM), with relative activity of 120.5 ± 2.1% and 116.1 ± 3.5% respectively, which shows that the mechanisms of metal ions in fibrinolytic activity is not clear and requires further study . Ueda et al. (2007), evaluating the enzyme produced by *Fusarium* sp. BLB found that PMSF (1 mM) and diisopropylfluorophosphate (DFP) 0.1 mM inhibited the enzyme activity by 41% and 79% respectively

The seaweed *Codium intricatum* producing enzymes CIP-I and CIP-II had completely inhibited the enzyme activity by DFP and 1-chloro-3-tosylamido-7-amino-L-2-heptanone (TLCK), both at a concentration of 1 mM. They were not affected by EDTA (10 mM) and cysteine (10 mM). These results suggest that the enzymes CIP-I and CIP II form part of the group of alkaline serine proteases (Matsubara et al., 1998). Matsubara et al. (1999) also evaluated *Codium latum* producing enzyme CLP, and found that the residual activity of the enzyme was completely inhibited with DFP (1 mM) and almost completely by TLCK (1 mM). In another study, Matsubara et al. (2000) also evaluated the performance of inhibitors in the catalytic activity of the enzyme (CDP) produced by *Codium divaricatum* and related that DFP (1 mM) and PMSF (1 mM) inhibited the enzyme activity completely, in contrast TLCK (1 mM), soybean trypsin inhibitor (SBTI) 200 µg/ml and aprotinin (4 TIU/mL) inhibited the enzyme activity almost completely.

The protease activity of mushroom serine fibrinolytic enzymes can be irreversibly inhibited by PMSF but no other protease inhibitors. Previous studies have showed that most fibrinolytic serine protease from traditional fermented foods belong to subtilisin of *Bacillus* origin such as nattokinase, subtilisin DFE and subtilisin QK-1 (Peng et al., 2005). The following table shows some of the properties (Table 7).

Others biochemical characteristic of fibrinolytic enzymes is the N-terminal amino acid sequence (Table 8).

Table 8 – Comparison of the N-terminal amino acid sequences of microbial fibrinolytic enzymes

Fibrinolytic enzyme	N-terminal amino acid sequence	Reference
Fu-P	QASSGTPATIRVLVV	Wu et al. (2009)
Fusarium sp. BLB	IVGTTAASGGDFPIIVSIYYQGRAR	Mitsuhiro et al. (2007)
bpDJ-2	TDGVEWNVDQIDAPKAW	Choi et al. (2005)
Rhizopus chinensis 12	SVSGIGLMHALG	Liu et al. (2005)
AMMP	MFSLSSRFFLYTLCLSAVAVSAAP	Lee et al. (2005)
Bacillokinase II	ARAGEALRDIYD	Jeong et al. (2004)
Subtilisin QK-2	AQSVPYGISQIKAPALHSQG	Ko et al. (2004)
Subtilisin DFE	AQSVPYGVSQIKAPALHSQGFTGS	Peng et al. (2003)
Subtilisin DJ-4	AQSVPYGVSQIKAP	Kim and Chio (2000)
CIP	X-TPLTQVLSGNAVLVEAVLVEA VKA	Matsubara et al. (2000)
CLP	VVGGDEPP	Matsubara et al. (1999)
SW-1	R/N/FP/DGMTMTAIANQNTQIN	Wang et al. (1999)
KA38	VYPFPGPOPN	Kim et al. (1997)
CK	AQTVPYGIPLIKAD	Kim et al. (1996)
NK	AQSVPYGISQIKAPALHSQGYTGS	Fujita et al. (1993)

The enzyme Subtilisin QK-2 (Ko et al, 2004) has the first 20 amino acids identical to the enzyme NK (FUJITA et al., 1993), however, differ both enzyme Subtilisin DFE (Peng et al., 2003) for having an Isoleucine at the eighth amino acid of the N-terminal portion, in which case the enzyme Subtilisin QK-2 and NK is a Valine. The N-terminal amino acid sequences are well described in the literature, however poorly understood. The amino acid sequence may be related to the stability of fibrinolytic enzymes due to the strong interaction between the amino acid residues.

The amino acid sequence also contribute to enzyme stability by binding to the substrate, making an enzyme more become fixed to the substrate and less sensitive to denaturation, thus increasing the chances of its catalytic activity.

Many fibrinolytic enzymes are being discovered in recent decades, among them those produced by micro-organisms are the most well studied. The search for low cost sources and effective for the control of thrombosis still requires much study. Microalgae can be an inexpensive way of fibrinolytic enzymes, since they are easy to handle and growing, not requiring expensive growth sources, on a large scale, do not require large expenditures of energy, since the light from the sun can be a tool for photosynthesis.

5. Conclusion

Although several fibrinolytic enzymes and plasminogen activators have been discovered from different microbial sources or expressed, the development of new enzymes with higher fibrin specificity, stability, less drawbacks and low cost of production is still ongoing, and much work needs to be done mainly concerning thrombolytic effects in vivo.

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6. Artigo 2

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Microalgae *Chorella vulgaris*: a new era in bioprospecting of fibrinolytic enzymes

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Abstract

Cardiovascular diseases are the leading causes of death worldwide and may be caused by the accumulation of fibrin in the blood vessels. In general, therapy for thrombosis is based in surgical operation, intake of antiplatelets, anticoagulants, or fibrinolytic enzymes. Microbial fibrinolytic enzymes have attracted much more attention than typical thrombolytic agents because of the expensive prices and the side effects of the latter. Various microorganisms producers of fibrinolytic enzymes are already described in the literature, including bacteria and fungi. The objective was to produce fibrinolytic enzymes from the microalgae *Chlorella vulgaris* using glycerol and agroindustrial residue corn steep liquor as a source of carbon and nitrogen, respectively. Assessing the culture conditions for the production of fibrinolytic enzyme, it was observed that using 0.5 % corn steep liquor in medium

Bold's Basal (BBM) liquid microalgae produced 745 U mL^{-1} of fibrinolytic enzyme. 2^2 plus star central composite experimental design combined with response surface methodology was employed, and the maximum cell concentration (X_m), the cell productivity (P_X), fibrinolytic activity (F_{act}) and ratio of fibrinolytic to protease activity (F_{act} / P_{act}) were selected as the response variables. X_m predicted by equation using 0.9 % of glycerin and 1.2 % of corn steep liquor, obtaining an estimated value of 1.52 g L^{-1}. The cell productivity, protease production and fibrinolytic enzyme could not be optimized, however, reached levels of 232 mg L^{-1} day^{-1}, 416 U mL^{-1} and 704 U mL^{-1}, respectively. The present study showed that *C. vulgaris* grown in culture medium using glycerol and agroindustrial residue corn steep liquor, it may be feasible to obtain high levels of fibrinolytic enzyme concentration, as well as being used as a resource in the fight against cardiovascular disease.

Keywords

Chlorella vulgaris, Fibrinolytic Enzyme, Corn Steep Liquor, Agroindustrial Waste

1. Introduction

Cardiovascular diseases, including acute myocardial infarction, ischemic heart disease, valvular heart disease, peripheral vascular disease, arrhythmias, high blood pressure and stroke, are the leading causes of death worldwide (Mine et al., 2005). According to the World Health Organization (2012), in 2030, almost 25 million people will die from cardiovascular diseases, mainly from heart disease and stroke

Thrombolytic agents, anticoagulants, antiplatelet and direct thrombolytics, are used for the treatment of thrombotic disorders (Mackman, 2008). One of the major pharmaceutical applications of microbial protease (fibrinolytic protease) is in the treatment of thrombosis, which is considered as one of the most widely occurring cardiovascular diseases in modern life (Mine et al., 2005). A variety of microbial fibrinolytic enzymes such as tissue plasminogen activator (t-PA), urokinase (u-PA),

and bacterial plasminogen activator streptokinase (s-PA) have been extensively studied and used as thrombolytic agents (Mukhametova et al., 2002). All these thrombolytic agents still suffer significant shortcomings, including requirement of large therapeutic dose, short plasma half-life, limited fibrin specificity, reocclusion and bleeding complications (Reddy, 1998).

Fibrinolytic enzymes from microbial sources have been reported from various species of *Bacillus, Streptomyces, Aspergillus* and others. Use of extracts of algae of the genus *Codium* for digestion of fibrin clot was reported by Matsubara et al. (1998, 1999, 2000).

Organic residues from agriculture and industries, e.g. soybean residue, cane molasses, glycerol and monosodium glutamate waste liquor (MGWL), had been exploited increasingly in bioprocesses because those were excellent substrates for heterotrophic micro-organisms growth by supplying the essential nutrients (Pandey et al., 2000). On the other hand, its application provides an alternative way to reduce the production cost and help to solve many environmental hazards. In general, this organic residues has been utilized to bacteria, yeast, fungi and, in limitated number, microorganism photosynthetic cultivation.

Cultivation of photosynthetic microorganism for production of various useful metabolites has been investigated mainly under photoautotrophic cultures. However, due to light limitation, the cell concentrations attainable in photoautotrophic cultures are usually very low with resultant high cost of down stream processing. A feasible alternative for photoautotrophic culture is to use a mixotrophic culture in which such as sugars and organic acids are used as carbon sources and CO_2 in the presence of light are simultaneously assimilated and both respiratory and photosynthetic metabolism operates concurrently. In contrast to photoautotrophic culture, mixorotrophic culture can be performed in conventional microbial bioreactors.

The carbon source can represent up to 60% of the production cost and for this reason the use of alternative sources of carbon which are more abundant and available as industrial and agricultural wastes (Villas Bôas and Esposito, 2000). The biomass produced by bioconversion of agro-industrial wastes has been a

valuable alternative to the traditional applications of such residues, since the substrates are widely available and low cost (Shojaosadati et al. 1999). These wastes are commonly disposed in the environment, causing an excessive accumulation of organic matter in nature, because although these materials are fully biodegradable, its production occurs in large quantities, so intensive and requiring a long period for these wastes recycled naturally. Brazil, as a country of great agricultural activity, produces large amounts of agro-industrial waste (Villas Bôas and Esposito, 2000). In the search for technologies that use industrial subproducts to obtain products with higher added value, biotechnology offers many alternatives to the rational use of these materials (Pandey et al., 2001).

The present study evaluated the fibrinolytic activity from cell extract of microalgae *Chlorella vulgaris* cultivated in low-cost agro-industrial residues, initiating a new era of bioprospecting of fibrinolytic enzymes from microalgae. This is the first report about of fibrinolytic enzyme from microalgae *C. vulgaris*.

2 Materials and methods

2.1 Microorganism

The microalgae *Chlorella vulgaris* UTEX 1803 was originally acquired by the Culture Collection of Algae at the University of Texas (Austin, TX, USA).

2.2 Medium and culture condition

During the preliminary test, the microalga was grown under constant aeration, initial inoculum of 50 mg L^{-1}, temperature at 27 ± 1 °C, light 26 ± 1 µmol photons m^{-2} s^{-1} with white lamps and erlenmeyer flasks (1 L) containing 400 mL of Bold's Basal medium (BBM) liquid proposed by Stein, J. (Ed.). The medium was supplemented with 0.5 % glycerol (Dynamic, PA) and corn steep liquor (CornProducts Brazil, Cabo - PE, Brazil) as described in Table 1. Then, it was performed a statistical design with different concentrations of glycerol and corn steep liquor using response surface methodology (RSM), where the microalga was grown under the same conditions and with illumination at 74 ± 3 µmol photons m^{-2} s^{-1} for evaluation of cell concentration, cell productivity and ratio of fibrinolytic to protease enzymes activities (Table 2).

Analytical methods

2.3 Determination of cell density

The cell density of the microalgae *Chlorella vulgaris* was assessed by turbidimetry at 685 nm (Xu, 2008).

2.4 Homogenization and extraction of bioactive

To obtain the crude extract, 50 mg / mL lyophilized biomass was weighed and homogenized in phosphate buffer (PB) 0.1 M pH 7.0, at room temperature for 30 minutes. The homogenate was centrifuged at 15,000 rpm for 10 minutes at 4 °C and the supernatant was used as the extract to evaluation the protease and fibrinolytic activities (Matsubara et al., 1998, 2000).

2.5 Glycerol and treatment of corn steep liquor

Glycerol was only just autoclaved at 121 °C for 15 minutes for sterilization and subsequently used in cultivation. For crude corn steep liquor, it was first centrifuged at 15,000 rpm, 4 °C for 10 minutes to remove solid particles, the supernatant was collected and then adjusted to pH 8.0 with KOH concentrated and subjected to autoclaving at 121 °C for 20 minutes. After autoclaving, the corn steep liquor was centrifuged again in the same condition before and the supernatant was stored in the freezer until used (LIGGETT and KOFFLER, 1998).

2.6 Protease activity

Protease activities were assayed using azocasein as substrate, using spectrophotometer (Alencar et al., 2003). One unit (U) of enzyme activity was defined as the amount of enzyme able to hydrolyze azocasein giving an increase of 0.001 units of absorbance per minute, at 450 nm of absorbance.

2.7 Fibrinolytic activity

The specific enzyme activity was evaluated using an assay of fibrin degradation (Wang et al., 2011; Wu, 2005). In this assay, 1 unit (fibrin degradation unit, FU) of enzyme activity is defined as a 0.01-per-minute increase in absorbance at 275 nm of the reaction solution.

2.8 Protein concentration

Protein concentrations were determined by using BCA or Micro BCA protein assay reagent Kit (BCA™ Protein Assay Kit, Thermo SCIENTIFIC). Bovine serum albumin was used as protein standard.

2.9 Experimental design and results analysis

The RSM was used to determine the influence of the two independent variables, glycerol concentration (C_{gly}) and corn steep liquor concentration (C_{CSL}), on the four response variables selected for this study, namely maximum cell concentration (X_m), cell productivity (P_X), activity (F_{act}) and ratio of fibrinolytic to protease activity (F_{act} / P_{act}). To this purpose, multivariable regression analyses were done under the conditions preliminarily determined by the experimental design (Table 2, $p < 0.05$). Such design was based on the methodology called ‘‘star planning,’’ proposed by Barros Neto et al. (1996), which consists of two factors in five levels of independent variables. The central point was fourfold repeated so as to check the reproducibility of results. The independent variables C_{gly} and C_{CSL} and its corresponding ranges were selected on the basis of the results of Liang et al. (2009) and Mahboob et al. (2012) respectively.

RSM was applied to the experimental data using Statistical software. Linear and second order polynomials were fitted to the experimental data to obtain the regression equations. The significance of regression coefficients of individual linear, quadratic and interaction term and regression coeficient of model were used in selecting the best models. A regression method was used to fit the second order polynomial equation (1) to the experimental data and to identify the relevant model terms. The same statistical software was used to generate the statistical and response plots.

Equation(1)

$$Y = b_0 + \sum b_i x_i + \sum b_{ii} x_{ii}^2 + \sum b_{ij} x_i x_j$$

(Eq. 1)

Were, Y is the response function, b_0 is an intercept, b_i, b_{ii} and b_{ij} are the coefficients of the linear, quadratic and interaction terms, respectively. And accordingly X_i and X_j represent the coded independent variables. The fitted polynomial equation is expressed as surface and contour plots in order to visualize the relationship between the response and experimental levels of each factor and to deduce the optimum conditions.

According to the analysis of variance, and regression coefficients of individual linear, quadratic and interaction terms were determined. The regression coefficients were then used to make statistical calculation to generate dimensional and contour graphic from the regression models. Statistica (Version 8.0, USA) software package was used to analyze the experimental data. *p* –values of less than 0.05 were considered to be statistically significant. The test for significance of the regression models, the test for significance on individual model coefficients and the lack-of-fit test were performed using the same statistical package.

3. Results and discussion

3.1 Selection of culture medium for the production of fibrinolytic enzyme

Protease and fibrinolytic activities of the cells extracts were showed in the Table 1. In the tests without corn steep liquor addition (1 and 4) were not observed protease activity while those with the corn steep liquor addition was obtained protease activity of 292 U mL^{-1} (test 2) and 1,483 U mL^{-1} (test 3). The removal of inorganic source ($NaNO_3$) of BBM increased proteases production (Table 1), which is not feasible for the purpose of the work. One of characteristics of the ideal thrombolytic agent is fibrin specific which allow direct degradation of fibrin at the clot surface. Furthermore, higher fibrin specificity would limit activation of circulating plasminogen and thus degradation of fibrinogen — attributes that would be expected to reduce the risk of bleeding (Van de Werf, 1999). The cell extract with corn steep liquor (0.5 %) and BBM (with NaNO3) had lower concentrations of protease and fibrinolytic higher (Table 1, test 2). Thus the BBM medium with $NaNO_3$ was used in the optimization conditions.

Table 1 – Conditions for the production of fibrinolytic enzyme using culture medium supplemented with glycerol and corn steep liquor.

Test	Culture medium	Protease activity (U mL^{-1})	Fibrinolytic activity (U mL^{-1})
1	BBM	NS	UN
2	BBM + Corn Steep Liquor 0.5 %	292.0[a]	741.5[a]
3	BBM[1] + Corn Steep Liquor 0.5 %	1,483[b]	1,099[b]
4	BBM + Glycerol 0.5 %	NS	UN

NS = not significant; UN = unrealized; BBM[1] without $NaNO_3$; [a,b] = Values with the same superscript are not significantly different according to the Tukey test ($p > 0.05$).

From the results, it was performed a statistical design using RSM, where different concentrations of glycerol and corn steep liquor were evaluated for optimization of biomass production, cell productivity, proteases and fibrinolytic enzymes production. Statistical design was performed with standard BBM added with glycerol and corn steep liquor, agroindustrial waste. High fibrinolytic enzyme production can reduce production costs and facilities the steps of *downstream*, since purification steps will be used in future.

3.2 Cell growth

Growth profiles of the *C. vulgaris* microalgae with 1 % glycerol and different corn steep liquor concentrations are showed in Figure 1. Increasing corn steep liquor concentration increase the maximum cell concentration. In mixotrophic microalgae culture, the metabolism assimilates CO_2 autotrophic and organic sources provided to it. According to the literature, some microalgae show much higher yield when cultivated mixotrophically which can be justified by the fact that the additional carbon source minimizes the consequences generated by self shading effect. The assimilation of inorganic carbon is hampered due to high cell

density, due to low light availability. When microalgae assimilate more carbon, have a higher production of carbohydrates, lipids and proteins.

Corn steep liquor is rich in protein, carbohydrates, and minerals, and the above results showed that the nutrients were adequate for cell growth. When growth becomes limited due to nutritional deficiency or shading effect, the microalgae utilize the organic carbon source, such as corn steep liquor, in its metabolic processes or to have altered them to produce enzyme as protease, so that they were detectable by the assay procedure. The cultures with 1 % and 1.7 % of corn steep liquor showed similar cell concentration, with a slight decrease of approximately 18% using 1.7 % of corn steep liquor. Stationary phase of cell curve growth using 0.3 % corn steep liquor was after 5 days, obtained X_m of 650 mg L^{-1}. In cultures with 1 % of corn steep liquor, X_m was 1,455 mg L^{-1} after 8 days, while that using 1.7 % of corn steep liquor, the X_m was 1,260 mg L^{-1} after 6 days. *C. vulgaris* growth using 1 % glycerol and 1 % corn steep liquor obtained highest cell concentration and can be quite viable when applied on an industrial scale. Mahboob et al. (2012) related that corn steep liquor and urea, which are low cost nitrogen sources, were highly stable for *C.vulgaris* growth.

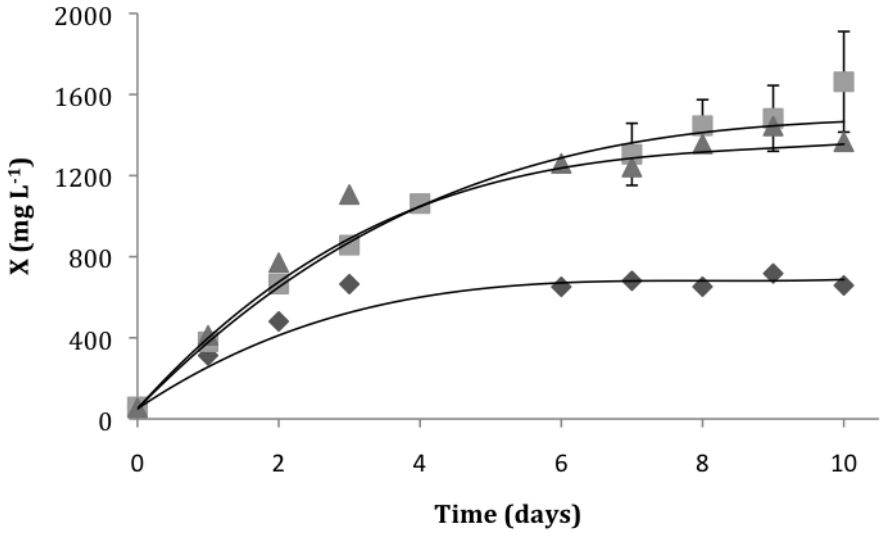

Fig. 1 – Growth profile of the microalgae *C. vulgaris* using 1 % glycerol and differents corn steep liquor concentration (CLS): (♦) CSL 0.3 %; (▲) CSL 1 %; (■) CSL 1.7 %.

The growth profile of the *C. vulgaris* cultivates in BBM with 1 % corn steep liquor and different glycerol concentrations is described in Figure 2. The addition of 1.7 % glycerol exhibited an inhibitory effect on the growth of microalgae, with growth similar to that obtained with 0.3 % glycerol. Konh et al. (2012) showed that *Chlorella vulgaris* can utilize glycerol as a sole carbon substrate for the production of biomass and biochemical components, such as photosynthetic pigments, lipids, soluble carbohydrates and proteins. The stationary phase of cell growth cultivated in 0.3 % glycerol was obtained after 9 days and X_m values was of 1,250 mg L^{-1}. When *C. vulgaris* was cultivated in BBM with 1 % and 1.7 % glycerol, the stationary phase reached after 8 days and X_m was of 1,455 and 1,200 mg L^{-1}, respectively. Liang et al. (2009) observed an inhibitory effect on *C. vulgaris* growth using 2 % glycerol, obtained X_m of 656 mg L^{-1} at 6 days of cultivation. Heredia-Arroyo (2011) mixotrophic growth of *C. vulgaris* using 80:20 % glucose:glycerol, observed an increase in cell concentration, while increased levels of glycerol, about 15 g / L, promoted inhibition.

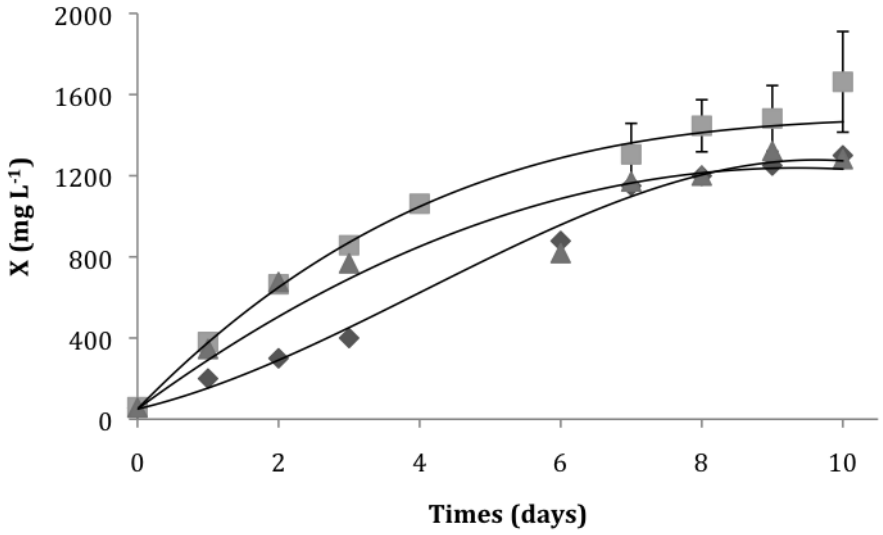

Fig. 2 – Growth profile of the microalgae *C. vulgaris* using 1 % corn steep liquor and differents glycerol concentration:. (♦) Glycerol 0.3 %; (■) CSL 1 %; (▲) CSL 1.7 %.

Cheng et al. (2013) optimized the growth of *C. protothecoides* under three variables independents ($NaNO_3$, $MgSO_4.7H_2O$ and proteose concentrations) obtaining the maximum biomass yield of 1,190 mg L^{-1} after 11 days of cultivation,

though use of proteose in industrial scale is not feasible. The present work showed that it is possible to use two industrial residues to obtain high microalgae concentration (average 1.45 g L^{-1}, using 1 % glycerol and 1 % corn steep liquor) since the costs for the cell production was minimal.

3.3 Protease and fibrinolytic production

The test 4 (Table 2) using 1.5 % glycerol and 1.5 % corn steep liquor resulting high protease and fibrinolytic concentrations of 416 U mL^{-1} and 583 U mL^{-1}, respectively. However, the best result was using 1.7 % glycerin and 1 % corn steep liquor (Test 5) which obtained relative low protease production of 261 U mL^{-1} and high fibrinolytic activity of 704 U mL^{-1}, showing that protease produced has the ability to directly degrade fibrin.

Currently, not there are papers reporting the use of enzymes produced by microalgae in combating cardiovascular disease. Matsubara et al. (1998, 1999, 2000) found that the purified enzyme extracts of three macroalgae of the genus *Codium* showed fibrinolytic activity. The enzyme extract of the alga *C. intricatum* showed specific activity of 691 U mg^{-1} for CIP-I and 533 U mg^{-1} for CIP-II. *C. divaricatum* showed specific activities of 6.3 U mg^{-1}. Abdel-Naby et al. (1992) found that several species of *Streptomyces* are producing fibrinolytic enzymes extracellular and that the species *Streptomyces* sp. NCR 411 was the best producer with activity of 16 U mL^{-1}. The same authors found that the use of $(NH_4)_2SO_4$ was favorable production of fibrinolytic enzymes and the increase of inorganic phosphate (KH_2PO_4) from 0.05 to 1.2 g L^{-1} stimulates the enzyme production (14.3 to 19.4 U mL^{-1}). We also assessed the effect of carbon source on the production of fibrinolytic enzymes, and it was found that the use of 50 g L^{-1} was better starch concentration for enzyme production with about 18 U mL^{-1} activity. Studies of *Bacillus lichniformis* B4 performed by Al-Juamily and Al-Zaidy (2012) evaluated high productivity of fibrinolytic enzyme (30 U mL^{-1}). The same authors also evaluated in isolated conditions different, enzymatic production, using solid lentils medium (26 U mL^{-1}) at pH 7.2 (66 U mL^{-1}), mannitol as a carbon source (46 U mL^{-1}), soy peptone as nitrogen source (50 U ml-L^{-1}) and shaking incubator at 180 rpm (96 U mL^{-1}). Juamily-Al and Al-Zaidy (2013) after purification

of fibrinolytic enzyme from *Bacillus lichniformis* B4, obtained fibrinolytic activity of 95 U mL^{-1}. Jayalakshmi et al. (2012) found that at pH 7.0 (36 h) *Bacillus subtilis* GBRC1 showed 1.74 U mL^{-1} of fibrinolytic activity. Using dextrin as carbon source in the fermentation liquid, the same author showed obtained 1.8 U mL^{-1} activity. Ammonium chloride was the best nitrogen source with maximum production of fibrinolytic enzyme 1.6 U mL^{-1} at 36 h of fermentation. Silva et al. (2013) from fermentation broth of *Streptomyces* sp. DPUA1576, obtained 109 U mL^{-1} fibrinolytic activity. This present study obtained higher fibrinolytic activity with extracts from the microalgae *C. vulgaris*, with activity between 159.0 to 704.4 U mL^{-1}, proving to be a source future in combating cardiovascular disease, specifically in direct degradation of fibrin clot.

3.4 Optimization of medium composition using RSM

A central composite design (CCD) with five coded levels for the two factors glycerol (*Gly*) and corn steep liquor (*CSL*) were used for this purpose. The independents variables and their levels, based on the preliminary results (Table 1), were observed on the X_m, cell productivity, ratio of fibrinolytic to protease activity. The experimental results were used to develop the mathematical models using response surface methodology (RSM). Response Surface optimization is more advantageous than the traditional single parameter optimization in that it saves time, space and raw material.

3.4.1 Maximum cell concentration and cell productivity

As showed in the Table 2, X_m varied between 650.0 to 1,480 mg L^{-1}. Based in these values, the regression analysis was applied to X_m in function of both C_{gly} and C_{CSL}. It is possible to get a quadratic polynomial equation, derived from Equation (2), to express the relationship between X_m and the selected independent variables. To get better fitting of the model, the interaction coefficients between the

independent variables were omitted, because they were proved to be not significant. The optimal conditions to maximize X_m (C_{gly} = 0.9%; C_{CSL} = 1.2%) were estimated by deriving Equation (2). The maximum cell concentration estimated by the model was 1,516 mg L^{-1} . Li et al. (2011) using 0.1% glycerol in photoheterotrophic cultivation of *C. minutissima* UTEX 2341, achieved a maximum cell concentration of 770 mg L^{-1} in 7 days of culture. Liang et al. (2009) noted that the growth of *C. vulgaris* under autotrophic conditions yielded 722 mg L^{-1} of algal biomass in 6 days of culture. Carbon, nitrogen and phosphorous sources are three main influencing important nutrients microalgae growth (Chen and Chen, 2006), and use of glycerol as a carbon source strongly stimulate its growth. About 45 % of microalgae organism is composed of carbon (Singh et al., 2011). The present work presented about twice the X_m obtained by Li et al. (2011) and Liang et al. (2009), demonstrating that the use of glycerol and corn steep liquor, an agro-industrial waste improve maximum cell concentration.

$$X_m = 1{,}448.5 - 53.9\, C_{gly} + 273.6\, C_{CSL} - 114.1\, C_{gly}^2 - 316.6\, C_{CSL}^2$$

Eq. 2

Table 2 – Experimental results of *Chlorella vulgaris* cultivation as a function of two independent variables, glycerol (C_{gly}) and corn steep liquor (C_{CSL}), and their responses variables (X_m, P_X, P_{act}, F_{act} and F_{act} / P_{act}).

Test	x_1[a]	x_2[b]	C_{gly}[c] (%)	C_{CSL}[d] (%)	X_m[e] (mg L^{-1})	P_X[f] (mg L^{-1} dia^{-1})	P_{act}[g] (U mL^{-1})	F_{act}[h] (U mL^{-1})	F_{act} (U mL^{-1}) / P_{act} (U mL^{-1})
1	- 1	- 1	0.5	0.5	881.2	146.0	218.0	232.0	1.314
2	-1	1	0.5	1.5	1325	220.0	182.0	159.0	0.703
3	1	- 1	1.5	0.5	680.7	75.00	229.0	476.5	1.992
4	1	1	1.5	1.5	1165	232.0	416.0	583.4	1.416
5	1.414	0	1.7	1.0	1200	150.0	261.0	704.4	2.907
6	- 1.414	0	0.3	1.0	1250	139.0	158.0	184.4	1.031
7	0	1.414	1.0	1.7	1260	213.0	271.0	199.3	0.904
8	0	- 1.414	1.0	0.3	650.0	130.0	249.0	313.0	1.065
9	0	0	1.0	1.0	1430	179.0	191.0	277.1	1.383
10	0	0	1.0	1.0	1480	185.0	181.0	277.1	1.602
11	0	0	1.0	1.0	1430	179.0	181.0	245.0	1.754
12	0	0	1.0	1.0	1480	185.0	191.0	245.0	1.358

[a]x_1 = variable coded for $C_{gly;}$ [b]x_2 = variable coded for $C_{CSL;}$ [c]C_{gly} = glycerol concentration (%); [d]C_{CSL} = corn steep liquor concentration (%); [e]X_m = maximum biomass concentration; [f]P_X = cell productivity; [g]P_{act} = protease activity; [h]F_{act} = fibrinolytic activity.

The statistical significance of the model equation was evaluated by analysis of variance (ANOVA), which showed that the regression is statistically significant at 97% ($p < 0.05$) confidence level. The adjust coefficient of determination (R^2) was calculated to be 0.95, indicating that the model could explain 95% of the variability.

Three-dimensional response surfaces were plotted on the basis of the model equation, to investigate the interaction among the variables and to determine the optimum concentration of each factor for maximum cell concentration (Fig. 3)

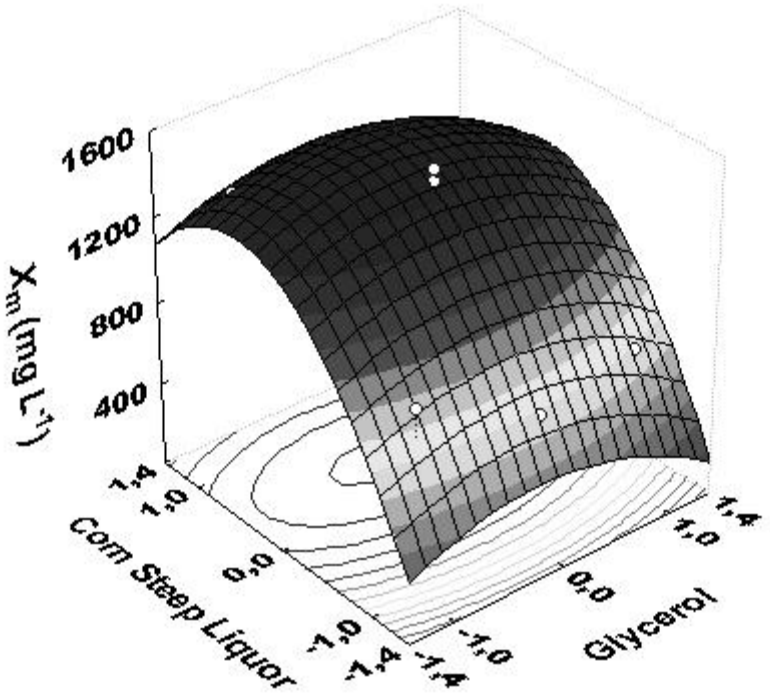

Fig. 3 - Response surfaces showing the mutual effect of glycerol and corn steep liquor (variable coded) in maximum cell concentration.

High glycerol and corn steep liquor concentrations increase cell productivity, obtaining higher values about 232 mg L^{-1} day^{-1} (Table 2; Figure 4). This result was obtained since there was an decrease in the cultivation time in the experiment with high C_{gly} and $C_{CSL.}$ By the way, cell productivity is calculated as function of cell concentration (X_m) and time cultivation, low time of cultivation provides higher cell productivity. Li et al. (2011) related that *C. minutissima* UTEX 2341 productivity of 110 mg L^{-1} day^{-1} was obtained by addition of 1 g L^{-1} of glycerol, photoheterotrophic

culture would also contributes to dramatic biomass increase. This study with *C. vulgaris* UTEX 18033 obtained cell productivity in little more than 50 % compared with Li et al. (2011). Cheng et al. (2013) obtained *C. protothecoides* productivity of 108 mg L^{-1} day^{-1}, 50 % lower than the present work, using 0.25 g L^{-1} proteose, a product of hydrolysis of proteins which increases the cost of cultivation of microalgae. Moazami et al. (2011) screening 147 strains of microalgae isolated from the Persian Gulf and Qeshm Island (Iran) and the strain with higher cell productivity were PTCC 6016 (*Nanochloropsis* sp.) with 46.5 mg L^{-1} day^{-1} and PTCC 6003 (*Nanochloropsis* sp.) of 32.6 mg L^{-1} day^{-1}, using growth medium based on sea salt and RM medium.

The second order polynomial equations (expressed in terms of coded values) fitted to the experimental data of the CCD for predicting *Px*, which showed *p*-value < 0.05 are given in Eqs.(3).

$$P_X = 182.9 - 5.4\,C_{gly} + 46.5\,C_{CSL} - 15.0\,C_{gly}^2 - 7.7\,C_{CSL}^2 + 20.7\,C_{gly}C_{CSL}$$

Eq. 3

The goodness of fit of the regression equation is tested by examining the adjusted determination coefficient, R^2_{Adj}. The values R^2_{Adj} (0.80 for Eqs.(3)) indicate a high degree of agreement between the observed and predicted values for cell productivity, suggesting that the proposed model equations provide satisfactory and accurate results.

Statistical testing of the model was conducted in the form of analysis of variance (ANOVA), which is required to assess the significance and adequacy of the model. Here, the ANOVA of the regression model demonstrates that the model is highly significant, as indicated by the calculated *p*-value ≤ 0.05 for cell productivity.

The response surface plot of the cell produtivity (P_X) is seen in Figure 4.

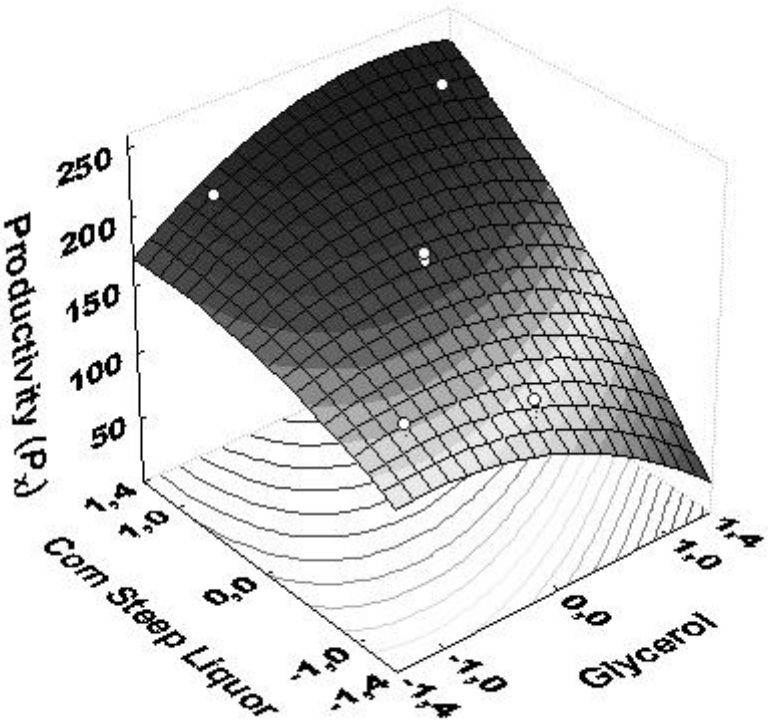

Fig. 4 - Response surfaces showing the mutual effect of glycerol and corn steep liquor (variable coded) in cell productivity.

3.4.2 Ratio between fibrinolytic and protease activities

As described previously, fibrinolytic enzymes need to have high substrate specificity to fibrin to be feasible their application and other proteases with broad substrate specificity should be avoided. Then higher the ratio between the fibrinolytic and protease activities, more specificy is enzyme produced. RSM design was applied to obtain higher ration betweent fibrinolytic and protease activities (Table 2).

By applying multiple regression analysis on the ratio betweent fibrinolytic and protease activities, the response variable and the independent variables were related by the following second-order polynomial equation:

$$F_{act} / P_{act} = 1.51 + 0.51\ C_{gly} - 0.15\ C_{CSL} + 0.21\ C_{gly}^{2} - 0.31\ C_{CSL}^{2}$$

Eq. 4

The *p*-values were used as a tool to check the significance of each coefficient. Regression coefficients of ratio between fibrinolytic and protease activities were estimated using coded units. All linear and quadratic coefficients C_{CSL} were significant ($p < 0.10$). Although the linear effects of C_{CSL} presents a descriptive level of $p = 0.12$, this was considered important for the determination model. The interaction coefficient was not significant and removed from the model.

The developed prediction equation shows that increasing of C_{CSL} increase F_{act} / P_{act} values, while that the increase of C_{gly} improve F_{act} / P_{act} values only in the high C_{CSL}, indicanting that the F_{act} / P_{act} values is more dependent of C_{CSL} than C_{gly}. High proteins and peptides concentrations are found in corn steep liquor (Hull, 1996) and it can induce the production of proteases by microalgae *C. vulgaris.* Ferrero et al (1996) related that protease production by *Bacillus licheniformis* MIR 29 was repressed in the glycerol presence although cell growth had been observed.

The effect of C_{CSL} and C_{gly} concentrations on the F_{act} / P_{act} values has been investigated by using Response Surface Methodology (RSM). Response surface were constructed for determining the optimum conditions for a required F_{act} / P_{act} values (Figure 5).

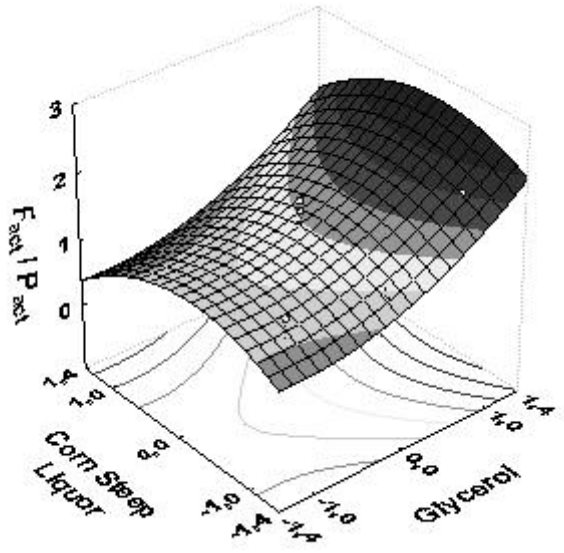

Fig 5 - Response surfaces showing the mutual effect of glycerol and corn steep liquor on ratio between fibrinolytic and protease activities (F_{act} / P_{act}).

The Statistical significance of the regression model was checked by analysis of variance (ANOVA) for the response surface quadratic model. The coefficient of determination (R^2) value of the regression for the response related to significant effects on the model was 0.87, which means that the sample variation of 87.0 % for ratio between fibrinolytic and protease activities was attributable to the factors. This indicates that the model was adequate for prediction within the range of experimental variables.

The optimum conditions to production of F_{act} / P_{act} values were C_{CSL} = 0.76% and C_{gly} =0.40 % obtained the predicted yield of 2,41. The close correlation was seen between the experimental and predicted values which validate the model. The predicted F_{act} / P_{act} values by optimal levels of the variable generated by the model was in close correlation with experimental value (Figure 6), which signifies the RSM methodology over tradidional optimization approach.

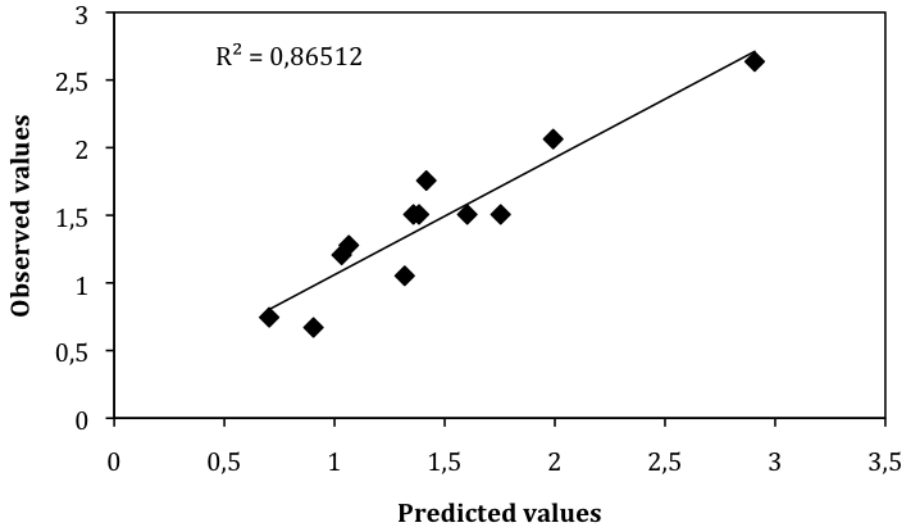

Figure 6. Linear relationship between the predicted and experimental values of ratio between fibrinolytic and protease activities (F_{act} / P_{act}).

4. Conclusion

The results showed that the use of corn steep liquor to production of fibrinolytic enzymes from the microalgae *Chlorella vulgaris* UTEX 1803 is potentially viable. The agro-industrial residues proved to be good for increasing cell concentration, cell productivity and fibrinolytic enzymes, obtaining great potential for future industrial and biotechnological applications in cardiovascular disease treatment.

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