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Queratinases de *Bacillus subtilis* S14:

produção, expressão e análise de enzimas mutantes

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LISTA DE ABREVIATURAS

BIGGA: N–benzoil–Ile–Glu–Gly–Arg–p–nitroanilIna

BRI: BioResource International

CCD: Central composite design (Delineamento completo rotacional)

DFP: Diisopropil–fluorofosfato

DNAS: *DNA Shuffling*

EDTA: Ácido etilenodiamino tetra–acético

EGTA: Ácido etilenoglicol tetra–acético

EP–PCR: *Error prone PCR*

E–64: *trans*–epoxisucinil–L–leucilamido(4–guanidino)–butano

IPTG: Isopropil–β–D–1–tiogalactopiranósideo

$K_L a$: Coeficiente volumétrico de transferência de oxigênio

KerS14: Queratinase *Bacillus subtilis* S14

ORF: Open reading frame (fase de leitura aberta)

PMSF: Fluoreto de fenilmetil sulfonil

SDS: Dodecil sulfato de sódio

SDS–PAGE: Eletroforese em gel de poliacrilamida com dodecil sulfato de sódio

TCA: Ácido tricloroacético

TLCK: Tosil–L–lisina clorometil cetona

TPCK: Tosil–L–fenilalanina clorometil cetona

Tris: Tris hidroximetil aminometano

rKerS14: Queratinase de *Bacillus subtilis* S14 recombinante

RSM: Response surface methodology (Metodologia de superfície de resposta)

rpm: Rotações por minuto

supKBs14: Sobrenadante queratinolítico de *Bacillus subtilis* S14

PCR: Polimerase chain recation (reação em cadeia da polimerase)

vvm: Volume por volume por minuto

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RESUMO

Enzimas industriais movimentam um mercado mundial estimado em sete bilhões de dólares anualmente. Entre essas enzimas, incluem-se as queratinases, enzimas capazes de catalisar a hidrólise de queratina e com aplicação potencial na indústria coureira. A queratinase KerS14, produzida pelo *Bacillus subtilis* S14 é capaz de depilar o couro sem causar danos ao colágeno. Embora tenha esta capacidade de grande interesse biotecnológico, sua baixa termoestabilidade a 50 °C é uma característica desvantajosa para a aplicação industrial da enzima. Visando aumentar a produção de queratinases pelo *B. subtilis* S14 e a termoestabilidade da KerS14, duas estratégias foram adotadas: mudanças nas condições de cultura do microrganismo selvagem e obtenção de enzimas mutantes recombinantes. Na primeira estratégia, *B. subtilis* S14 foi cultivado em meio farinha de pena 1,5% e $CaCl_2$ 1%. O sobrenadante da cultura foi caracterizado e 60% da atividade queratinolítica permaneceu depois de armazenada por nove dias a temperatura de 50 °C. Em paralelo, a ORF que codifica a KerS14 foi amplificada e clonada em vetor de expressão. Os resíduos de aminoácidos G61, S98 e P239 da KerS14 foram modificados por mutagênese sítio dirigida , as enzimas mutantes foram expressas in *Escherichia coli* e purificadas. A enzima recombinante (rKerS14) foi mais termoestável do que a enzima selvagem. Além disso, foi verificado que o fibrinogênio e fibrina são hidrolisados pela rKerS14, mostrando que a enzima também tem potencial para desenvolver drogas para o tratamento de doenças cardiovasculares.

ABSTRACT

Industrial enzymes have a world market of more than seven billion dollars per year. Keratinases are among these enzymes. They have a potential for use in tannery. The keratinase KerS14, produced by *Bacillus subtilis* S14, differ from other keratinases because it can depilate leather without damaging collagen. Despite this great biotechnological potential, its low thermal stability at 50 °C is an undesirable property for industrial application. In order to increase keratinases production by *B. subtilis* S14 and KerS14 thermal stability, two strategies were adopted: changes in wild–type microorganism growth conditions and producing recombinant mutant enzymes. In first strategy, *B. subtilis* S14 were grown in feather meal 1.5% and $CaCl_2$ 1 %. The supernatant obtained after fermentation was characterized and its keratinolytic activity remained in 60% after nine days of storage at 50 °C. In parallel, the KerS14 ORF was amplified and cloned into an expression vector. The KerS14 amino acid residues G61, S98 and P239 were modified and mutant enzymes were expressed in *Escherichia coli* and purified. It was verified that recombinant enzyme (rKerS14) is more thermal stable than the wild–type enzyme. In addition, it is able to hydrolyze fibrinogen and fibrin which is useful to develop drugs for the treatment of cardiovascular diseases.

1. INTRODUÇÃO

1.1 Enzimas Industriais

A produção e comercialização de enzimas industriais são crescentes. Em 2002, o mercado foi estimado em 1,5 bilhões de dólares (KIRK *et al*., 2002). Nos anos seguintes, o mercado aumentou para 5,1 bilhões de dólares em 2009 (SANCHES & DEMAIN, 2009) e estimado em 7 bilhões de dólares em 2013 (FERNANDES, 2010). Enzimas são utilizadas em diversas áreas da indústria, tanto em processos de fabricação como fazendo parte da composição do produto adquirido pelo consumidor final (Tabela 1). Em 2003, o mercado global de enzimas foi dividido nas seguintes áreas: alimentação (incluindo laticínios, cervejaria, panificação, sucos de fruta) (45%), detergente (34%), têxteis (11%), coureira (3%), polpa e papel (1,2%) (SINGH, 2009).

Dentre as enzimas comercializadas estão celulases, lipases, amilases e principalmente peptidases. As peptidases correspondem à maior parte deste mercado, sendo responsáveis por aproximadamente 60% de todas as enzimas industriais comercializadas no mundo (WARD *et al*., 2009). As três maiores empresas produtoras de enzimas do mundo, Novo Nordisk, Genecor International e DSM N.V. possuem 41%, 21% e 8% do mercado mundial de peptidases, respectivamente (WARD *et al*., 2009). Peptidases são utilizadas principalmente na formulação detergentes e indústrias de alimentos. Do total de enzimas utilizadas em detergentes, as peptidases correspondem à maior parte, correspondendo a 89% do mercado mundial de peptidases (GUPTA *et al*., 2002). Em 2002, a União Europeia utilizou 900 toneladas da subtilisinas, obtidas de espécies de *Bacillus* (MAURER, 2004).

Na indústria alimentícia, as peptidases são aplicadas em laticínios, utilizadas como amaciantes de carne e flavorizantes (Tabela 1), na formulação de preparados para

alimentação infantil e no aumento da digestabilidade de alimentos (FERNANDES, 2010). Outra aplicação relevante de peptidases é no processo de síntese do adoçante aspartame (SANCHES & DEMAIN, 2009). Peptidases também são utilizadas como auxiliares da digestão (WARD *et al*., 2009) e medicamento anti–inflamatório, como a estreptoquinase, capaz de remover coágulos pulmonares e utilizada no tratamento de trombose (PENG *et al*., 2005).

Tabela 1. Enzimas industriais, classificação, área de aplicação e funções (adaptado de AEHLE, 2007).

Enzima (Número E.C.)	Áreas de aplicação	Função
Glicose oxidase (1.1.3.4)	Panificação	Espessante; aumento da resistência do glúten
	Indústria cervejeira	Aumento da meia vida da cerveja
	Indústria láctea	Coagulação do leite
Lacase (1.10.3.2)	Papel e celulose	Branqueamento de papel
	Têxtil	Prevenção da recoloração no processo de estonagem
Lipases (3.1.1.3)	Indústria láctea	Coagulação do leite
	Formulação de detergentes	Remoção de resíduos de gordura
α–amilases (3.2.1.1)	Panificação	Evitar endurecimento da massa
	Detergentes	Remoção de resíduos de amido
	Indústria cervejeira	Precipitação do mosto e fermentação
β–amilase (3.2.1.2)	Indústria cervejeira	Precipitação do mosto
Transglutaminase (2.3.2.13)	Laticínios	Aumento da textura do iogurte
Catalase (1.11.1.6)	Indústria cervejeira	Aumento da meia vida da cerveja
	Indústria láctea	Preservação do leite
	Têxtil	Renoção de peróxido de hidrogênio

Enzima (Número E.C.)	Áreas de aplicação	Função
Celulases (3.2.1.4)	Indústria cervejeira	Fermentação
	Formulação de detergentes	Remoção de partículas sólidas, reavivamento de fibras
(3.2.1.91)	Têxtil	Acabamento de algodão
	Papel e celulose	Branqueamento da celulose
Xilanases (3.2.1.8)	Panificação	Incremento da estabilidade da massa
	Alimentação animal	Aumento da digestibilidade de cereais na ração
	Papel e celulose	Branqueamento da celulose
Pectinases (3.1.1.11)	Sucos de frutos	Remoção de *peeling* em frutas
(4.2.2.10)	Sucos de frutos	Remoção de *peeling*
(3.2.1.67)	Têxtil	Lavagem de algodão
Subtilisina (3.4.21.62)	Formulação de detergentes	Remoção de resíduos proteicos
	Alimentação animal	Aumento da digestibilidade de proteínas na ração
Orizina (3.4.21.63)	Alimentação animal	Aumento da digestibilidade de proteínas na ração
Quimiosina (Renina) (3.4.23.4)	Indústria láctea	Coagulação do leite

Peptidases modificadas geneticamente também são comercializadas. Em 1991, a primeira subtilisina modificada geneticamente foi lançada no mercado, tendo como diferencial maior estabilidade à oxidação causada por peróxido de hidrogênio, característica desejada para a formulação de detergentes para uso em lavagem de roupa (MAURER, 2004) (Tabela 2).

Tabela 2. Subtilisinas geneticamente modificadas formuladas em produtos para uso como detergentes.

Enzima	Produto	Fabricante	Referencia
Subtilisina	Everlase®	Novozymes	MAURER, 2004
Subtilisina	Kannase®	Novozymes	MAURER, 2004
Subtilisina	Ovozyme®	Novozymes	AEHLE, 2008
Subtilisina	Properase®	Genencor	MAURER, 2004

Dentre as peptidases, as queratinase são importantes para alguns ramos industriais. Entre suas aplicações, está o uso na indústria coureira, devido à capacidade de depilar do couro. Como demonstrado anteriormente, a queratinase KerS14, foco da presente tese, é capaz de depilar o couro sem danificar o colágeno (MACEDO *et al*., 2005). Além disso, depilação total é obtida sem o uso de sulfeto ou qualquer outro agente químico, evitando assim os principais danos causados ao meio ambiente pelos processos usuais de curtimento de peles (MACEDO *et al*., 2005; GIONGO *et al*., 2007; PILLAI *et al*., 2011; DETTMER *et al*., 2012).

Outra aplicação importante é a utilização de queratinases para obtenção de farinha de pena, utilizada na formulação de ração animal. A produção da farinha de pena é realizada em condições de alta pressão e temperatura, ocasionando perdas na qualidade nutricional. A utilização de queratinases é uma alternativa para evitar a

degradação de aminoácidos de interesse nutricional (GRAZZIOTIN *et al*., 2006; SHIH, 2012).

Novas utilizações de queratinases têm sido propostas, como a degradação de príons por queratinases produzidas pelo *B. licheniformis* PWD–1 (LANGEVELD *et al*., 2003) e *P. aeruginosa* KS–1 (SHARMA & GUPTA, 2010). Outra proposta de aplicação é o controle do nematoide *Meloidogyne incognita*, praga em diversas culturas agrícolas, propondo pela primeira vez o uso da enzima como pesticida (YUE *et al*., 2011).

1.2 Queratinases comercializadas

A primeira queratinase de origem microbiana comercializada foi a Versazyme®, produzida pela empresa BioResource International (BRI). Este produto originou-se a partir da queratinase produzida pelo *B. licheniformis* PWD–1 (WILLIAMS *et al*., 1990), sendo aplicada para obtenção de farinha pena (ODETALLAH *et al*., 2005; WANG *et al*., 2008a). A BRI também comercializa a Valkerase®, produto a base de queratinase utilizado como aditivo que aumenta o valor nutricional de ração para animais (GUPTA *et al*., 2012). Outra queratinase disponível no mercado é a Prionzymer®, lançada recentemente pela Genecor International em parceria com o governo do Reino Unido. Este produto é utilizado com o objetivo de degradar príons, com aplicação na descontaminação de instrumentos cirúrgicos (GUPTA *et al*., 2012). Também há no mercado uma preparação comercial chamada PURE100Keratinase, produzida pelas empresas Zurko Bioreseach e Proteos Biotech, com o propósito de aplicação dermatológica (GUPTA *et al*., 2012).

1.3 Classificação de peptidases e queratinases

Peptidases formam a subclasse de enzimas hidrolases capazes de clivar ligações peptídicas e, de acordo com o Comitê de Nomenclatura Enzimática (EC) da União

Internacional de Bioquímica e Biologia Molecular (NC–IUBMB; www.chem.qmul.ac.uk/iubmb/enzyme/), classificadas como E.C 3.4._._.

As peptidases são divididas em dois grandes grupos, de acordo com seu modo de ação: exopeptidases e endopeptidases. As exopeptidases atuam em ligações peptídicas próximas das extremidades N ou C–terminal dos peptídeos, sendo denominadas amino ou carboxilpeptidases, respectivamente (RAO *et al*., 1998).

As exo peptidases que atuam na região N–terminal são denominadas aminopeptidases (EC 3.4.11._) quando liberam um único aminoácido. Quando liberam um dipeptídeo ou um tripeptídeo, são denominadas dipetidilpeptidases ou tripeptidilpeptidases (EC 3.4.14._), respectivamente. As exopeptidases que atuam na região C–terminal são denominadas carboxipeptidases quando liberam um resíduo de aminoácido e são divididas em sub–subclasses de acordo com seu mecanismo catalítico: tipo serino carboxipeptidases (EC 3.4.16._), tipo metalocarboxipeptidases (EC 3.4.17._) e tipo cisteíno carboxipeptidases (EC 3.4.18._). Ainda há as exopeptidases específicas para dipeptídeos, como a dipeptidases (EC 3.4.13._), ou a omega peptidase (EC 3.4.19._) capazes de remover resíduos terminais que são substituídos, ciclados ou ligados por ligações isopeptídicas (RAO *et al*., 1998).

As endopeptidases são classificadas em sub–subclasses: ácido glutâmico endopeptidases, serino endopeptidases, cisteína endopeptidases, ácido aspártico endopeptidases, metaloendopeptidases e treonina endopeptidases. Recentemente, foi proposta a adição de uma nova sub–subclasse de peptidases, classificada como asparginopeptídeo liases (RAWLINGS *et al*., 2011).

1.3.1 Ácido glutâmico endopeptidases (EC 3.4.23.19)

Seu sítio ativo é formado por Glu–Gln, que desempenha papel importante na ligação do subtrato e na catálise (FUJINAGA *et al*., 2004). É uma sub–subclasse recente de peptidases, presente em fungos filamentosos e bactérias (KONDO *et al*., 2010). Diferenciam–se das ácido aspártico endopeptidases por não serem inibidas por pepstatina e na ausência de similaridade com enzimas semelhantes a pepsina (KONDO *et al*., 2010).

1.3.2 Serino endopeptidases (EC 3.4.21._)

As serino endopeptidases estão amplamente distribuídas, apresentando funções diversas (SIEZEN & LEUNISSEN, 1997). A sub–subclasses tem como característica a tríade catalítica, formada pelos aminoácidos serina, histidina e asparagina. Outra característica é o sítio catalítico que possui uma serina, presente no motivo Gly–Xaa–Ser–Yaa–Gly (RAO *et al*., 1998). São inibidas por diisopropilfluorofosfato (DFP) e fluoreto defenilmetilsulfonil (PMSF) (SIEZEN & LEUNISSEN, 1997). Entre as serino endopeptidases estão a quimiotripsina (EC 3.4.21.1), a tripsina (EC 3.4.21.4) e as subtilisinas (EC 3.4.21.62). Conforme descrito no item 1.1, as subtilisinas são de grande importância biotecnológica.

1.3.3 Cisteína endopeptidases (EC 3.4.22._)

Possuem como sítio ativo Cys–His ou His–Cys e são inibidas por ácido *p*–cloromercuribenzóico. A papaína é a cisteíno endopeptidase melhor descrita e de relevância comercial, sendo muito utilizada como amaciante de carne (RAO *et al*., 1998).

1.3.4 Ácido aspártico endopeptidaes (EC 3.4.23._)

Denominadas peptidases ácidas, devido a sua atividade no intervalo de pH 3–4. Possuem no sítio catalítico o ácido aspártico, sendo o sítio ativo é formado pelos motivos Asp–Ser–Glu ou Asp–Thr–Glu e são inibidas por pepstatina (RAO *et al*.1998; DUNN, 2002). Entre as ácido aspártico endopeptidases estão a pepsina, fundamental nos processos a digestão de alimentos em animais e a quimiosina, também utilizada na indústria de laticínios (Tabela 1).

1.3.5 Metalo endopeptidases (E.C. 3.4.24._)

Metalo endopeptidases são enzimas em que o sítio catalítico envolve a presença de íon metálico divalente, na maioria dos casos o íon zinco, mas também os íons cobalto, ferro e manganês e o aminoácido glutamato. Apresenta o motivo His–Glu–Xaa–Xaa–His, característico da sub–subclasse (GOMIS–RÜTH, 2009). Os agentes quelantes EDTA e o–fenantrolina são capazes de se ligar ao metal do sítio ativo, inibindo a atividade da enzimática (RAO *et al*., 1998).

1.3.6 Treonina endopeptidases (EC 3.4.25._)

Possui o aminoácido treonina presente no sítio ativo. Esta sub–subclasse foi estabelecida em 1995 sendo isolado do proteassoma de *Saccharomyces cerevisiae* e também está presente em proteassoma de procariotos (RAWLINGS *et al*., 2012).

1.3.7 Aspargino peptídeoliases (EC 4.3.2._)

Descrito recentemente, se trata de uma família de classificada como amidina liase (EC 4.3.2._), não sendo classificada como hidrolases como as demais peptidases. Tem no sítio ativo uma asparginase, que é o agente nucleofílico da reação enzimática (RAWLINGS *et al*., 2011).

1.4 Queratinases

As queratinases são denominadas quanto à capacidade de degradar a queratina e não pelo mecanismo de catálise, existindo queratinases classificadas como serino endopeptidases e metaloendopeptidases (BRANDELLI *et al*., 2010). Embora a classificação de queratinases com o EC 3.4.99.11 seja descrita na literatura (GUPTA & RAMMANI, 2006) esta é inadequada de acordo com o a Comissão de Nomenclatura da IUBMB, (http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/4/99/11.html), sendo suprimida em 1992.

1.5 Micro–organismos produtores de queratinases

A maior parte das queratinases conhecidas pertence à sub–subclasse serino endopeptidases e é produzida por linhagens de *Bacillus* (BRANDELLI *et al*., 2010). Dentre as espécies, estão o *Bacillus licheniformis* (WILLIAM *et al.*, 1900), *B. subtilis* (MACEDO *et al*., 2005, GIONGO *et al*., 2007) *Bacillus pumilis* (SON *et al*., 2008) e *Bacillus amyloliquefaciens* (GIONGO *et al*., 2007). Também há estudos de metaloendopeptidases queratinolíticas produzidas por *Bacillus* sp. (LEE *et al*., 2002; TORK *et al*., 2013).

O gênero *Streptomyces* também é pesquisado devido ao potencial de produção de queratinase termoestáveis (BRANDELLI *et al*., 2010). Entres os membros do gênero, estão o *Streptomyces fradiae* (NICKERSON *et al*., 1963), *Streptomyces pactum* (BÖCKLE *et al*., 1995), *Streptomyces albidoflavus* (BRESSOLLIER *et al*., 1999) e *Streptomyces gulbargensis* (SYED *et al*., 2009). Outro gênero bacteriano estudado é o *Chryseobacterium*, produzindo queratinases classificadas como metaloendopeptidase (RIFFEL *et al*., 2007; WANG *et al*., 2007; CHAUDHARI *et al*., 2013).

Há relatos de outros gêneros bacterianos produtores de queratinases, contudo o número de trabalhos publicados é inferior em relação aos gêneros citados anteriormente, como *Nocardiopsis* (SAHA *et al*., 2012), *Vibrio* (SANGALI & BRANDELLI, 2000), *Microbacterium* sp. (THYS *et al*., 2006), *Xanthomonas* (DE TONI *et al*., 2002), *Pseudomonas* (SHARMA & GUPTA, 2010) e *Stenotrophomonas* (CAO *et al*., 2009).

Em fungos, as queratinases têm despertado interesse pela capacidade destes micro–organismos em colonizar e degradar substratos queratinosos, além do papel de queratinases na infecção causada por fungos dermatófitos (BŁYSKAL, 2009). As queratinases de fungos dermatófitos, como *Microsporum*, *Trychophyton* e *Doratomyces microsporum* têm sido isoladas, algumas com importância médica (BRANDELLI, 2008). O estudo de queratinases produzidas pelos gêneros *Aspergillus, Penicillium, Chrysosporium, Fusarium, Microsporum, Trichophyton* e *Acremonium* possui maior destaque na literatura (BŁYSKAL, 2009).

1.6 Produção de queratinases

A produção de queratinases por micro–organismos ocorre em meio cultivo líquido, também denominado fermentação submersa, processo também utilizado para a produção de outros produtos microbianos. Dentre as vantagens da fermentação submersa em relação à semi–sólida, estão a padronização da produção e obtenção de produtos de difícil síntese química, como enzimas e aminoácidos (ANDERSON, 2009).

Os estudos realizados sobre a produção de queratinases são geralmente em fermentação submersa na escala de frasco de Erlenmeyer. Nestes, são avaliados os constituintes do meio de cultura (fontes de carbono, nitrogênio e micronutrientes) e as condições de cultivo (pH, temperatura, homogeneização do meio de cultura e demanda de oxigênio) (GUPTA *et al*., 2002; GUPTA *et al*., 2006; BRANDELLI *et al*., 2010).

Diversos meios de cultivo foram propostos, podendo ser divididos em meios com substratos queratinosos e meio com substratos não queratinosos (BRANDELLI *et al*., 2010). Os substratos queratinosos mais utilizados para a produção de queratinases são o pelo bovino (MACEDO *et al*., 2005), cabelo (CAI & ZHENG, 2009; MAZOTTO *et al*., 2010) e penas (FAKHFAHK–ZOUARI *et al*., 2010; CEDROLA *et al*., 2011; AGRAHARI & WADHWA, 2012). A farinha de pena também é utilizada, sendo adequada para a produção de queratinase por *Microbacterium* sp. (THYS *et al*., 2006), *Bacillus* (FAKHFAHK–ZOUARI *et al*., 2010; MAZOTTO *et al*., 2011; DAROIT *et al*., 2011) e *Streptomyces* sp. (TATINENI *et al*., 2007).

Entre os substratos não queratinosos, o farelo de soja foi mais adequado do que penas para a produção de queratinases pelo *B. subtilis* P13 (PILLAI *et al*., 2011). Além disso, resíduos agroindustriais em que proteínas não são o constituinte majoritário serviram para a produção de queratinases, como bagaço de cana de açúcar (AWAD *et al*. 2011) e quitina de camarão (WANG *et al*., 2008b).

Outros constituintes também são adicionados nos meios de cultivo visando à produção de queratinases, tais como carboidratos (mono, di ou polissacarídeos), fontes de nitrogênio (uréia, peptonas e extrato de levedura) e sais. Contudo, os vários relatos demonstram que a adição destes suplementos pode tanto trazer aumento na produção de queratinases em alguns microrganismos como levar a decréscimo da produção em outros. O efeito dos constituintes do meio de cultura na produção de queratinases depende da concentração dos mesmos, bem como do microrganismo estudado, sendo necessário determinar as condições ótimas de produção de queratinases caso a caso (BRANDELLI *et al*., 2010).

1.7 Expressão heteróloga de queratinases

Várias queratinases foram clonadas e expressas, com o objetivo de aumentar a produção ou a melhorar degradação de substratos queratinosos (BRANDELLI *et al*., 2010). A maior parte das queratinases expressas é provinda de *Bacillus* (GUPTA *et al*., 2012), mas também há queratinases expressas de *Streptomyces* (LI *et al*., 2013) e *P. aeruginosa* (LIN *et al*., 2009). A expressão heteróloga de queratinases pode mudar as propriedades da enzima recombinante em relação à selvagem (HU *et al*., 2013). Além disso, nem sempre o rendimento da enzima recombinante é adequado, devido à formação de corpos de inclusão (GUPTA & RAMNANI, 2006). Dentre os micro–organismos utilizados para a expressão de queratinases recombinantes estão *E. coli* (LIN *et al*., 2009a), *B. subtilis* (LIU *et al*., 2013) e a levedura *Pichia pastoris* (LIN *et al*., 2009b).

1.8 Mutações em enzimas

1.8.1 Mutação sítio dirigida

Nestas técnicas, informações sobre a estrutura e funções da proteína alvo estão disponíveis, podendo ser baseadas na literatura e/ou com a utilização de ferramentas de bioinformática (FERNANDES, 2010). As mutações são escolhidas e inseridas no gene, criando modificações pontuais (LABROU, 2010).

1.8.1.1 Mutagênese sítio dirigida por PCR

Nesta técnica de mutagênese sítio dirigida, se utilizam quatro primers: dois com a mutação desejada e outros dois para amplificar o gene mutante. No primeiro PCR, os primers 1 e 3 se anelam na sequencia desejada, assim como os 2 e 4. Os primers 2 e 3 possuem o códon mutante desejado. Dois produtos de DNA dupla fita são obtidos. Estes

produtos são desnaturados e anelados, gerando heterodúplex contendo a mutação desejada. Os finais sobrepostos de ambos são completados usando uma DNA polimerase. Um segundo PCR é realizado para amplificar todo o gene com a mutação desejada (LABROU, 2010).

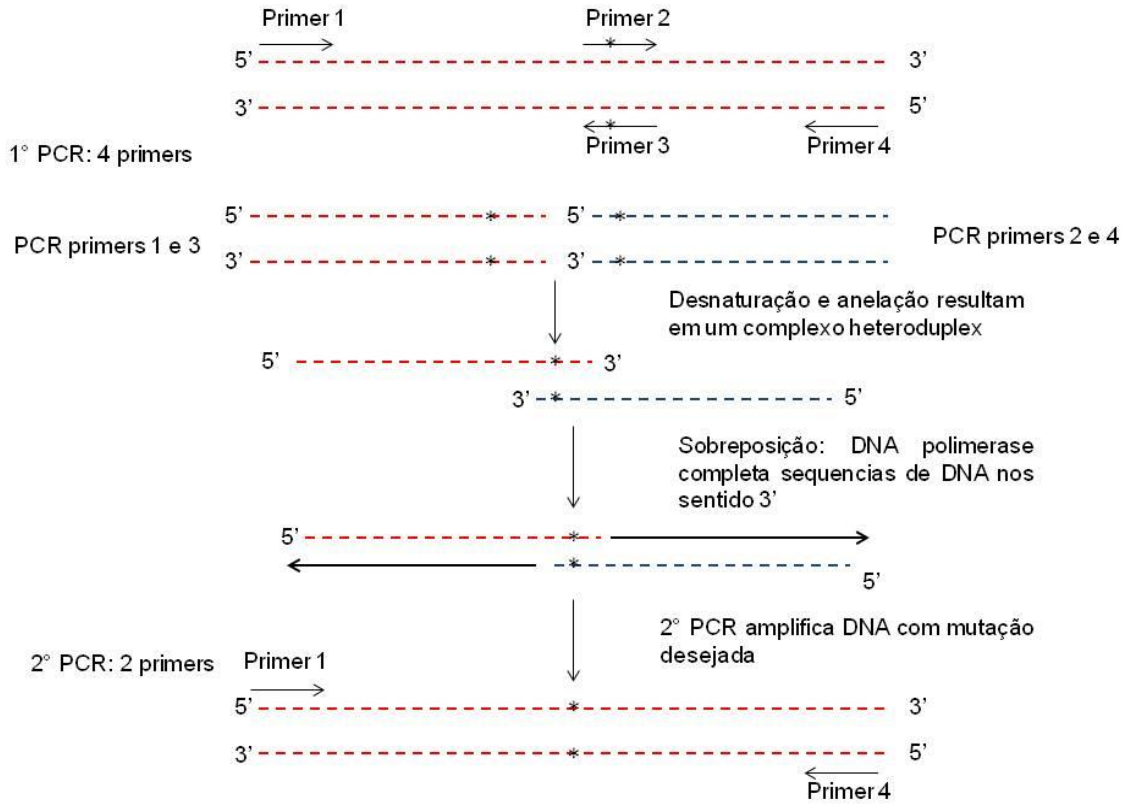

Figura 1. Mutagênese sítio dirigida por PCR. * Local da mutação no gene.

1.8.1.2 Mutagênese sítio dirigida por plasmídeo molde seguido de digestão por DpnI

Nesta técnica, dois *primers* complementares ao DNA molde (no caso um plasmídeo) são sintetizados contendo um códon mutante desejado. Uma PCR é efetuada, gerando novas copia com o códon mutante. A enzima de restrição DpnI é adicionada ao produto da reação e digere a fita molde de DNA, restando somente copias

mutantes do plasmídeo. Este pode então ser utilizado na transformação de células competentes (LABROU, 2010) (Figura 2).

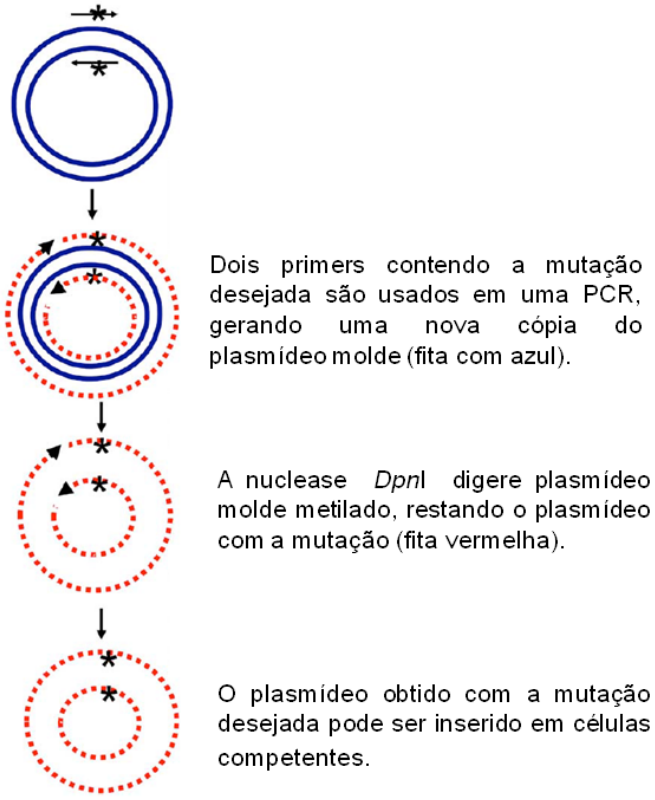

Figura 2. Mutagênese sítio dirigida em plasmídeo molde seguido de digestão por DpnI.

1.8.2 Evolução direcionada

As técnicas de evolução direcionada são ideais quando não se tem conhecimento de informações detalhadas da proteína alvo (VALETTI & GIRALDI, 2004). Nestas técnicas, um grande número de mutantes são gerados e avaliados nas condições ambientais desejadas (EIJSINK *et al*., 2005). As mutações promissoras são selecionadas e se necessário uma nova uma etapa de mutação (round) e avaliação é efetuada. Entre os métodos de evolução direcionada estão o *error prone*–PCR (EP–PCR) e DNA *shuffling* (DNAS), podendo ser combinados entre si (VALETTI & GIRALDI, 2004; EIJSINK *et al*., 2005) ou conjunto com técnicas de mutagênese sítio dirigida (Tabela 3).

Tabela 3. Aumento da termoestabilidade de enzimas utilizando técnicas de mutagênese.

Enzima	Técnicas	N° de clones /rounds	Aumento da termoestabilidade (%) a temperatura de (°C)	Referência Bibliográfica
Xilanase	EP–PCR + DNAS	10644/3	90 (60)	RULLER *et al*. (2008)
Subtilisina	EP–PCR + Mutagênese Sítio Dirigida	864/1	35 (60)	MIYAZAKI & ARNOLD (1999)
Lipase	EP–PCR + DNAS + Mutagênese Sítio Dirigida	7000/4	90 (60)	AHMAD *et al*. (2008)
Amilosacarose	EP–PCR + Mutação Sítio Dirigida	60000/1	100 (50)	EMOND *et al*. (2008)

1.9 Gênero *Bacillus* e *B. subtilis*

As características do gênero são a morfologia de bastonetes, positivos quanto à coloração de Gram, aerobióticos facultativos (podem ser aerobióticos obrigatório), formadores de endósporo e produtores de catalase. *Bacillus* é de grande importância antrópica, visto que está presente em muitas áreas da atividade humana, causando efeitos negativos na indústria de alimentos, como o *Bacillus cereus* responsável por contaminar alimentos e na agricultura, como o *Bacillus anthracis*, responsável pela doença carbúnculo (MADIGAN *et al*., 2010). Sua importância econômica é verificada na produção de bioinseticidas (*Bacillus thuringiensis*) (BEAVOA *et al*., 2011), produção de alimentos fermentados (*B. subtilis*) (PENG *et al*., 2005) e produção de enzimas (*B. subtilis*, *B. licheniformis, Bacillus amyloliquefaciens*) (SCHALLMEY *et al*., 2004; GUPTA *et al*., 2012) e probióticos (*B. cereus, B. subtilis*) (CUTTING, 2011). Dentre todas as espécies do gênero, o *B. subtilis* é estudado intensamente, devido a sua importância econômica e fácil manipulação (PIGGET, 2009).

O *B. subtilis* S14, microrganismo foco da presente tese foi isolado de solo (GAVA, 1999) e caracterizado como produtor de enzimas capazes de degradar a queratina de grande potencial na depilação do couro (GAVA, 1999; MACEDO *et al*., 2005). Além disso, *B. subtilis* S14 produz a KerS14, queratinase que foi purificada e caracterizada como subtilisna (MACEDO *et al*., 2008).

1.10 Queratina

Produzidas pelas células epiteliais de vertebrados superiores, as queratinas tem funções mecânicas e de proteção, (KORNIŁŁOWICZ–KOWALSKA & JUSTYNA, 2011). São as proteínas formadoras de cabelo, chifres, penas, unhas, peles, pelos, calosidades, escamas e apêndices epidérmicos, sendo de difícil degradação pela maioria das peptidases devido a presença de interações hidrofóbicas, pontes de hidrogênio e, fundamentalmente, pontes dissulfeto (VOET & VOET, 1990; BRANDELLI *et al*., 2010). São classificadas como α–queratina, β–queratina (VOET *et al*., 2000) e γ–queratina (KORNIŁŁOWICZ–KOWALSKA & JUSTYNA, 2011).

As α–queratinas são encontradas apenas em mamíferos (NELSON & COX, 2011), como componente majoritário de cabelos, unhas, garras e cascos. Possui uma concentração elevada de resíduos cisteína, que formam pontes dissulfeto, estabilizando a estrutura quartenária da queratina (NELSON & COX, 2011). Esta característica faz com que a α–queratina seja insolúvel e resistente à lise enzimática (KORNIŁŁOWICZ–KOWALSKA & JUSTYNA, 2011), recebendo também a denominação de queratina dura (VOET *et al*., 2000).

As β–queratinas são denominadas devido às folhas β–pragueadas que a formam. Na sua composição há maior quantidade de resíduos dos aminoácidos serina, glicina, prolina e alanina e menor de cisteína, tendo, em comparação com as α–queratinas,

menor quantidade de pontes dissulfeto em sua composição (KORNIŁŁOWICZ–KOWALSKA & JUSTYNA, 2011). Devido a esta característica é considerada queratina "mole" e está presente em penas e escamas (VOET *et al*., 2000).

A γ–queratina é uma proteína não estrutural, de característica amorfa, com conteúdo rico em pontes de dissulfeto, podendo ser encontrada na cutícula capilar, formado por proteínas globulares de massa molecular próximo a 15 kDa (KORNIŁŁOWICZ–KOWALSKA & JUSTYNA, 2011).

1.11 Degradação de queratina

No meio ambiente, a degradação da queratina ocorre pela ação de micro–organismos. Contudo, não foi estabelecido qual modo a degradação ocorre (KORNIŁŁOWICZ–KOWALSKA & JUSTYNA, 2011). A primeira teoria considera que apenas a ação de queratinases esteja envolvida na degradação da queratina. Contudo, preparações de queratinases frequentemente são ineficientes em hidrolisar queratina, supostamente devido à grande quantidade de pontes dissulfeto presentes nas moléculas de queratina (BRANDELLI *et al*., 2010).

Outra teoria propõe um mecanismo em dois estágios, sulfitólise e proteólise. Tal teoria foi proposta ao se estudar a degradação de queratina pelo fungo *Microsporum gypseum*. As pontes de dissulfeto são quebradas pelo sulfeto inorgânico produzido pelo fungo, facilitando a ação subsequente das queratinases. Alternativamente, alguns autores propõem que a sulfitólise e a proteólise ocorrem concomitantemente (KORNIŁŁOWICZ–KOWALSKA & JUSTYNA, 2011).

Em bactérias também é proposto um mecanismo de degradação da queratina em dois estágios. Foi observado que durante a decomposição da queratina, ocorre a redução de pontes dissulfeto, com a liberação de peptídeos com cisteína (KORNIŁŁOWICZ–

KOWALSKA & JUSTYNA, 2011). O comprovado aumento no número de grupos tióis livres durante o crescimento bacteriano contribuiu para validar esta hipótese (BRANDELLI *et al*., 2010). Em *Stenotrophomonas* foi comprovada a ocorrência de uma enzima similar a dissulfeto redutase, capaz de reduzir as pontes dissulfeto na queratina, removendo as ligações covalentes entre os resíduos de cisteína seguido da ação de uma protease não queratinolitica (KORNIŁŁOWICZ–KOWALSKA & JUSTYNA, 2011).

A participação das dissulfeto reductases na degradação da queratina tem sido mais explorada nos últimos anos. RAHAYU *et al.* (2012) purificou três dissulfeto reductases produzidas pela linhagem *Bacillus* MRS. Além disso, foi descrita uma queratinase com atividade peptidásica e de dissulfeto reductase produzida por *Bacillus halodurans* (PRAKASH *et al*., 2010).

2. OBJETIVO GERAL

Aumentar a produção de queratinases pelo *B. subtilis* S14 e obter uma variante com maior termoestabilidade do que a enzima nativa.

2.1 Objetivos específicos

– Estabelecer condições otimizadas de cultivo submerso para a produção de queratinase pelo *B. subtilis S14*;

– Caracterizar o sobrenadante da cultura otimizada;

– Clonar a ORF que codifica a KerS14 em vetor de expressão;

– Realizar mutações na ORF previamente clonada;

– Obter as enzimas queratinolíticas mutantes recombinantes;

– Avaliar a termoestabilidade das enzimas obtidas.

3. RESULTADOS

Os resultados obtidos neste trabalho serão apresentados na forma de dois subcapítulos. O subcapítulo 3.1 trata do manuscrito sobre a determinação de um meio de cultura otimizado para a produção de queratinases e escalonamento para bioreator. O subcapítulo 3.2 consta a clonagem da ORF que codifica a KerS14 em vetor de expressão, a realização de mutações na mesma ORF visando o aumento da termoestabilidade e a expressão e caracterização das enzimas recombinantes obtidas.

ARTIGO I

Thermostable keratinases production by *Bacillus subtilis* S14

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Abstract

Bacillus subtilis S14 is a producer of keratinases with potential application in the leather industry, because it produces KerS14, a keratinase that does not damage bovine collagen. This work presents the establishment of a medium composed by feather meal and $CaCl_2$ to produce thermostable keratinases by *B. subtilis* S14. The supernatant obtained from the optimized medium, named supKBs14, was characterized. supKBs14 remains thermostable at 50 °C for 216 hours and has optimum temperature of 50 °C. Moreover, supKBs14 hydrolyzes horns, retaining its enzymatic activity after the process. In addition, the scale–up to 5L bioreactor was performed. Maximum keratinase production was obtained with aeration rate of 1 vvm, agitation of 400 rpm and oxygen mass transfer rate of 24.48 h^{-1}. These results reveal that it is possible to obtain a simple medium to produce keratinases on a bioreactor scale in short fermentation times, with potential application in biotechnological processes that require thermostability at 50 ºC for a long period, as well as in horn degradation.

Keywords: Keratinase production, Feather meal, *Bacillus subtilis* S14, Thermal stability, Horn degradation

1. Introduction

Industrial enzymes accounted for a global market estimated at 5.1 billion dollars in 2009 (*1*). Enzymes of this type have several industrial applications, including food (45%), detergent (34%), textiles (11%), leather (3%), pulp and paper (1.2%) industries (*2*). Keratinases are included among these enzymes. They are proteases able to degrade keratin, the main constituent present in epidermis and its appendices, such as hair, feathers and horn. Keratinous wastes, like chicken feather and horn pile up in the environment, raising environmental concerns that drive the development of new biotechnological recycling approaches (*3*). Keratinases have potential in keratinous waste processing and in the food supplement industry, among other applications such as detergent additive and in leather industry (*4*).

A diversity of microorganisms is able to produce keratinases, like fungi and bacteria (*4*). Species of *Bacillus*, such as *Bacillus pumilis* (5), *Bacillus licheniformis* (6) and *Bacillus subtilis* are keratinase producers (7).

Considering that microbial enzyme production is highly dependent on medium composition, the selection of a suitable medium is a critical step to achieve a preparation with high enzyme activity. The Response and Surface Methodology (RSM) is a useful approach to determine medium composition for enzyme production (8), and is a suitable method to design optimal biotechnological processes (9). RSM has been used to optimize culture media for the production of a variety of enzymes, like amylases, lipases and proteases (9).

Several studies have investigated keratinase production in submerged fermentation on a flask scale (*10,11,12,13,14,15,16*), but few have addressed the process on a bioreactor scale (*17,18*). Bioreactor studies are necessary to scale–up

enzyme production. An important parameter to determine in scale–up studies is the oxygen mass transfer coefficient ($K_L a$). It is useful to estimate an adequate oxygen supply, critical to microorganism growth and enzyme production (*19*).

In a previous work, it was shown that *B. subtilis* S14 produces KerS14, a keratinase that has the singular ability to hydrolyze keratin without damaging collagen. Clearly, this property pointed this enzyme as being potentially useful in the leather industry (*7, 20*). To shed more light on a method to obtain suitable amounts of this enzyme, high enough to reliably test its industrial applications, the present work aims (i) to select a suitable culture medium for *B. subtilis* S14 keratinase production, (ii) to characterize the keratinolytic activity obtained, (iii) to evaluate a possible application of this keratinase in bovine horn degradation and (iv) select conditions to produce keratinases on a bioreactor scale.

2. Material and Methods

2.1 Microorganism: maintenance and culture preparation

Bacillus subtilis S14, was previously isolated (*7*) and maintained at 4 °C on Luria–Bertani medium (LB) (1% peptone, 0.5% yeast extract, 1% NaCl) agar plates. The microorganism was pre–inoculated with a loopful in 50 mL LB medium and cultivated for 9 h at 180 rpm, 37 °C. The pre–inoculum optical density (OD) at 600 nm was adjusted to 0.5. A standard inoculum used in all experiments was prepared adding 0.25 mL of pre–inoculum cell suspension to 50 mL of liquid culture media.

2.2 Selection of culture media for keratinase production in Erlenmeyer flasks

B. subtilis S14 was cultivated under 23 different conditions (Table 1). Feather meal, horn and bovine hair were used as keratin sources. In some experiments glucose,

lactose and starch were used as carbon source. As the medium feather meal without carbohydrates was the appropriate to keratinase production, other media components were tested. Inorganic nutrients ($CaCl_2$, $MgSO_4$, K_2HPO_4, NaCl and $ZnSO_4$) were utilized as additives to feather meal media. Unless specified otherwise, all cultures were grown at 180 rpm, 37 °C for 24 h, in 250 mL Erlenmeyer flasks filled with 50 mL of medium, adjusted in pH 8.0. Moreover, keratinase production was evaluated at different pH values (4.0 and 6.5) adjusted with 0.1 M HCl or NaOH, in a 15 g/L feather meal media. Buffered feather meal media using sodium phosphate, pH 8.0, was also tested. The feather meal medium at 15 g/L, pH 8.0 was defined as 100%, in order to facilitate the comparison with the results obtained in a previous work about *B. subtilis* S14 (*11*).

2.3 Experimental design and statistical analysis

Once one medium (feather meal 15 g/L + $CaCl_2$ 5 g/L) was selected, it was optimized using RSM in a 2x2 plus star configuration factorial central composite design (CCD). The independent variables, feather meal and $CaCl_2$, were tested at five levels (Table 2), with four–star points and five replicates at the central point. All media had their pH adjusted to 6.5, before inoculum addition.

A second order polynomial equation (Eq. 1) was fitted to the data by a multiple regression procedure, where *Y* is the predicted response, b_0 is a constant; X_1 is feather meal; X_2 is $CaCl_2$; b_1 and b_2 are linear coefficients; b_{11} and b_{22} are quadratic coefficients; and b_{12} is the interaction coefficient.

Eq 1. $Y = b_0 + b(X_1) + b_{11}(X_1)^2 + b_2(X_2) + b_{22}(X_2)^2 + b_{12}(X_1X_2)$ (1)

Keratinase production data were analyzed by analysis of variance (ANOVA) to estimate *t* values and *P* values, using Statistica 7.0 software (Statsoft, Tulsa, OK, USA).

The variance explained by the model is given by the multiple coefficient of determination (r^2).

2.4 Keratinolytic activity

Keratinolytic activity was measured using azokeratin as substrate, prepared according to Tomarelli (*21*). Assays were conducted for 30 min at 50 °C, incubating the sample (100 µL) with 500 µL of 1.5% (*w/v*) azokeratin suspension in 50 mM phosphate buffer, pH 8.0. Reactions were stopped adding 500 µL of a 30 % (*v/v*) trichloroacetic acid solution. Controls were prepared adding the trichloroacetic acid solution before the sample. Tubes were centrifuged (10000 rpm, 10 min) and activity was calculated by the increase in absorbance at 440 nm. One unit of enzyme activity was defined as the amount of enzyme that increases solution absorbance by 0.1 per hour under the conditions described above.

2.5 Characterization of the keratinolytic activity in *B. subtilis* S14 culture supernatant

The culture grown in 15 g/L feather meal 10.5 g/L $CaCl_2$ medium, was centrifuged (10000 rpm for 10 min), and the cell free *B. subtilis* S14 keratinolytic supernatant, named supKBs14, was used as enzymatic sample in our study.

The effect of pH upon supKBs14 activity was tested in the 3.5–10.5 range. Buffers used were 50 mM citrate phosphate (pH 3.5–7.0), 50 mM Tris HCl (pH 7.0–9.0), 50 mM sodium phosphate (pH 8.0) and 50 mM carbonate–sodium carbonate (pH 9.5–10.5).

To determine the effect of the temperature on supKBs14 activity, tests were conducted at 22, 30, 37, 45, 50, 65 and 75 °C.

Thermal stability was also tested, incubating supKBs14 for 216 h at 4, 25, 37 and 50 °C. After incubation, supKBs14 activity was assayed as described in item 2.4. Activity at 0 h was taken as 100%.

The effect of surfactants SDS, Tween 80, Tween 20 and Triton X–100 was also evaluated. supKBs14 was pre–incubated with inhibitors or other substances at 50 °C for 30 min before residual keratinolytic activity was measured. Activity in the absence of inhibitors was used as control and taken as 100% of activity.

supKBs14 inhibition profile was analyzed in keratinolytic assays following the protocol described at item 2.4, in the presence of different inhibitors: EDTA, EGTA, β–mercaptoethanol PMSF, E–64, TLCK, TPCK, pepstatin and chymostatin, using concentrations recommended by Beyno and Bond (*22*).

The effect of metal ions on keratinolytic activity was tested at two concentrations (1 mM and 10 mM). The ions used were Na^{+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, Hg^{2+}, Cu^{2+} Co^{2+} and Fe^{2+}. supKBs14 was incubated with ions at 50 °C for 30 min immediately before keratinolytic activity was measured. The keratinolytic activity in absence of metallic ions was considered as 100% of activity.

2.6 Bovine horn degradation assay

Pieces of bovine horn (0.22 g) were autoclaved (121 °C, 1 atm, 30 min) in glass tubes and incubated with supKBs14. To test the effect of $CaCl_2$ on supKBs14 activity, three treatments were tested: (i) 15 mL of supKBs14; (ii) 15 mL of supKBs14 with 1% of $CaCl_2$ and (iii) 15 mL of with 2% of $CaCl_2$. As control, an autoclaved aliquot of supKBs14 was used (121 °C, 1 atm, 30 min). Bovine horn keratin–degradation assays were done at 50 °C and 140 rpm, for 216 h. Samples (0.8 mL) were collected at different times during the assay and then analyzed for keratinolytic activity. Horn pieces

were weighted before and at the end of the assay to calculate degradation. All the experiments were performed in triplicate.

2.7 Fermentation in bioreactor

Fermentations were performed in a 5 L stirred–tank bioreactor (Biostat B model B) filled with 4 L of optimized medium, feather meal 15 and $CaCl_2$ 10.5 g/L. Inocula were performed by addition of 40 mL of a 12-hours *B. subtilis* S14 culture in LB. Temperature was maintained at 37 °C and aeration was maintained at 1 vvm. The agitation rate was evaluated at three different rates: 200, 400 and 600 rpm. All fermentations lasted 24 hours. Samples (10 mL) were taken at a two–hour interval and centrifuged at 10000 rpm for 30 min in order to obtain a clear supernatant. The supernatant was analyzed for keratinolytic activity as described in 2.4.

2.7.1 Oxygen mass transfer coefficient ($K_L a$) determination

Dissolved oxygen concentration data in the fermentation medium was measured using a polarographic electrode. The $K_L a$ was determined as described by (*19*).

3. Results and discussion

3.1 Selecting a culture media for keratinase production in Erlenmeyer flasks

Compared to bovine horn and hair, feather meal is the best keratin source concerning keratinase production by *B. subtillis* S14 (Table 1). This agrees with other studies which showed that *Bacillus spp.* produces higher keratinolytic activity when feather meal is the sole carbon and nitrogen source, when compared with any other keratinaceous material (*5, 10, 11*).

Addition of carbohydrates such as glucose, lactose and starch decreased keratinase production by *B. subtilis* S14 (Table 1), suggesting that when it has a carbohydrate as a

carbon source this organism does not mobilize its metabolism to obtain carbon from keratin. The same is true for protease production by *Bacillus* sp. (*11*), *B. horikoshii* (*12*), and *B. cereus* (*13*). On the other hand, sucrose or corn flour *B. subtilis* strain KD–N2 (*14*) and *B. pumilis* (*5*) keratinolytic activity, indicating that this carbon supply is enough and bacteria increase keratin degradation in order to obtain more nitrogen (*11*,*12*,*13*).

Keratinase production by some microorganisms depends on the presence of some salts (*4*). In this sense, $CaCl_2$ increased *B. subtilis* S14 keratinase production, contrarily to $MgSO_4$, K_2HPO_4, NaCl and $ZnSO_4$, which decreased keratinases production (Table 1). So, an appropriate combination of feather meal and $CaCl_2$ is critical to achieve a high level of keratinase production by *B. subtilis* S14 and should be considered in order to optimize keratinase production.

Keratinase production is higher when the initial feather meal medium pH is 6.5 (Table 1). *B. subtilis* S14 growing at pH 8.0 in a buffered feather meal medium produces lower keratinase amounts, when compared to a setting of identical media, but non–buffered (Table 1).

Table 1. Keratinase production by *B. subtilis* S14 in different culture media.

Culture media composition*	Keratinolytic Activity ($U.mL^{-1}.h^{-1}$)	Relative Activity (%)
FM (15) pH 8.0	7.85	100
FM (15) pH 6.5	8.35	106
FM (15) pH 4.0	0	0
FM (15) + $CaCl_2$ (5)	9.22	117
FM (15) + $MgSO_4$ (5)	1.88	24
FM (15) + K_2HPO_4 (5)	6.54	87
FM (15) + NaCl (5)	7.73	98
FM (15) + $ZnSO_4$ (5)	0	0
FM (15) Buffer	6.63	84
FM (15) Buffer + Starch (5)	4.91	62
FM (15) Buffer + Glucose (5)	4.12	52
FM (15) Buffer + Lactose (5)	3.37	43
FM (15) + Starch (5)	4.79	61
FM (15) + Glucose (5)	0.6	7
FM (15) + Lactose (5)	7.03	89
H (15)	0	0
H (15) + Starch (5)	1.47	22
H (15) + Glucose (5)	2.42	31
H (15) + Lactose (5)	0.32	4
BH (15)	0	0
BH (15) + Starch (5)	0	0
BH (15) + Glucose (5)	0.81	10
BH (15) + Lactose (5)	0	0

* (FM) Feather Meal; (H) Horn; (BH), Bovine Hair. Numbers is parenthesis refers to concentrations in g/L

3.2 Optimizing culture medium for keratinase production

Tuning optimal conditions for keratinase production as a function of feather meal and $CaCl_2$ (independent variables) was done using CCD with RSM. Table 2 shows the keratinolytic activity achieved in each experimental condition. The effect of feather meal and $CaCl_2$ upon keratinase production is shown in Fig. 1. Maximum activity was observed in the central point, with feather meal and $CaCl_2$ initial concentrations of 15 and 10.5 g/L, respectively.

Table 2. Coded values and concentration (in parenthesis) of independent variables for a full factorial design and enzymatic activity ($U.mL^{-1}.h^{-1}$).

Run	Feather meal (X_1) (g/L)	$CaCl_2$ (X_2) (g/L)	Keratinolytic activity ($U.mL^{-1}.h^{-1}$)
1	–1 (6.5)	–1 (3.8)	5.0
2	1 (23.5)	–1 (3.8)	8.2
3	1 (6.5)	1 (17.2)	6.1
4	1 (23.5)	1 (17.2)	5.8
5	0 (15)	0 (10.5)	11.2
6	0 (15)	0 (10.5)	11.3
7	0 (15)	0 (10.5)	11.1
8	0 (15)	0 (10.5)	10.7
9	0 (15)	–1.41 (1)	3.6
10	0 (15)	1.41 (20)	11.5
11	–1.41 (3)	0 (10.5)	3.4
12	1.41 (27)	0 (10.5)	6.2

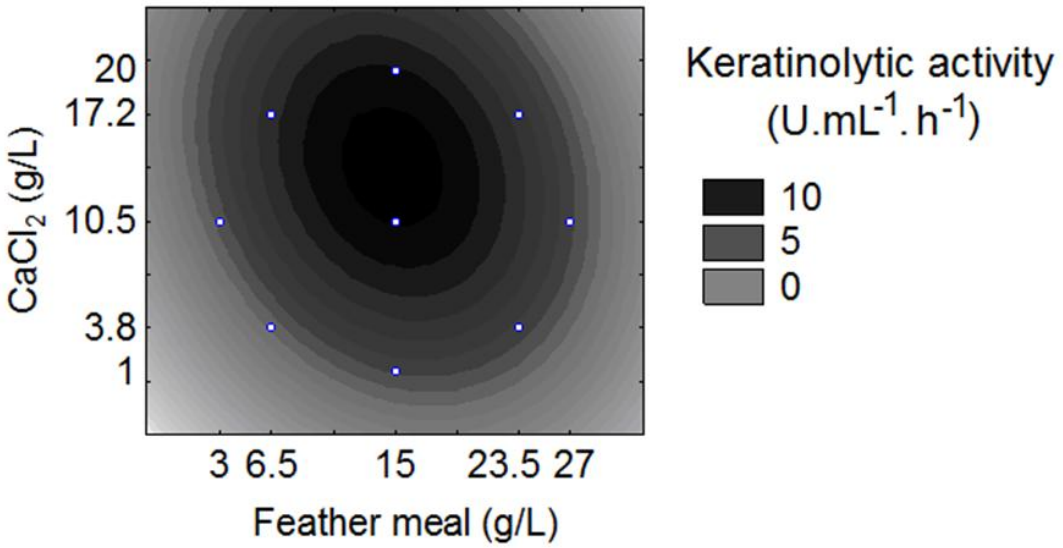

Fig 1. Response surface methodology (RSM) plot for the combinatory effects of feather meal (X_1) and $CaCl_2$ (X_2) upon keratinase production by *B. subtilis* S14.

This effect of feather meal upon keratinase production by *B. subtilis* S14 (Table 3) is not ubiquitous among *Bacillus* spp., because it does not affect keratinase production by *B. pumilus* A1 (*10*). Addition of $CaCl_2$ increases keratinase production by *B. subtilis* S14 similarly to what is observed with metallopeptidase production by *B. cereus* (*15*). At the end, the optimized medium leads to a keratinase production increase of 41% (11.1 $U.mL^{-1}.h^{-1}$), relative to the starting condition (feather meal 15 g/L at pH 8.0) (Table 1).

Table 3. Effects of feather meal and $CaCl_2$ upon keratinase production by *B. subtilis* S14.

Factor	Effect	Standard Error	*t*–value	*p*–value
Mean	11.1	0.12	91.31	<0.0000[a]
Feather meal concentration (L)	0.66	0.17	3.87	0.0304[a]
Feather meal concentration (Q)	–6.22	0.20	–32.24	<0.0000[a]
$CaCl_2$ concentration (L)	3.54	0.17	20.54	0.0002[a]
$CaCl_2$ concentration (Q)	–3.48	0.19	–18.03	0.0003[a]
1Lx2L	–1.78	0.24	–7.34	0.0052[a]

[a] = significant factors $p< 0.05$

Results from the statistical analysis are given in Table 3. The independent variables (feather meal and $CaCl_2$) are significant for a confidence level of 95% at linear and quadratic levels. Considering the linear level, feather meal and $CaCl_2$ affect keratin production positively (0.66 and 3.54, respectively). However, considering the quadratic level these effects are negative for both independent variables (–6.22 and –3.48, respectively) (Table 3), demonstrating that a large amount of feather meal and $CaCl_2$ are not necessary conditions to obtain a high keratinolytic activity.

The ANOVA analysis afforded to establish a second–order polynomial function (Eq. (2)) which describes enzyme production as a function of the independent variables (Table 4).

$$Y=11.1+(0.33).(X_1)–(3.11).(X_1)^2+(1.77).(X_2)–(1.74).(X_2)^2 +0.9(X_1).(X_2) \quad (2)$$

Where Y is the keratinolytic activity; X_1 and X_2 are feather meal and $CaCl_2$ concentrations, respectively.

The coefficient of determination (R^2) for the model is 0.88. This indicates that the model explains 88% of the response variability. Calculated *F* is 9.34, while the *F*

tabulated is 4.39 (Table 4), at the confidence level of 95%. Thus, the model is predictive, since real the *F* value is higher than the *F* tabulated value.

Table 4. Analysis of variance (ANOVA) of central composite design (CCD).

Source of variations	Sum Squares	Degree Freedoms	Mean Squares	F value Cal	F value Tab
Regression	99.00	3	19.80	9.34	4.39
Residual	12.72	9	2.12		
Lack of Fit	12.54	3	4.18		
Pure Error	0.1176	3	0.059		
Total SS	111.7	11			

*F–Value tabulated 95%, R^2=0.88. F–ratio=2.12

3.3 Characterization of the supernatant from *B. subtilis* S14 growth in the optimized medium

The keratinolytic activity in the supernatant of a *B. subtilis* S14 culture produced in the optimized medium (named supKBs14) was observed in the pH range 5.5–10.5, with maximum activity at pH 8.0 (Fig. 2). This activity in a wide pH range seems to be useful for industrial processes, because it abolished the need for a pH control system. Additionally, activity in alkaline pHs is critical for some biotechnological purposes like detergent formulation. Some keratinases produced by other *Bacillus* have similar properties (*15, 16*). The supKBs14 potential as additive in detergent formulation is underscored, because it maintains most of its protease activity even in the presence of surfactants (Table 5). In the presence of SDS 1%, Tween 80 1% and Triton X–100 1% supKBs14 activity is conserved by more than 50%. Moreover, supKBs14 is totally active in the presence of Tween 20 1% (Table 5).

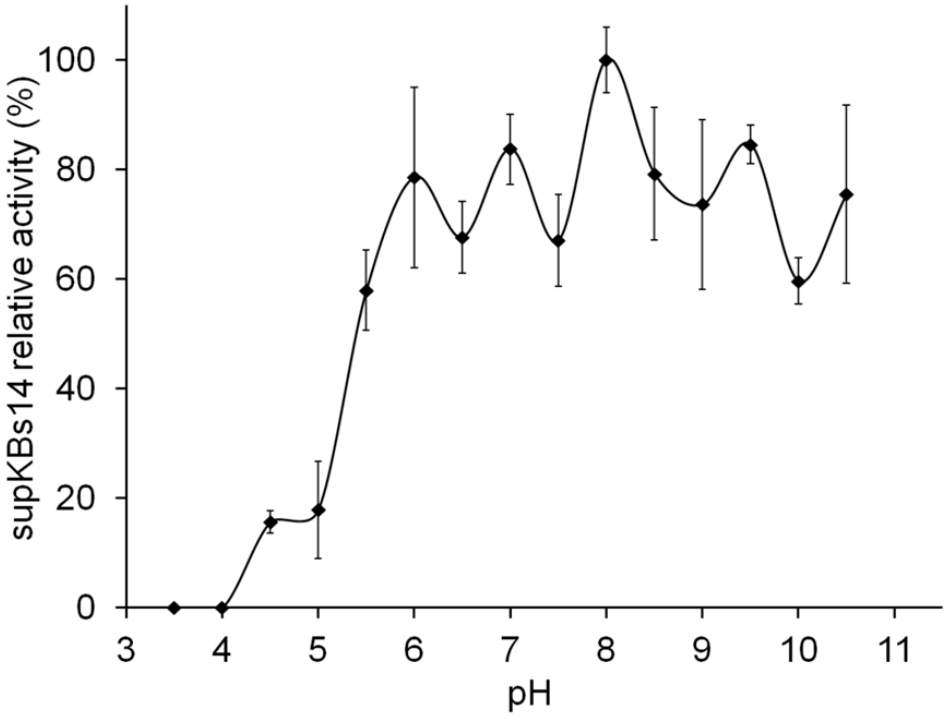

Fig. 2. Effect of pH on supKBs14 keratinolytic activity. Enzymatic activity was assayed at 50 ºC at indicated pHs.

Table 5. Effect of protease inhibitors, chelators, surfactants and reducing agents upon supKBs14 activity.

Treatment	Concentration	Residual activity (%)
Control	–	100
PMSF	0.2 mM	36
E–64	10 µM	84
TLCK	50 µM	93
TPCK	50 µM	88
Pepstatin	1 µM	72
Chymiostatin	50 µm	64
EDTA	1 mM	101
EDTA	10 mM	83
EGTA	1 mM	112
EGTA	10 mM	59
SDS	0.1%	60
SDS	1%	69
β-Mercaptoethanol	1%	74
β–Mercaptoethanol	10%	48
Tween 80	0.1%	83
Tween 80	1%	62
Tween 20	0.1%	96
Tween 20	1%	101
Triton X–100	0.1%	72
Triton X–100	1%	58

Similarly to other kerantinases, supKBs14 optimum temperature is 50 °C (Fig. 3) (*6,16,23*), an optimum temperature suitable for the application of the enzyme as a detergent additive.

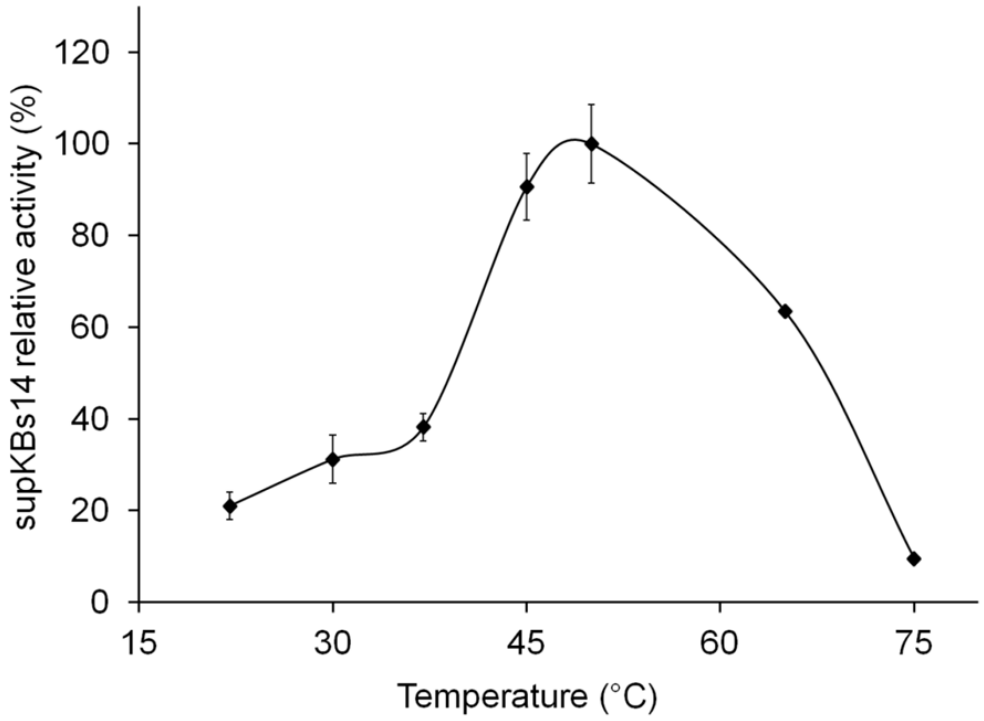

Fig. 3. Effect of temperature on supKBs14.

supKBs14 thermal stability was investigated at different temperatures (4°C, 25°C, 37°C and 50 °C). At 4 °C and 25 ºC supKBs14 activity is stable for more than 200 hours (Fig. 4), which is a relevant property concerning shelf life. At 37 °C, supKBs14 activity is reduced by 25% after 12 h, remaining at this level for at least 216 hours (Fig. 4). At 50 °C, the enzymatic activity was reduced to 58% after 12 h, remaining at this value for 216 h (Fig. 4). These results suggest that *B. subtilis* S14 produces a number of keratinolytic enzymes, one of which is thermostable for over 12 h. This is a differential relatively to other *Bacillus* isolates, which produce keratinases

displaying a thermal stability lower than supKBs14 (*24*). supKBs14 properties indicate that it may be useful for long–time industrial processes at 50 ºC.

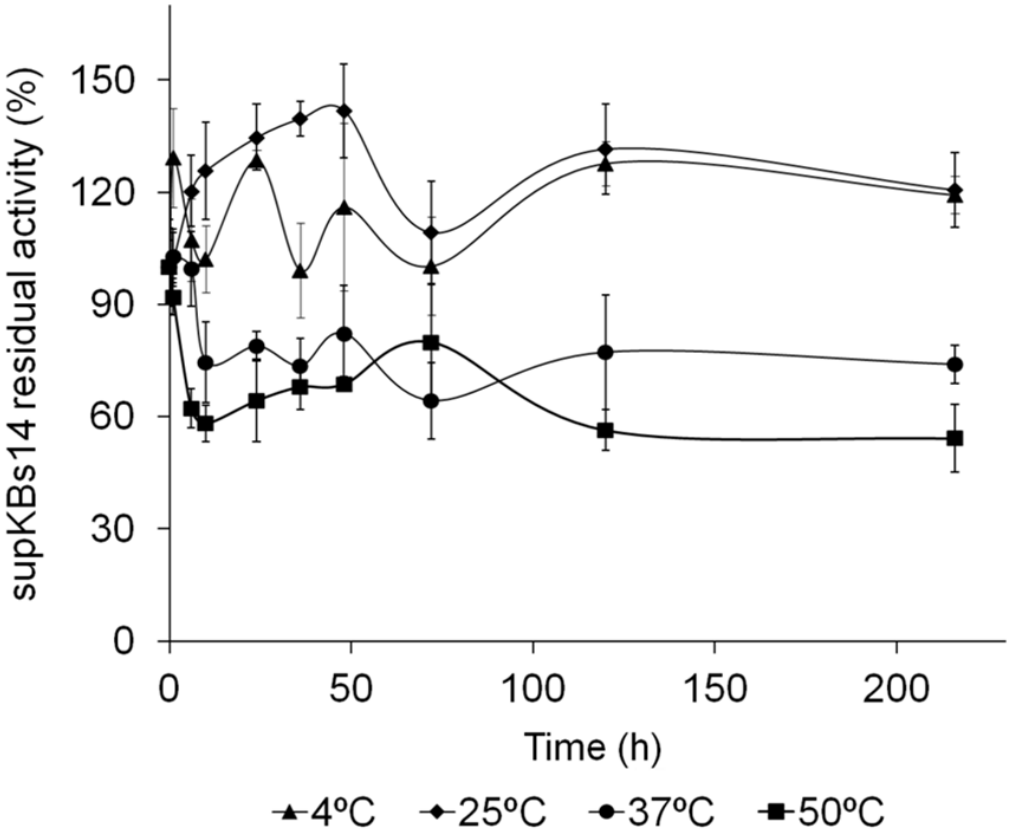

Fig. 4 Thermostability on supKBs14 activity.

PMSF, a serine–protease inhibitor, reduced supKBs14 activity by 64%. Also, other peptidase inhibitors tested (E–64, TLCK, TPCK,) inhibit supKBs14 activity, although at a lower level when compared to PMSF (Table 5). PMSF inhibits several keratinases (*10,16,24*). PMSF, E–64, TLCK and chymostatin inhibit pure KerS14, but pepstatin and TPCK had no effect on it (*20*), suggesting again that supKBs14 has more than one keratinase.

Most of the metal ions investigated (Hg^{2+}, Cu^{2+}, Co^{2+}, Mg^{2+}, Zn^{2+}) inhibit supKBs14 activity upon azokeratin (Table 6). Contrarily, Ca^{2+}, Na^{+} and Fe^{2+} improved keratinolytic activity. Similarly, other *Bacillus* proteases are also activated by Ca^{2+}

(*20*,*25*). Diversely, Na^{+} activates some keratinases (*26*). Hg^{2+} inhibits supKBs14 activity only at 10 mM, indicating that it is not a cysteine–endopepdidase, as the previously characterized KerS14 (*20*) and one *B. pumilus* keratinase (25). Although in the present study Co^{2+} reduced supKBs14 activity (Table 6), it increases remarkably the activity of the purified KerS14 (*20*). These results support the conclusion that other enzymes account for more total *B. subtilis* S14 total keratinase activity than the previously characterized KerS14 (*20*).

Table 6. Effect of salts upon supKBs14 activity.

Salts	Concentration (mM)	Residual activity (%)
Control	–	100
NaCl	1	127
NaCl	10	87
$CaCl_2$	1	115
$CaCl_2$	10	92
$HgCl_2$	1	103
$HgCl_2$	10	35
$CuCl_2$	1	67
$CuCl_2$	10	63
$CoCl_2$	1	45
$CoCl_2$	10	35
$MgCl_2$	1	69
$MgCl_2$	10	53
$ZnSO_4$	1	95
$ZnSO_4$	10	53
Fe_2SO_4	1	110
Fe_2SO_4	10	87

3.5 Horn degradation

Although horn does not induce keratinase production by *B. subtilis* S14 (Table 1), supKBs14 is able to degrade horn (Table 7). After 216 h at 50 ºC under agitation, horn is degraded by 33%. Contrarily, horn degradation by supKBs14 is not affected by Ca^{2+}, similarly to the activity on azokeratin.

Table 7. Residual horn dry weight after hydrolysis by supKBs14.

Treatment	Residual dry horn weight (%)
Control inactive supKBs14*	$94.6^{a} \pm 2.5$
supKBs14	$66.8^{b} \pm 2.6$
supKBs14 + $CaCl_2$ 1%	$76.1^{c} \pm 5.2$
supKBs14 + $CaCl_2$ 2%	$78.1^{c} \pm 3.7$

Means ±standard error with different letters are significantly different according the Tukey test at $p = 0.05$.

*supKBs14 autoclaved

3.6 supKBs14 production in bioreactor

In all fermentations performed in this work, keratinolytic activity increased during the first 10 hours and then tended to stabilize (Fig. 5). On the other hand, in other *Bacillus* species, protease production stabilizes after 20 hours of fermentation (*27*,*28*). Concerning agitation, keratinase production by *B. subtilis S14* is higher at an aeration rate of 400 rpm (Fig. 5), as observed for *B. licheniformis* NCIM–2042 (*29*) and *B. licheniformis* UV–9, at 500 rpm (*30*). Fermentations carried out at lower agitation are advantageous, because energy consumption is a significant fraction of the total operational costs (*29*).

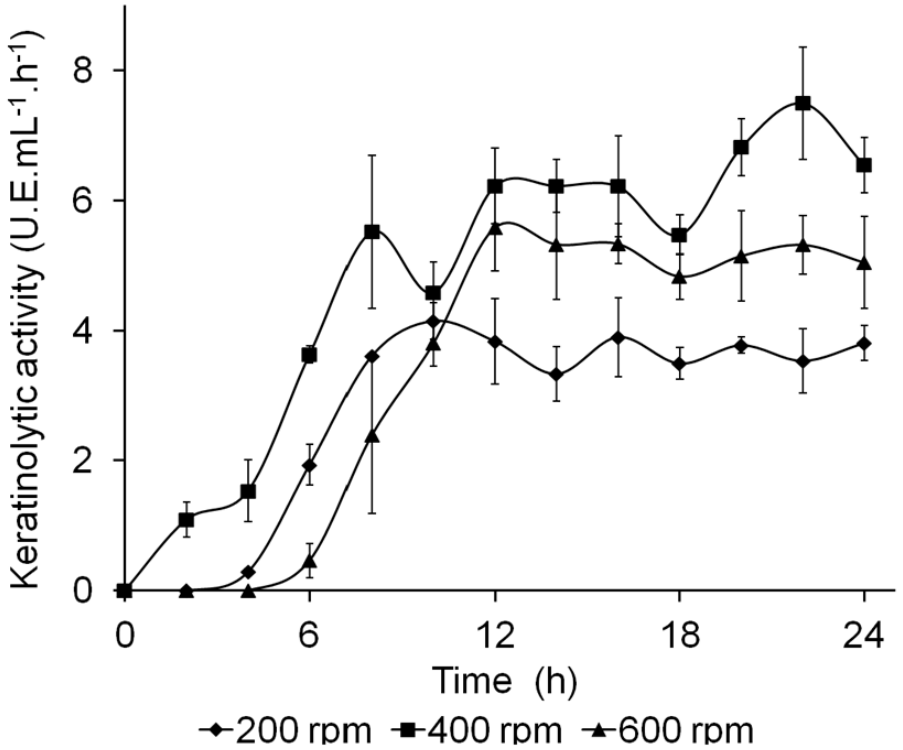

Fig. 5. Keratinase production in bioreactor fermentation.

K_La values at 200 rpm and 600 rpm are 15.48 h^{-1} and179.64 h^{-1}, respectively. At the best fermenting condition for keratinase production (400 rpm), K_La value is 24.48 h^{-1}. This K_La is lower than the values observed for protease production by *B. licheniformis* NCIM–2042 (62 h^{-1}) (*29*) and *B. licheniformis* UV–9 (72 h^{-1}) (*30*). These fermentations were performed with a media composed only by soluble components (*29, 30*), while in the present work feather meal, an insoluble material, was present, which suggests that oxygen transfer rate in a feather meal medium is less efficient than in a totally soluble medium.

Despite some reports of keratinase production in bioreactor (*17,18*), this is the first time that keratinase production in a feather meal medium is investigated in a fermentation scale–up work.

4. Conclusion

This work established a simple culture medium able to produce keratinolytic activity by a *Bacillus* strain on a bioreactor scale with a desirable thermostable activity in only 24 hours of fermentation. The remarkable supKBs14 stability for more than 216 hours at 37 and 50 ºC suggests that the enzymes produced are potentially useful for applications in processes requiring long incubation periods. supKBs14 activity at 8–10.5 pH range, and the fact that it keeps more than 50% of its activity in the presence of ionic and non–ionic surfactants are remarkable characteristics which indicate its use as a detergent additive. As supKBs14 properties (culture supernatant) differ from those presented by KerS14 (a protease purified from the same culture supernatant), it is clear *B. subtilis* S14 produces other keratinases in addition to KerS14 (*7*,*20*).

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ARTIGO II

A recombinant subtilisin with keratinolytic and fibrin(nogen)olytic activity

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Abstract

KerS14 is a keratinase with great potential in tannery, since it degrades keratin without damaging collagen. The enzyme was previously characterized and described as bellowing to the subtilisin group. Although KerS14 has this biotechnological potential, its thermal stability is low. In order to improve this characteristic, in this present work the KerS14 ORF was cloned and expressed into pET–5a vector with a His–tag at C–terminal, and some ORF nucleotides were mutated in order to have mutants G61C, S98C, P239R and G61C_S98C. These mutations were selected because they are related to improving thermal stability of another subtilisin. The four mutated and the non–mutated enzymes were expressed, purified and characterized regarding their thermal stability, optimum temperature and pH. The presence of the His–tag showed to increase the non-muted enzyme thermal stability and to decrease the thermal stability of the muted enzymes. In addition, rKerS14 demonstrated a remarkable fibrin(nogen)olytic activity. This indicates that rKerS14 has potential in tannery and in treatment of cardiovascular diseases also.

Keywords: *Bacillus subtilis* S14, rKerS14, keratinase, fibrin(nogen)olytic activity, His–tag

1. Introduction

The subtilisins are serine proteases with applications in laundry, detergent and pharmaceutical [1]. Several subtilisins are available in the marked, as Savinase^{TM} and Esperase^{TM}, and they are used as additives in detergent preparations [2]. Additionally, some subtilisins have great biotechnological potential in tannery because their keratinolytic activity [3] or fibronolytic activity [4]. Also, other subtilisins have potential as drugs in the treatment of cardiovascular disease [4]. Subtilisins amounts for a representative part of industrial enzymes, a total global market estimated in US$ 7 billion in 2013 [5].

Bacillus subtilis S14 produces KerS14, a keratinase classified as subtilisin. This enzyme was well characterized, and has a unique feature: it hydrolyzes keratin and does not act upon collagen. So, it has a great potential for use in tannery replacing sulfides as dehairing agent [3,6]. Despite this potential, its thermal stability does not fit the requirements for application in processes requiring temperatures around 50 °C.

A strategy to improve thermal stability in enzymes is site–directed mutation. This methodology was applied successfully to improve different features in several enzymes, as oxidative stability in amylase [7], increasing substrate affinity in β–glucosidase [8] and increasing thermal stability in lipases [9] and subtilisins [10,11]. One of these techniques site directed mutagenesis techniques is double stranded DNA template and selection of mutants with the restriction enzyme DpnI. In this technique, two oligonucleotides primers contain a mutant codon with a mismatch sequence are used in a PCR to amplify a plasmid template. The plasmid amplified carries the desired mutation and the template plasmid is eliminated using DpnI digestion since it is derived from *Escherichia coli* and it is methylated [12].

In order to have a KerS14 with a stronger thermal stability, the open reading frame (ORF) was cloned in an expression vector and mutated in some amino acid residues. The recombinant KerS14, named rKerS14 and four mutants were expressed in an active form. The activities of the recombinants enzymes were characterized concerning thermal stability, optimum temperature and optimum pH. Also, rKerS14 fibrinolytic and fibrinogenolytic activities make it as a potentially useful to treat cardiovascular disease.

2. Materials and Methods

2.1 KerS14 ORF amplification

Bacillus subtilis S14 was maintained as described by [3]. To extract bacteria DNA, *B. subtilis* S14 was growth in Luria–Bertani (LB) medium and genetic material was extracted by alkaline lysis. The primers AACATAGAGAAGCAAAATGTGG (forward) and AACTCGAGTTGTGCAGCTTGTAC (reverse) were design based on KerS14 amino acid sequence (AQSVPYGISQIKAPALHSQGYTGS——VAVINSG) [3,6]. Within the genetic material, a PCR was done, starting at 94 °C during 5 min and after followed by 40 cycles, with 30 s at 94 °C, 30 s at 54 °C and 60 s at 72 °C and an additional 5 min of final extension at 72 °C. Reaction product was analyzed in 1% agarose gel.

2.2 KerS14 ORF cloning

PCR product was linked in the cloning vector pCR® 2.1–TOPO® and transformed in Top 10 *Escherichia coli* competent cells, by heat–shocking. The cells were plated in LB agar medium containing kanamycin (50 $\mu g.mL^{-1}$) and grown at 37 °C. The clones were selected, grown in LB medium with kanamycin and the plasmid DNA extracted by alkaline lysis. The success of the transformation was confirmed by DNA digestion and

nucleotide insert sequence analyzed. The plasmid–ORF harbored was named pCR–TOPO/KerS14.

2.3 KerS14 ORF subcloning into expression vector

The ORF sequence was subcloned into pET–5a vector via BamHI and NheI sites. Besides this, a His–tag coding sequence was added to reverse prime. The following primers were used: sense, 5'–TTCCTTT*GCTAGC*ATGAGAAGCAAAAAATTGTG GATCAGC–3' and reverse 5'–AAAAAAA*GGATCC*TTAGTGGTGGTGGTGGTGGTGTTGTGCAGCTGCTTGTAC GTTGATTATC–3' (Nhe I and BamHI restriction sites are in italics and bases that coding His–tag amino acids are underlined). A PCR was performed using 10 ng of pCR–TOPO/KerS14 as template. Reaction conditions were described in item 2.1. The amplified DNA was digested with BamHI and NheI, dephosphorylated with CIAP and purified with Geneclean II kit. In parallel, 50 ng of plasmid pET–5a received the same treatment as described above. For ligation, digested insert and digested vector were ligated with T4 DNA ligase. The resulting plasmid was named pET–5a/KerS14 and was utilized to transform XL1 Blue *E. coli* cells by electroporation. Cells were selected in LB agar ampicillin (50 $\mu g.ml^{-1}$), growth in LB medium with ampicillin and the plasmid DNA extracted by alkaline lysis. The success of the ligation was confirmed as described in item 2.2.

2.4 Site–directed mutagenesis

In order to obtain rKerS14 mutants, the double–stranded DNA templates and DpnI treatment technical was utilized. The amino acids to be changed were selected based in KerS14 homology with subitilisin E [10,11]. The plasmids used as DNA template and primers are showed in Table 1. PCR and digestion by Dpn I were performed as described by [13]. Digested mutants plasmids were utilized to transfected

E. coli TOP 10 cells. Plasmidial DNA was extracted as described in item 2.2. The insert region in plasmid was sequenced and analyzed to confirm mutation.

Table 1. Templates and synthetic oligonucleotides used for site–directed mutagenesis and respective mutants expressed enzymes.

Plasmid template	Primer (underline: codon mutant)	Plasmid obtained	Mutant expressed enzyme
pET–5a/KerS14	G61C 5'–AAACCCATACCAGGACTGCAGTTCTCACGGTAC–3' G61C_ANTISENSE 5'–GTACCGTGAGAACTGCAGTCCTGGTATGGGTTT–3'	pET–5a/KerS14G6C1	rKerS14G61C
pET–5a/KerS14	S98C 5'–CATTATATGCAGTAAAAGTGCTTGATTGCACAGGAAGCGGCCA–3' S98C_ANTISENSE 5'–TGGCCGCTTCCTGTGCAATCAAGCACTTTTACTGCATATAATG–3'	pET–5a/KerS14S98C	rKerS14S98C
pET–5a/KerS14	P239R 5'–AATTCTTTCTAAGCACCGGACTTGGACAAACGCGC–3' P239R_ANTISENSE 5'–GCGCGTTTGTCCAAGTCCGGTGCTTAGAAAGAATT–3'	pET–5a/KerS14P239R	rKerS14P239R
pET–5a/KerS14S98C	G61C 5'–AAACCCATACCAGGACTGCAGTTCTCACGGTAC–3' G61C_ANTISENSE 5'–GTACCGTGAGAACTGCAGTCCTGGTATGGGTTT–3'	pET–5a/KerS14G61C_S98C	rKerS14 G61C_S98C

2.5 rKerS14 and mutants expression

Plasmids were transfected into *E. coli* BL21 (DE3) strain and plated onto LB agar plates containing ampicillin (50 $\mu g.ml^{-1}$). Cells grown at 37 °C and 180 rpm in LB medium containing ampicillin until an optical density of 0.6–0.8, at 600 nm. After, expression of rKerS14 and its mutants were achieved by addition of 1 mM IPTG and incubating the culture temperature at 25 °C. The inducted cells were growth during 6 h. Cells were harvested by centrifugation (10000 rpm, 15 min, 4 °C) and the supernatant discarded. The pellet was ressuspended in 50 mM Tris–HCl pH 8.0 (2 mL) and disrupted by addition of lisozyme at a final concentration of 1 $mg.mL^{-1}$. The material was incubated overnight at 4 °C and centrifuged (10000 rpm, 15 min, 4 °C). Two fractions were obtained: the soluble fraction, correspondent to the supernatant and the insoluble fraction, correspondent to the pellet. The soluble fraction was dialyzed against 50 mM imidazole in 20 mM sodium phosphate, pH 7.4 with 500 mM NaCl buffer. The insoluble fraction was harvested and submitted to a western blot analysis.

2.6 Insoluble fraction western blot analysis

Protein samples were subjected to 12% SDS–PAGE under reducing conditions. Gels were transferred to a nitrocellulose membrane for western blot analysis. Membranes were blocked with 5% non–fat dry milk–PBS and incubated with anti His–tag antibody conjugated diluted 1:1000. Development was performed in a dark chamber with nitro blue tetrazolium and 5–bromo–4–chloro–3–indolyl phosphate.

2.7 Soluble fraction purification

The soluble fraction was dialyzed in 50 mM imidazole in 20 mM sodium phosphate, pH 7.4 with 500 mM NaCl buffer and filtered through a 0.22 μm sterilized membrane. Soluble enzymes were purified by Ni^{2+}–affinity chromatography. Elution was done with 300 mM imidazole in 20 mM sodium phosphate, pH 7.4 with 500 mM

NaCl. Fractions displaying protease activity were dialyzed against 50 mM Tris–HCl, pH 8.0.

2.8 Purified rKerS14 protein sequencing

To confirm rKerS14 purification, a pure rKerS14 preparation was resolved by 12% SDS–PAGE. The unique band was cut from the gel, treated with trypsin digestion and the fragments analyzed by MS/MS as described by Shevchenko et al., [2007][14].

2.9 Enzymatic assay

To determinate enzymatic activity in samples, 20 µL of the preparations were incubated in 150 µL of 50 mM Tris–HCl buffer, pH 8.0 and 30 µL of 2 mM N–Succinyl–Ala–Ala–Pro–Phe–*p*–nitroanilide (AAPP) in presence of 1 mM $CaCl_2$. Assays were performed for 3 h at 37 °C. The amount of released *p*–nitroaniline was measured spectrophtometriacally at 405 nm and compared to a *p*–nitroaniline standard curve. One enzyme unit was defined as the production of 1 µmol of *p*–nitroaniline per min in the above conditions. The protein content of the purified enzyme preparation was determined by the BCA assay kit (Pierce, Rockford, USA).

2.10 rKerS14 and mutants enzymes activities characterization

2.10.1Thermal stability

The thermal stability of enzymes was determinate incubating at 50 and 60 °C, during 60 and 15 min, respectively. The enzymatic activity at time 0 min was taken as 100%.

2.10.2 Effect of the temperature in enzymes activities

The effect of the temperature in enzymes activities was tested at 22, 30, 37, 42, 50 and 65 °C. The enzymatic activity in temperature of 37 °C assay was taken as 100% for each enzyme.

2.10.3 Effect of the pH in enzymes activities

To determinate the optimal pH of the enzyme, it was incubated in a pH range from 5 to 10. The buffer systems were as follows: 50 mM sodium citrate buffer, pH 5.0–6.0; 50 mM potassium phosphate buffer, pH 7.0; 50 mM Tris–HCl buffer, pH 8.0–9.0; 50 mM carbonate–sodium carbonate buffer, pH 10. The enzymatic activity in 50 mM Tris–HCl buffer pH 8.0 was taken as 100% for each enzyme.

2.10.4 rKerS14 catalytic kinetics determination

rKerS14 K_m, K_{cat} and K_m/K_{cat} were determinate using N–Benzoil–Ile–Glu–Gly–Arg–*p*–nitroanilide (BIGGA) as substrate (ten concentrations over the range 0.025–0.9 mM). Catalytic kinetics was calculated by a nonlinear regression of a Michaelis–Menten curve using GraphPad Prism 6.

2.11 rKerS14 keratinolytic activity

This assay was performed as described by [6], using azokeratin as substrate.

2 .12 rKerS14 fibrin(nogen)olytic activity

These assays were performed as described by [15]. Briefly, 0.6 µg of rKerS14 was incubated with 200 µg of fibrin during 6 h at 37 °C, in 50 mM Tris–HCl 8.0 (final volume 100 µL). The reactions were stopped denatured by heating in the presence of 5% β–mercaptoethanol and subjected to SDS–PAGE.

In order to determine fibrinogen degradation, fibrin was mixed with 1 mU of human plasmin. rKerS14 was added to the formed clot in a final volume of 100 µL and incubated at 37 °C. Reaction was stopped at different times with the addition of 100 µL of denaturing solution (8 M urea, 4% SDS, 4% β–mercaptoethanol). The reactions were analyzed by SDS–PAGE.

3. Results and discussion

3.1 PCR and cloning of KerS14 ORF

PCR using a primer based on a partial KerS14 amino acid sequence produced an approximately 1200 base pairs segment. This segment was successful cloned into pCR2.1–TOPO vector. This construction was named pCR–TOPO/KerS14 and sequencing confirmed an insert of 1146 nucleotides, predicting a 381 amino acids ORF (Fig. 1). Comparing this nucleotide sequence with sequences deposited in GenBank, it has a 99% identity with genes from other *B. subtilis* strains proteases (GenBank accession no. YP_004207041.1; ABJ98765.1; ABY65903.1; ABJ98766.1; AFC89901.1; YP_004207041.1; ABU93240.1; AAX35771.1; ABJ98765.1; AAX35772.1; ZP_03590715.1; ADK11043.1; CAA74536.1; ABJ99976.1; ACE63521.1 and ABY65903.1). As all subtilisins produced by *Bacillus*, the ORF sequence indicates KerS14 is synthesized as a pre–pro–protein (Fig. 1) [16,17,18].

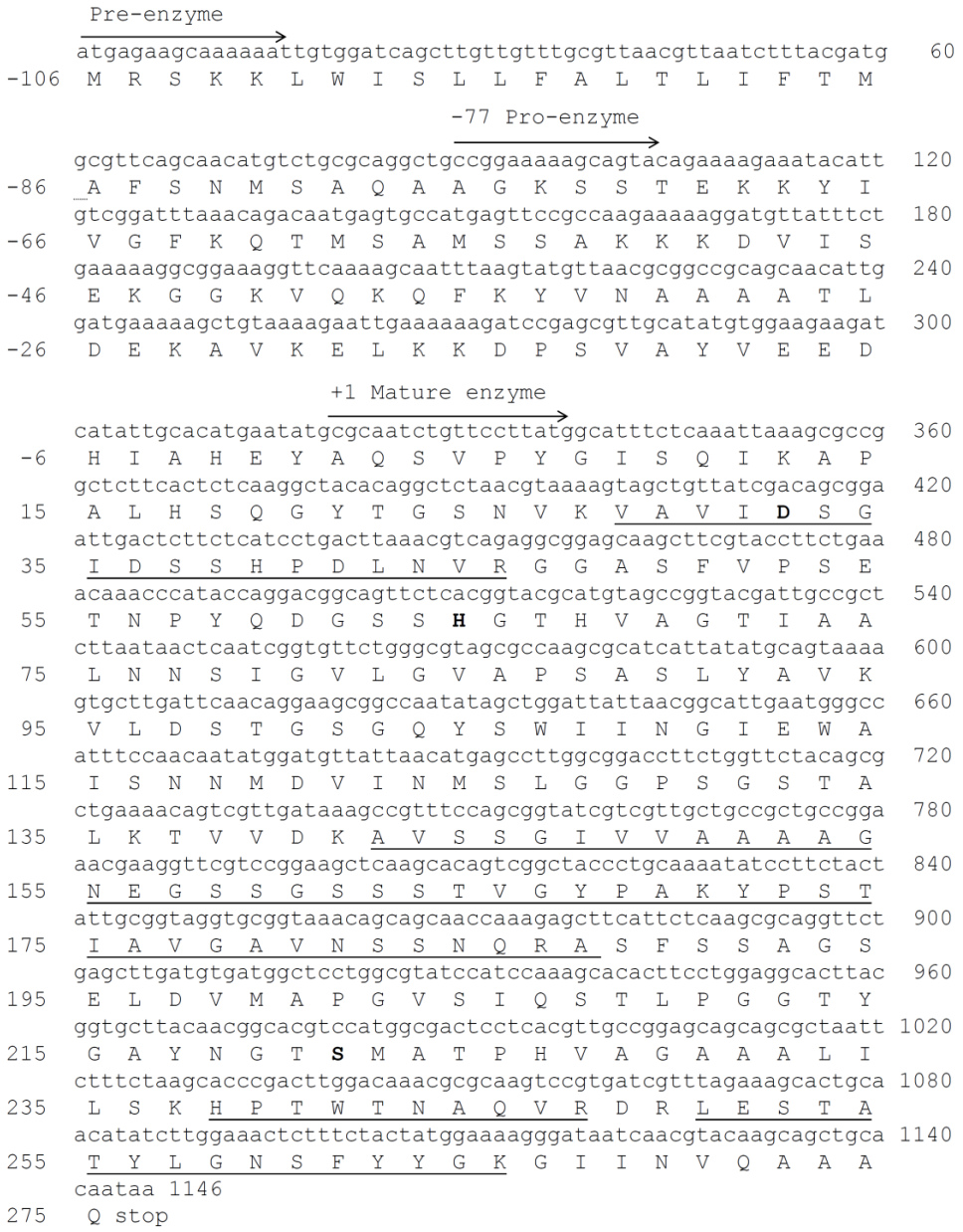

Fig. 1. Nucleotides (lowercase letters) and deduced amino acid sequence (capital letter) of the KerS14 ORF. Bold letters: active site amino acids. The amino acid sequences of five internal peptides determined by LC–MS/MS are underlined.

3.2 Subcloning KerS14 ORF and site directed mutagenesis

KerS14ORF was successfully cloned into pET–5a. The new construction was named pET–5a/KerS14 and used as template for site directed mutagenesis. After mutagenesis, the four mutated plasmids were named pET–5a/KerS14G61C, pET–5a/KerS14S98C, pET–5a/KerS14P239R and pET–5a/KerS14G61C_S98C, according the mutation (Table 1).

3.3 Expression of rKerS14 and its mutants

Expression of the rKerS14 was observed in insoluble (confirmed by western–blot, data not showed) and soluble form. Enzyme expression was detected at 6 and 18 h after induction by IPTG. More than 80% of the rKerS14 was detected in the insoluble fraction as inclusion bodies with a molecular weight of 40 kDa, corresponding to pre, pro and mature enzyme. Possibly due to be in inclusion bodies, the enzyme did not folded properly and consequently there had no enzymatic activity and is was not able to autoprocess.

As typical for subtilisin [18] in its soluble form, rKerS14 undergoes autoprocess and the pro and pre–peptides are excised and the mature protein is formed. In its mature form, rKerS14 has a molecular weight of 25 kDa. Enzyme mutants were obtained in the same way, partially as inclusion bodies without activity and partially soluble and active.

3.4 Purification of rKerS14 and its mutants

Solubles fractions were purified using in one step procedure using Ni^{2+}–affinity chromatographic (Fig. 2). This chromatographic procedure allowed a total recovery of the soluble enzymes (Table 2). rKerS14 identity was confirmed by LC–MS/MS. Five internal tryptic peptides (VAVIDSGIDSSHPDLNVR, AVSSGIVVAAAAGNEGSSGSSSTVGYPAK, KYPSTIAVGAVNSSNQRA,

HPTWTNAQVR and LESTATYLGNSFYYGK) were identified by fragmentation analysis (Fig 1).

Table 2. Summary of the purification step of rKerS14 and mutants enzymes.

Enzyme	Step	Total protein (mg)	Total activity (U)	Specific activity ($U.mg^{-1}$)	Yield (%)	Purification (fold)
rKerS14	Soluble fraction	4.7	0.52	0.11	100	1
	Ni^{2+}–affinity chromatography	0.03	0.52	17.33	100	156
rKerS14G61C	Soluble fraction	4.97	0.35	0.07	100	1
	Ni^{2+}–affinity chromatography	0.036	0.35	9.72	100	138
rKerS14S98C	Soluble fraction	5.17	0.032	0.0062	100	1
	Ni^{2+}–affinity chromatography	0.024	0.032	1.33	100	215
rKerS14P239R	Soluble fraction	3.08	0.028	0.009	100	1
	Ni^{2+}–affinity chromatography	0.016	0.028	1.75	100	123
rKerS14G61C_S98C	Soluble fraction	4.75	0.072	0.015	100	1
	Ni^{2+}–affinity chromatography	0.048	0.072	1.5	100	100

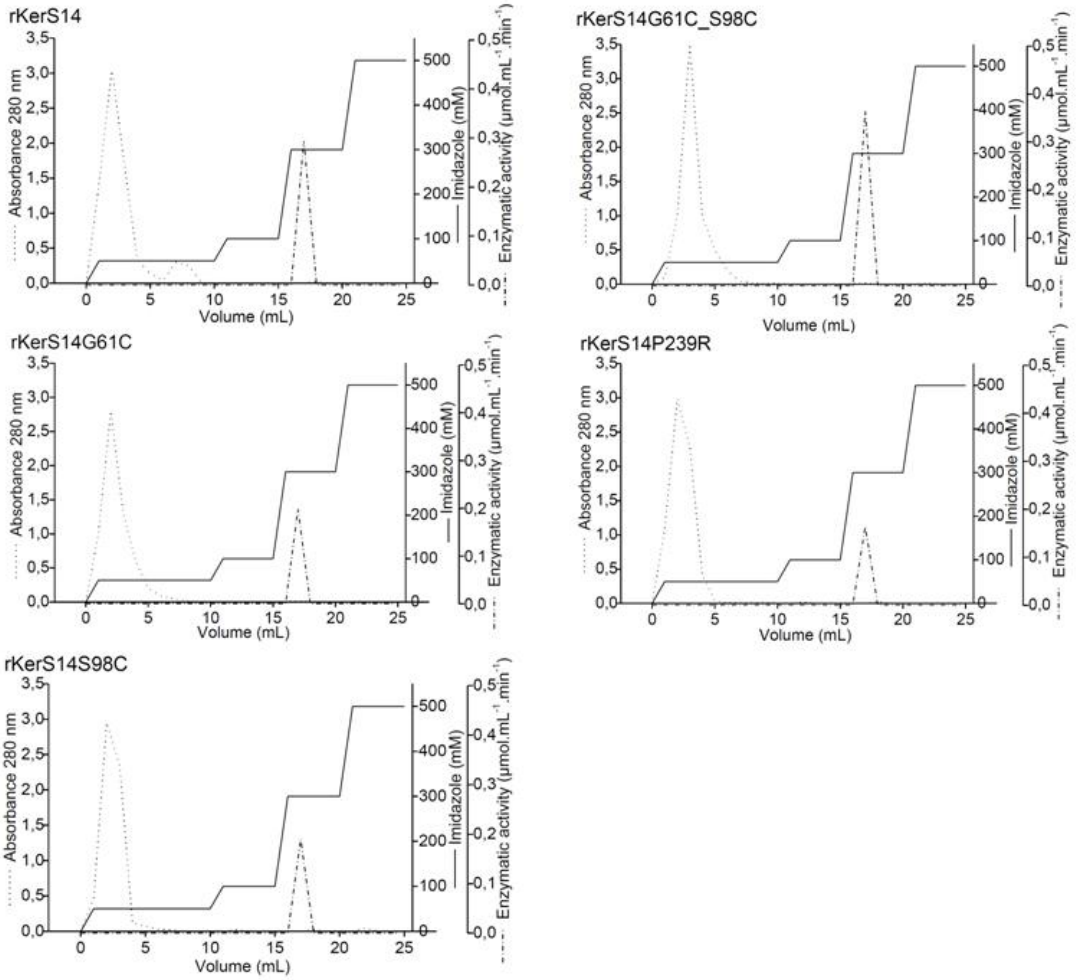

Fig. 2. Chromatograms of rKerS14 and its mutants on Ni2+–affinity column.

3.5 Enzyme characterization

3.5.1Thermal stability

rKers14 maintained 70% of its enzymatic activity at 50 °C for 30 min (Fig. 3a) and at 60 °C full activity remains for 10 min (Fig. 3b). Contrarily, native KerS14 is fully inactivated when incubated at 50 °C for less than 10 min [6]. So, rKerS14 is more stable than the wild–type enzyme. This indicates the His–tag tail at C–terminal increase rKerS14 thermal stability. Contrarily to rKerS14, removal of His–tag form the protein ILYd4 decreases its thermal stability [19]. Surprising, although all mutations done (G61C; S98C; P239R; G61C_S98C) were known to increase subtilisin E thermostability [10,11] they

decreased rKerS14 thermal stability (Fig. 3). These results impel to hypothesize that interactions among His-tag and other rKerS14 regions change the molecule conformation and consequently the mutations selected do not increase thermal stability.

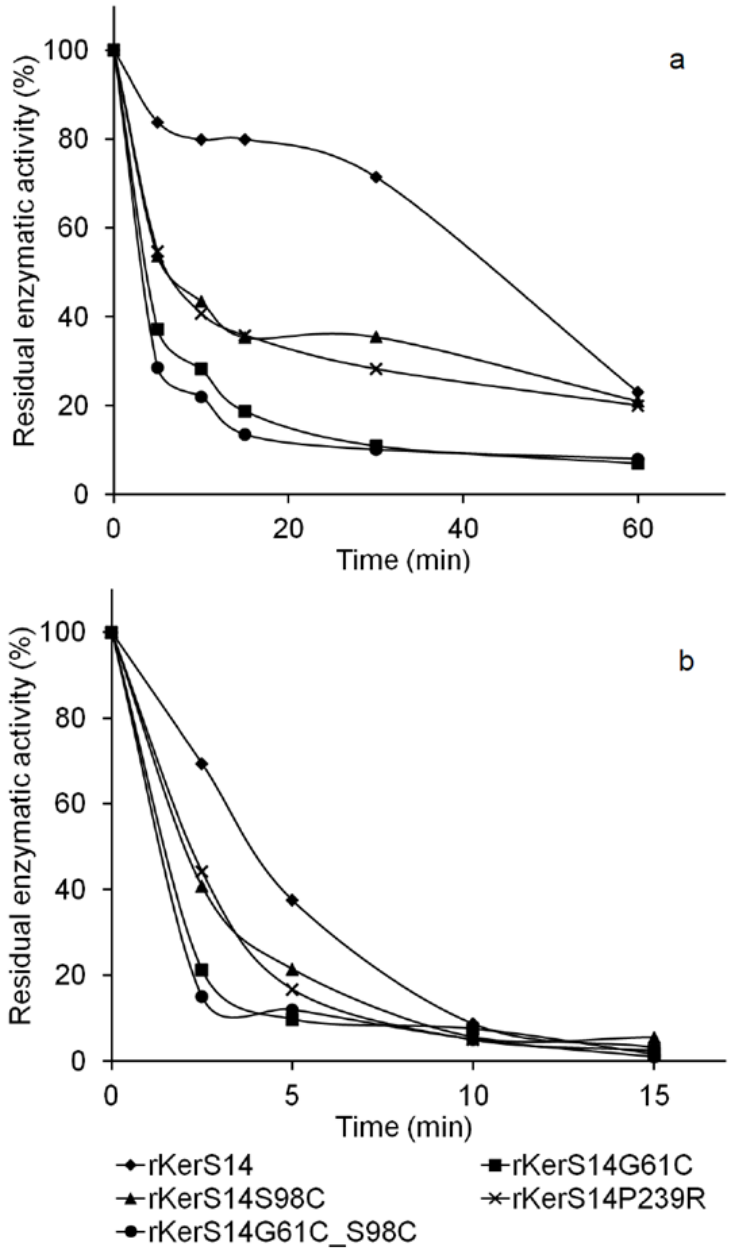

Fig. 3. Residual proteolytic activity over incubation time at 50 (a) and 60 °C (b) for purified rKerS14 and mutants.

3.5.2 Effect of temperature upon enzyme activity

rKerS14 and its mutants have a higher activity at temperatures lower than 37 °C (Fig. 4a). This contrast with other recombinant keratinases [20,21], and also the native KerS14 [6], which have a maximal activity at temperatures higher than 50 °C.

3.5.3 Effect of pH upon enzyme activity

The optimal pH for rKerS14 and all its mutants recombinant enzymes is 7.0, except rKerS14G61C that has an optimum pH at 9.0 (Fig. 4b). This characteristic differs from the wild type enzyme, which has an optimum activity at pH 8.0 [6]. It is well known that mutations in several subtilisins lead to changes in optimum pH [22].

Since non–mutated rKerS14 has an optimum pH different from the native enzyme the His-tag is imputed as responsible for the change in optimum pH. Indeed, a change in optimum pH was described in another recombinant keratinase, its optimum pH is 8.5 instead of 7.5 in the native, being this difference credited to the presence of a Trx–tag in the recombinant enzyme [23]. This reinforces the idea that the difference between KerS14 and rKerS14 is caused the His–tag at C–terminal motif.

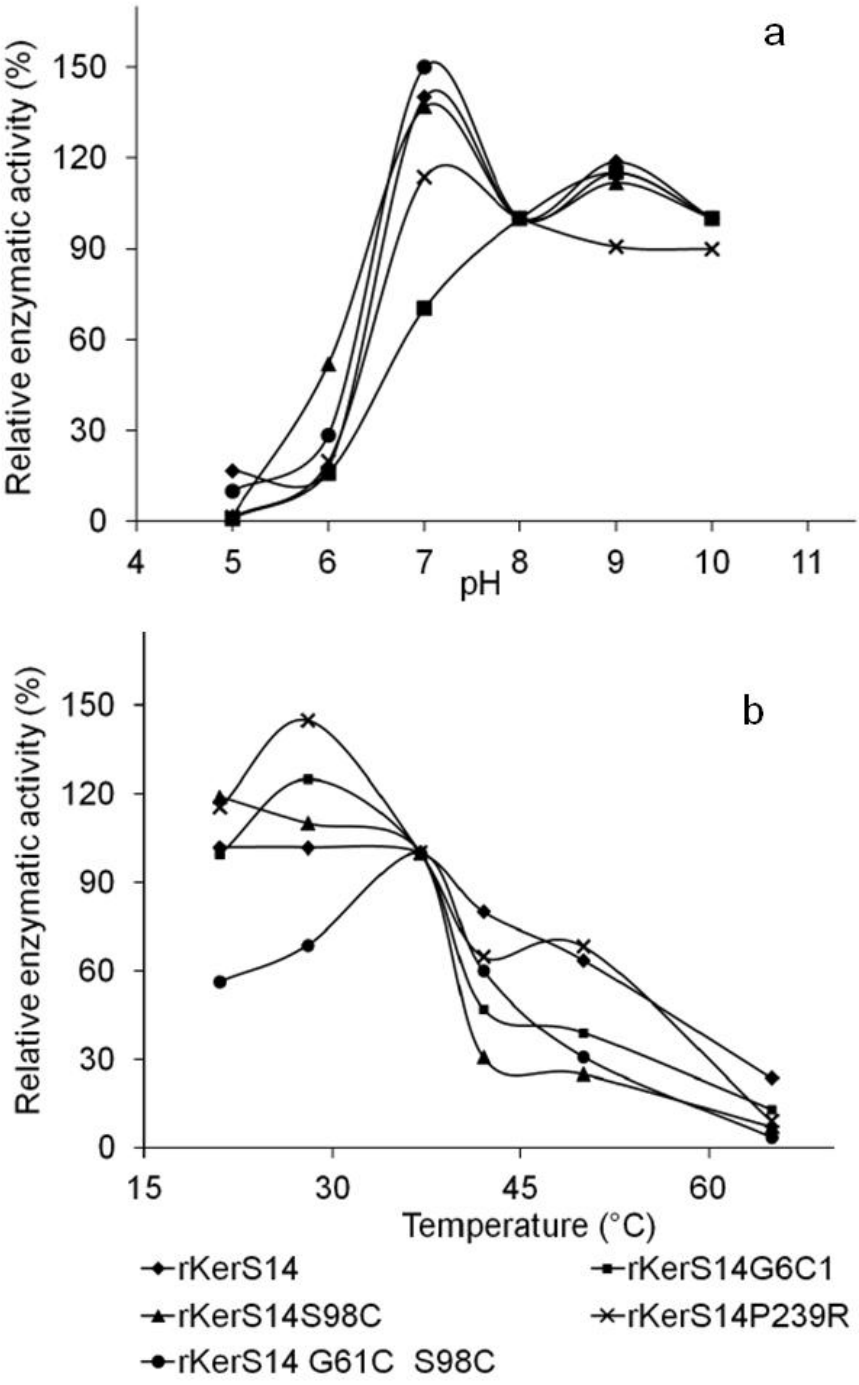

Fig. 4. Effect of temperature (a) and pH (b) upon recombinant enzymes activity.

3.5.4 rKerS14 catalytic properties

rKerS14 K_m upon BIGGA as substrate is 13.6 mM. This value is almost 60 fold higher than KerS14 (K_m = 0.23 mM) [6]. As rKerS14 K_{cat} is higher (77.65 s^{-1}) than the native enzyme (4 s^{-1}) [6] the native and recombinant enzymes have similar K_{cat}/K_m (0.017 $M^{-1}.s^{-1}$ and 0.008 $M^{-1}.s^{-1}$, respectively).

3.6 rKerS14 keratinolytic activity

rKerS14 is a true keratinase since it is able to hydrolyse azokeratin (0.15 $U.mL^{-1}.h^{-1}$). Several other keratinases maintain they keratinolityc activity when produced in a recombinant form [20,23,24,25].

3.7 rKerS14 fibrin(nogen)olytic activity

rKers14 hydrolyses fibrin and fibrinogen. It hydrolyzes fibrin α and β–chain, although more active upon α–chain, and does not hydrolyses γ–chain at all (Fig. 5). Upon fibrinogen rKerS14 is more active than upon fibrin and is equally active upon β and α–chain and is also inactive upon γ–chain. Fibrin and fibrinogen degradation by *Bacillus spp.* enzymes were already reported [15,26]. Similarly as other fibrinolytic enzymes, rKerS14 has potential to be useful in the treatment of cardiovascular diseases.

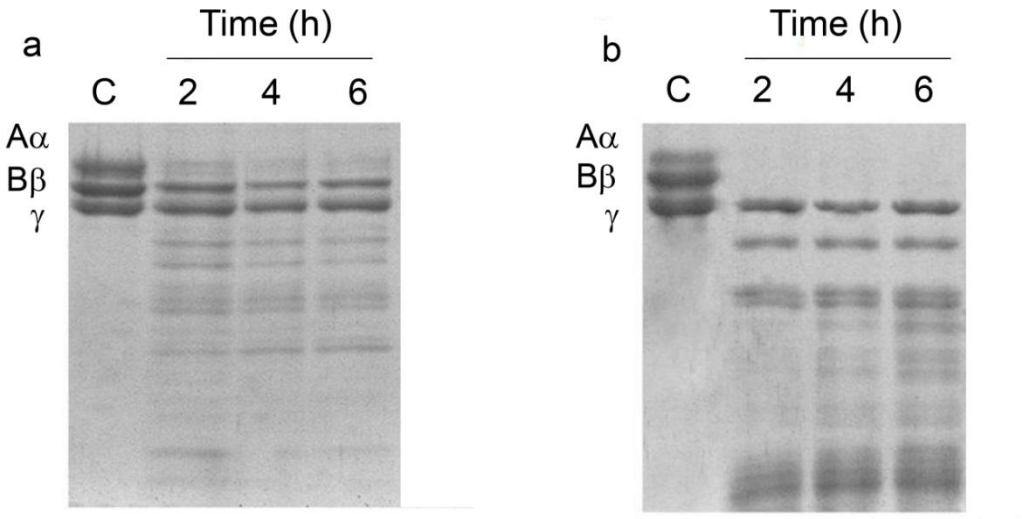

Fig. 5. Fibrin (a) and fibrinogen (b) degradation by rKerS14 monitored by SDS–PAGE. C: Control: fibrin (2.5 μg) or fibrinogen (2.5 μg). Other lanes are fibrin incubated with rKerS14 for 2, 4 and 6 h.

4. Conclusions

A recombinant keratinase was successfully expressed, with a thermal stability superior than the wild type. The presence of a C–terminal His–tag in the recombinant enzyme has an apparent contradictory effect: it decreases thermal stability in G61C, S98C, P239R and G61C_S98C rKerS14 mutants and increase it in the non mutated recombinant enzyme. rKerS14 has a remarkable fibrin(nogen)olytic activity. As far as we know, this is the first enzyme displaying keratinolytic and fibrin(nogen)olytic activity, making rKerS14, besides its use in tannery, also potentially useful to develop new drugs for cardiovascular diseases.

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4. DISCUSSÃO GERAL

KerS14 é uma enzima com alto potencial biotecnológico pois é capaz de depilar couro bovino sem agredir o colágeno, característica importante para a indústria coureira. Apesar deste potencial, não havia uma composição de meio de cultura adequada para a produção dessa enzima, permanecendo, portanto, necessidade de definir um meio de cultivo adequado para isso. Também, KerS14 possui baixa termoestabilidade a 50 °C, o que desfavorece sua aplicação na industria.

Com o objetivo de definir um meio de cultivo que permitisse a produção de queratinases pelo *B. subtilis* S14 de modo reprodutível, foram avaliados substratos queratinosos (farinha de pena, chifre e pelo bovino) e a combinação destes com outras fontes de nutrientes. Após etapa de seleção, em que a combinação de farinha de pena e $CaCl_2$ foi escolhida, foram determinadas as concentrações ideais de ambos no meio de cultura (farinha de pena 1,5% e $CaCl_2$ 1,05%), sendo este considerado o meio de cultura otimizado para a produção de queratinases. A adição de sais é capaz de aumentar a produção de queratinases, como cloreto de amônia (LO *et al*., 2012) e cloreto de sódio (TATINENI *et al*., 2007). O meio de cultivo otimizado permitiu que a produção de queratinases pelo *B. subtilis* S14 fosse reprodutível, conforme análise estatística discutida no subcapítulo 3.1.

O sobrenadante do cultivo otimizado, denominado supKBs14, foi caracterizado. O supKBs14 possui termoestabilidade superior ao sobrenadante do cultivo do *B. subtilis* S14 em soro de queijo e pelo bovino. supKBs14 manteve a atividade queratinolítica em 58% após ser incubado a 50 °C durante 216 horas, enquanto que não foi detectada atividade queratinolitica após 12 horas de incubação a 50 °C no outro sobrenadatante (MACEDO, 2002). O aumento significativo da termoestabilidade no supKBs14 pode decorrer da

presença de $CaCl_2$ no supKBs14, que pode conferir maior estabilidade às enzimas presentes no mesmo, como relatado no caso de outras peptidases (SMITH *et al*., 1999, MANNI *et al*., 2008; MORADIAN *et al*., 2009)

O perfil de atividade do supKBs14 em relação a inibidores de peptidases e íons sugerem a presença de outra enzima queratinolítica além da KerS14. A inibição causada pelo EDTA e EGTA na atividade queratinolítica do supKBs14 sugere que *B. subtilis S14* produz uma metaloendopeptidase com atividade queratinolítica, como observado em outras linhagens de *Bacillus* que produzem queratinase (LEE *et al*., 2002; TORK *et al*., 2013).

O supKBs14 possui atividade enzimática entre pH de 8–10.5, característica promissora para a formulação de detergentes. Outro potencial biotecnológico demonstrado pelo supKBs14 reside na degradação de chifre bovino. A hidrolise de substrato queratinosos é de relevância para a obtenção de biofertilizantes e também de hidrolisados proteicos para alimentação animal (BRANDELLI *et al*., 2012).

Embora a produção de queratinases esteja estabelecida, a maioria dos estudos é efetuada em cultivos utilizando frascos de Erlenmeyer. Estudos de escalonamento para a produção de enzimas em bioreatores são fundamentais para obtê-las em quantidades adequadas para uso industrial. Dentre as condições de fermentação realizadas neste trabalho, a fermentação a 1 vvm de aeração e 400 rpm de agitação foi a que levou a produção de maior atividade queratinolítica. Além disso, foi determinado o coeficiente volumétrico de transferência de oxigênio ($K_L a$), considerado principal parâmetro para o desenvolvimento e operação de bioreatores (MARQUES *et al*., 2009). O valor de $K_L a$ de 24.48 h^{-1} estabelecido poderá ser utilizado em estudos posteriores visando a produção de queratinases em bioreatores com volume superior a 5 L. Embora existam relatos de produção de queratinases em bioreatores (WANG & SHIH, 1999; RANINGER &

STEINER2003), este é o primeiro estudo em que foi determinado o $K_L a$ para a produção de queratinases em bioreatores.

Portanto, agora foi estabelecido um meio de cultivo padronizado e realizado o escalonamento da produção de queratinases para bioreator de 5 L. Além disso, a caracterização do supKBs14 revelou que o sobrenadante possui atividade queratinolítica termoestável e novas potenciais aplicações.

O subcapítulo 3.2 relata a clonagem da ORF, determinação da sequência de nucleotídeos e a expressão da KerS14 recombinante (rKerS14), bem como o efeito de mutações selecionadas visando o aumento da termoestabilidade da enzima recombinante.

A obtenção da sequência de nucleotídeos da KerS14 permitiu predizer a respectiva sequência de aminoácidos. Foi verificada similaridade entre as sequências de aminoácidos da KerS14 e da subtilisina E, sendo que a única diferença encontra se no resíduo 268, Isoleucina na KerS14 e a uma leucina na subtilisina E. Mutações nos resíduos de aminoácidos G61, S98 e P239 foram descritas como determinantes para conferir maior termoestabilidade à subtilisina E (TAGAKI *et al.*,1989; TAGAKI *et al.,* 1989) e portanto foram realizadas na ORF que codifica a KerS14 para testar a hipótese de que o mesmo acontece com a KerS14.

Entretanto, estas mutações não levaram a aumento da termoestabilidade das enzimas recombinantes em relação a rKerS14. Uma hipótese que explique essa diferença de comportamento é a presença cauda de histidina na região C–terminal da rKerS14 modificar a conformação da proteína. Conforme discutido na literatura, a adição de caudas de histidina ou de tioredoxina pode resultar em mudanças nas propriedades das proteínas recombinantes (LEE *et al*., 1999; WU *et al*., 2012; WANG *et al*., 2012; HU *et al*., 2013).

Outras possíveis consequências da adição da cauda da histidina na rKerS14 foram o aumento da termoestabilidade, a mudança do pH ótimo da atividade enzimática e o decréscimo na afinidade pelo substrato N–benzoil–Ile–Glu–Gly–Arg–p–nitroanilIna (BIGGA).

A atividade da KerS14 sobre o BIGGA, substrato utilizado na detecção do fator Xa, da cascata de coagulação sanguínea foi previamente relatada, sugerindo que a enzima nativa tenha potencial fibrinolítico (MACEDO *et al*., 2008). E efetivamente, rKerS14 é capaz de degradar fibrina e fibrinogênio, demonstrado a possibilidade da enzima ser útil para a remoção de coágulos. Outras subtilisinas isoladas de *Bacillus* foram descritas como fibrinolíticas, com propriedades biquímicas semelhantes com a KerS14 (WANG *et al*., 2008; WENG *et al*., 2009).

O estabelecimento de um meio de cultivo para a produção de queratinases termoestáveis e o escalonamento para bioreator de 5 L são avanços para o aprimoramento da produção de queratinases pelo micro–organismo *B. subtilis* S14, enquanto que a obtenção da rKers14, queratinases com atividade fibrinolítica amplia o potencial de aplicação das enzimas queratinolíticas produzidas pelo *B. subtilis* S14.

5. CONCLUSÕES

– Foi determinado um meio simples e eficaz para a produção de queratinases termoestáveis em bioreator pelo microrganismo *B. subtilis* S14.

– rKerS14 é mais termoestável que os mutantes obtidos e que a enzima nativa KerS14.

– rKerS14 é uma queratinase que também possui com ação sobre fibrina e fibrinogênio e tem potencial aplicação no tratamento de doenças cardiovasculares.

6. PERSPEPECTIVAS

Em ambos os subcapítulos da presente tese foram demonstradas que as queratinases de *B. subtilis* S14 possuem potenciais aplicações em áreas distintas e de grande relevância econômica, como a formulação de detergentes, hidrólise de substratos protéicos e tratamento de doenças cardiovasculares. Estes dados se somam a capacidade de depilar o couro sem causar danos ao colágeno, descrita anteriormente. Realizar experimentos em que as queratinases de *B. subtilis* S14 são formuladas e testadas nestas diferentes áreas será fundamental e permitirá a obtenção de produtos em que as queratinases serão o princípio ativo.

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8. ANEXO

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Formação Acadêmica

Doutorado em Biologia Celular e Molecular, Centro de Biotecnologia UFRGS, 2009-2013. Bolsista do: Conselho Nacional de Pesquisa, CNPq, Brasil.

Mestrado em Microbiologia Agrícola e do Ambiente, UFRGS, 2006 – 2008. Bolsista do: Conselho Nacional de Pesquisa, CNPq, Brasil.

Bacharelado e Licenciatura Ciências Biológicas, UFRGS, 2001 – 2005.

Formação complementar

2009 – How to write a good english lenguage paper. (Carga horária: 15h). Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

2005 – Planejamento Experimental e Otimização de Processos. (Carga horária: 30h). Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

2003 – Curso Plano de Negócios. 23 de agosto – 5 de setembro de 2003. SEBRAE– RS

Estágios

2004 – 2005 ─ Bolsista de Iniciação Científica na UFRGS, sob orientação da Profa. Dra. Marilene Henning Vainstein no projeto "Biologia Molecular de Fungos Entomopatogênicos – *Metarhizium anisopliae*". Carga horária semanal: 20 horas.

2002 – 2003 ─ Bolsista de Iniciação Científica no Geyer Medicamentos, sob orientação de Gláucia Porto Prates e Sydnei Mitidieri, no Projeto "Produção de Probióticos". Carga horária semanal: 20 horas.

2001 – 2002 ─ Estágio voluntário, sob Orientação da Profa. Dra. Marilene Henning Vainstein no projeto "Biologia Molecular de Fungos Entomopatogênicos – *Metarhizium anisopliae*". Carga horária semanal: 20 horas.

Prêmios e Títulos

2005 ─ Destaque do XVII Salão de Iniciação Científica da UFRGS, Sessão Microbiologia B, Porto Alegre.

2004 ─ 3º Lugar na Maratona do Empreendorismo da UFRGS, SEI, UFRGS.

Organização de eventos

Workshop Adesão Microbiana e Superfícies, Porto Algre, Brasil, 2011.

III Curso de Férias do Programa de Pós– Graduação em Biologia Celular e Molecular da UFRGS, 2010.

II Jornada Acadêmica de Biologia da UFRGS, 2005.

Orientação de Alunos

Co–orientação do trabalho de conclusão de curso de graduação em Farmácia – UFRGS, aluna Fernanda Cortez Lopes.

Produção Científica

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6. SILVA, L. A. D., SILVA, W. O. B., MACEDO, A. J. & TERMIGNONI, C. Seleção e otimização de meios de cultivo para a produção de queratinases pelo *Bacillus subtilis* S14 utilizando técnicas de planejamento experimental e superfície de resposta. In: 25 Congresso Brasileiro de Microbiologia, Porto de Galinhas. CD-ROM, 2009.

7. SILVA, L. A. D., SILVA, W. O. B., MACEDO, A. J. & TERMIGNONI, C. Otimização da produção de queratinases pelo *Bacillus subtilis* S14 e caracterização do sobrenadante de cultivo com aplicação industrial. In: IX Reunião Anual do Programa de Pós–Graduação em Biologia Celular e Molecular, Porto Alegre. Livro de Resumos, p. 72, 2009.