UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM MICROBIOLOGIA

AGRÍCOLA E DO AMBIENTE

POTENCIAL QUERATINOLÍTICO E CARACTERIZAÇÃO DE UMA QUERATINASE EXTRACELULAR DE *Bacillus* sp. P45

DANIEL JONER DAROIT

Porto Alegre, Rio Grande do Sul, Brasil
Julho de 2011

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Potencial queratinolítico e caracterização de uma queratinase extracelular de *Bacillus* sp. P45[1]

Autor: Daniel Joner Daroit
Orientador: Adriano Brandelli

RESUMO

Resíduos ricos em queratina são produzidos em grande quantidade por atividades agroindustriais, como é o caso das penas oriundas de abatedouros de aves, e seu acúmulo pode ocasionar problemas ambientais. Microrganismos queratinolíticos e suas queratinases são objeto de interesse biotecnológico, apresentando inúmeras aplicações. Este estudo avaliou o potencial queratinolítico de *Bacillus* sp. P45, a otimização da produção de queratinases, e a caracterização de uma queratinase extracelular. Esta linhagem degradou aproximadamente 90% das penas após 72 h de cultivo em caldo contendo 1% (m/v) de penas inteiras, sendo observadas a produção de proteases e queratinases extracelulares e o aumento do conteúdo de proteínas solúveis e de grupos tiol. A produção de queratinases extracelulares foi avaliada utilizando diferentes substratos, e o maior rendimento ocorreu em meio contendo farinha de penas (FP). A adição de carboidratos inibiu a produção de enzimas em meio FP, possivelmente através de repressão catabólica; contudo, a adição de NH_4Cl ocasionou incremento na produção. Utilizando experimento fatorial 2^2 observou-se que a variável FP foi mais significativa para a produção de enzimas do que NH_4Cl, sendo estabelecidas as concentrações 43-50 g/L de FP e 1,8-8,6 g/L de NH_4Cl para produção máxima de queratinases. A queratinase bruta apresentou atividade ótima a 50 °C e pH 7,0, sendo amplamente inibida por EDTA. Uma queratinase extracelular foi purificada e caracterizada como uma serino protease similar à subtilisina. Esta enzima, com aproximadamente 26 kDa, apresentou atividade ótima a 55 °C e pH 8,0, baixa estabilidade térmica e a capacidade de hidrolisar diversos substratos protéicos, como caseína e farinha de penas; a hidrólise de queratinas nativas ocorreu de forma restrita, possivelmente pela presença de pontes dissulfeto. Tetrapeptídeos contendo aminoácidos aromáticos e hidrofóbicos na posição P1 foram hidrolisados preferencialmente. A enzima apresentou maior atividade e estabilidade térmica na presença de cálcio (3 mM) ou magnésio (4 mM). A inativação térmica seguiu, aparentemente, uma cinética de primeira ordem, e os parâmetros cinéticos e termodinâmicos obtidos indicam que o íon cálcio é essencial para a estabilidade térmica da enzima.

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Keratinolytic potential and characterization of an extracellular keratinase from *Bacillus* sp. P45[1]

Author: Daniel Joner Daroit
Advisor: Adriano Brandelli

ABSTRACT

Keratin-rich wastes are produced in high amounts by agroindustrial activities, as in the case of feathers derived from poultry processing, and its accumulation might result in environmental problems. Keratinolytic microorganisms and its keratinases are biotechnologically interesting targets, presenting several applications. This study evaluated the keratinolytic potential of *Bacillus* sp. P45, the optimization of keratinase production, and the characterization of an extracellular keratinase. This strain degraded almost 90% of feathers after 72 h of cultivation in broth containing 1% (m/v) of whole feathers, with the observed production of proteases and keratinases, and the increase on the contents of both soluble protein and thiol groups. Extracellular keratinase production was evaluated with different growth substrates, and the higher enzyme yield occurred in media containing feather meal (FM). Addition of carbohydrates to FM medium inhibited enzyme production, possibly through catabolite repression; however, addition of NH_4Cl caused a higher keratinase production. From a 2^2 factorial design it was observed that the FM variable was most significant for enzyme production than NH_4Cl, with 43-50 g/L of FM and 1.8-8.6 g/L of NH_4Cl as the concentrations for maximal keratinase production. Crude keratinase showed optimum activity at 50 °C and pH 7.0, being largely inhibited by EDTA. An extracellular keratinase was purified, and characterized as a subtilisin-like serine protease. This enzyme, with approximately 26 kDa, presented optimum activity at 55 °C and pH 8.0, low thermal stability, and the ability to hydrolyze various protein substrates, such as casein and feather meal; only a limited hydrolysis of the native keratin substrates was observed, possibly due to the presence of disulfide bridges. Tetrapeptides containing aromatic and hydrophobic amino acid residues at position P1 were preferably hydrolyzed. The enzyme showed higher activity and thermal stability in the presence of calcium (3 mM) or magnesium (4 mM). Thermal inactivation followed an apparent first-order kinetics, and the obtained kinetic and thermodynamic parameters indicate that calcium ion is essential for enzyme thermal stability.

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1 INTRODUÇÃO

Enzimas microbianas são utilizadas em inúmeras aplicações industriais, e a demanda por enzimas mais estáveis, altamente ativas e específicas, cresce rapidamente. Com isso, diversos produtores comerciais de enzimas disponibilizam preparações enzimáticas adequadas para diferentes processos biotecnológicos. As proteases formam um grupo de enzimas fisiologicamente importantes que catalisam a hidrólise de ligações peptídicas em proteínas e peptídeos, também apresentando potencial biotecnológico para aplicação em diversas áreas comerciais e industriais.

As queratinas, componentes da pele e também penas, pêlos e outros apêndices epidérmicos, são proteínas altamente insolúveis e resistentes, de difícil degradação por proteases como pepsina e tripsina. A recalcitrância das queratinas e o aumento na geração de resíduos queratinosos, principalmente devido ao crescimento na produção avícola mundial, fazem destes resíduos um potencial problema ambiental.

Neste sentido, microrganismos queratinolíticos e queratinases microbianas vêm sendo explorados quanto à sua aplicação no bioprocessamento de materiais queratinosos, representando uma tecnologia alternativa para o manejo destes resíduos. Concomitantemente, a

bioconversão desta biomassa também origina aminoácidos e proteínas que podem ser utilizados na produção de rações e fertilizantes, entre outros produtos de elevado valor agregado. Portanto, a utilização da diversidade microbiana e de sua capacidade metabólica coloca-se como um enorme potencial a ser explorado. Além do manejo de resíduos queratinosos, queratinases microbianas podem ser potencialmente empregadas em indústrias de alimentos, têxteis, coureiras, como suplemento em rações, como componentes de detergentes, e em aplicações biomédicas e farmacêuticas.

Um dos principais obstáculos para a produção comercial de enzimas é representado pelo alto custo dos substratos utilizados em meios de cultivo. Portanto, a busca por substratos alternativos pode contribuir para a diminuição do custo de produção destas enzimas e, por conseguinte, ocasionar a redução dos custos dos processos nos quais tais enzimas são empregadas. Como a produção de queratinases é geralmente induzida por queratinas, os próprios resíduos queratinosos oriundos de atividades agroindustriais podem ser utilizados como potenciais substratos de baixo custo e amplamente disponíveis para a produção de queratinases microbianas.

Neste contexto, os objetivos deste estudo foram: a) avaliar o potencial queratinolítico de *Bacillus* sp. P45, bactéria previamente isolada do intestino do peixe *Piaractus mesopotamicus*; b) otimizar as condições de produção de proteases extracelulares em cultivos submersos; c) purificar e caracterizar uma protease queratinolítica extracelular com potencial relevância biotecnológica; d) investigar a estabilidade cinética desta enzima em diferentes temperaturas, na ausência ou presença de íons metálicos.

2 REVISÃO BIBLIOGRÁFICA

2.1 Proteases

Proteases, também denominadas proteinases ou peptidades, são enzimas que hidrolisam ligações peptídicas em proteínas e peptídeos. As proteases formam um complexo grupo de enzimas, que varia enormemente em suas características físico-químicas e catalíticas (RAO et al., 1998).

Estas enzimas participam de processos metabólicos essenciais, e sua importância é evidenciada pela ocorrência em todas as formas de vida (GUPTA et al., 2002a), representando aproximadamente 2% do total dos produtos codificados por genes (RAWLINGS & BARRETT, 1999; PÓLGAR, 2005). Proteases realizam modificações altamente específicas e seletivas em proteínas, desempenhando funções cruciais em processos fisiológicos. As proteases extracelulares atuam de forma importante na nutrição através da hidrólise de proteínas, facilitando a absorção dos produtos pelas células, sendo também importantes em processos patológicos e de antagonismo (RAO et al., 1998; PAGE & DI CERA, 2008a,b).

A classificação destas enzimas não se dá, de forma satisfatória, através de métodos utilizados para a maioria das enzimas, uma vez que todas as proteases catalisam, basicamente, a mesma reação, ou seja, a hidrólise de

ligações peptídicas (BARRETT et al., 2001). Contudo, as proteases são usualmente classificadas segundo critérios majoritários, como (i) tipo de reação catalisada, em endo ou exoproteases, dependendo do local de clivagem em relação às regiões terminais do substrato; (ii) pH de atividade ótima, em proteases ácidas, neutras ou alcalinas; (iii) natureza química e mecanismo do sítio catalítico, em serino, cisteíno, aspartil, treonino ou metaloproteases; e (iv) relações evolutivas no que diz respeito à sua estrutura (RAO et al., 1998; RAWLINGS & BARRET, 1999; EKICI et al., 2008; PAGE & DI CERA, 2008a,b).

Além das inúmeras funções celulares, as proteases são também relevantes para a biologia, medicina e biotecnologia. Proteases são responsáveis por 40% do total das vendas mundiais de enzimas, e o crescente interesse no estudo de proteases microbianas extracelulares deve-se às suas múltiplas aplicações industriais. Estas enzimas podem ser utilizadas como componentes de detergentes, na indústria de alimentos, couros, em processos de tratamento de resíduos e biorremediação, na síntese de peptídeos, na indústria de produtos farmacêuticos, entre outras aplicações (ANWAR & SALEEMUDDIN, 1998; KUMAR & TAKAGI, 1999; GUPTA et al., 2002a; SUMANTHA et al., 2006; SANCHEZ & DEMAIN, 2011). As serino proteases produzidas por espécies de *Bacillus*, particularmente aquelas relacionadas à subtilisina, são extensamente investigadas, tanto para fornecer informações sobre mecanismos de atuação e especificidade catalítica, quanto pela sua importância industrial (BRYAN, 2000; GUPTA et al., 2002b; SCHALLMEY et al., 2004).

2.2 Queratinas

As queratinas, proteínas estruturais fibrosas e insolúveis encontradas em vertebrados, são os principais componentes da pele e seus apêndices (pêlos, penas, unhas, cascos, chifres e escamas), e são os membros mais abundantes da superfamília dos filamentos intermediários (FUCHS & WEBER, 1994; COULOMBE & OMARY, 2002; GUPTA & RAMNANI, 2006). Estas proteínas não são efetivamente degradadas por proteases comuns, como tripsina, pepsina e papaína (BRANDELLI, 2008). As características de insolubilidade, estabilidade e resistência à degradação proteolítica das queratinas são conseqüências do empacotamento compacto das cadeias protéicas em estruturas de α-hélice (α-queratina) ou β-folhas (β-queratina), e do elevado nível de enovelamento da cadeia polipeptídica. Adicionalmente, alto grau de ligações inter e intracadeia ocorrem na cadeia polipeptídica através da formação de ligações dissulfeto, pontes de hidrogênio e interações hidrofóbicas (ONIFADE et al., 1998; PARRY & NORTH, 1998; FRASER & PARRY, 2008).

Aparentemente, o conteúdo de ligações dissulfeto é o principal determinante das características de resistência e estabilidade das diferentes queratinas. Nesse sentido, as queratinas são classificadas em ‘rígidas’ ou ‘leves’ de acordo com o teor de cisteína: as primeiras possuem alto conteúdo de cisteína e ligações dissulfídicas, enquanto que as últimas possuem menor conteúdo de cisteína (SCHROOYEN et al., 2001). Queratinas da epiderme (*stratum corneum*) são classificadas como α-queratinas ‘leves’, apresentando conteúdo de cisteína inferior a 10% em sua estrutura (GRADISAR et al., 2005).

As α-queratinas ‘rígidas’, componentes de pêlos, lã e unhas apresentam 10,5-17% de cisteína, enquanto que a queratina das penas encontra-se predominantemente sob a forma de β-queratina, com conteúdo de cisteína entre 4,2 e 7,6% (FRASER & PARRY, 2008; BRANDELLI et al., 2010).

2.3 Queratinas como resíduos agroindustriais

Pelos e penas são os principais resíduos queratinosos produzidos por atividades agroindustriais, compostos por aproximadamente 90% de queratina. No caso das penas, estas representam 7-10% do peso corporal total das aves (BRANDELLI, 2008). Com o aumento da produção avícola mundial, grande quantidade de penas tem sido gerada como resíduo do processamento. Estima-se que aproximadamente 5 milhões de toneladas de penas sejam produzidas anualmente como efluente da produção de carne de frango (POOLE et al., 2009).

Estes resíduos são usualmente incinerados, um processo que, além do seu alto custo, apresenta desvantagens tanto ecológicas, devido às elevadas emissões de CO_2, quanto de desperdício de energia (MARCONDES et al., 2008; MATSUI et al., 2009). Resíduos queratinosos também são depositados em aterros sanitários ou outros ambientes. O acúmulo localizado destes resíduos dificulta sua degradação e, além disso, a degradação anaeróbia da queratina resulta na produção de substâncias tóxicas, como sulfeto de hidrogênio e amônia (SINGH, 2002).

Alternativamente, as penas são submetidas à cocção sob pressão e moagem, dando origem ao produto denominado ‘farinha de penas’, que é

usualmente adicionado como suplemento em rações animais. No entanto, este processo apresenta alto custo devido ao aporte de energia térmica e mecânica. Além disso, o processamento provoca a destruição de aminoácidos essenciais (como metionina, lisina e triptofano), originando um produto de baixa digestibilidade e baixa qualidade nutricional, limitando sua utilização (ONIFADE et al., 1998; KARTHIKEYAN et al., 2007).

Particularmente, a indústria avícola enfrenta o desafio de produzir produtos de alta qualidade, de uma forma que satisfaça as expectativas dos consumidores, regulamentações ambientais, e que maximize os lucros (FREEMAN et al., 2009). Logo, o desenvolvimento de métodos de baixo custo para a disposição das penas pode reduzir consideravelmente problemas e riscos financeiros, ambientais, e de saúde pública (VASILEVA-TONKOVA et al., 2009). Estes fatores, combinados ao aumento da necessidade de conservação e reciclagem de energia, têm estimulado a busca por alternativas para o manejo destes resíduos recalcitrantes (BRANDELLI, 2008).

2.4 Microrganismos queratinolíticos e queratinases microbianas

Apesar da recalcitrância das queratinas, incluindo sua resistência à proteólise, estas proteínas são decompostas na natureza, evidenciado a existência de microrganismos queratinolíticos, particularmente bactérias e fungos, com a capacidade de degradar e utilizar estas proteínas como nutrientes (LUCAS et al., 2003; RIFFEL & BRANDELLI, 2006; ELÍADES et al., 2010; BACH et al., 2011). Estes microrganismos produzem enzimas denominadas queratinases, que compõem uma classe de proteases capazes

de degradar substratos queratinosos mais eficientemente do que outras enzimas proteolíticas (ONIFADE et al., 1998).

Microrganismos queratinolíticos mesófilos ou termófilos são encontrados em diversos locais, com ou sem histórico de deposição ou presença de materiais queratinosos, incluindo ambientes aeróbicos ou anaeróbicos. Logo, a produção de queratinases por microrganismos parece ser comum na natureza em diversas condições ecológicas, indicando a participação destes microrganismos na ciclagem do carbono, nitrogênio e enxofre das moléculas de queratina. Neste sentido, a exploração da diversidade microbiana pode, potencialmente, fornecer queratinases apropriadas para aplicações biotecnológicas (BRANDELLI et al., 2010).

No grupo das bactérias Gram-positivas, representantes do gênero *Bacillus* destacam-se como importantes produtores de queratinases (WERLANG & BRANDELLI, 2005; GIONGO et al., 2007; CORRÊA et al., 2010; NAGAL & JAIN, 2010). Entre as espécies descritas como queratinolíticas podem ser citadas *B. licheniformis* (WILLIAMS et al., 1990; MANCZINGER et al., 2003; FAKHFAKH et al., 2009), *B. subtilis* (MACEDO et al., 2005; BALAJI et al., 2008; PILLAI & ARCHANA, 2008), *B. cereus* (GHOSH et al., 2008), *B. pumilus* (KUMAR et al., 2008; FAKHFAKH-ZOUARI et al., 2010a), *B. pumilis* (KIM et al., 2005; SON et al., 2008), *B. megaterium* (PARK & SON, 2009), *B. pseudofirmus* (GESSESSE et al., 2003; KOJIMA et al., 2006), e *B. halodurans* (TAKAMI et al., 1999; PRAKASH et al., 2010a). Outras bactérias queratinolíticas Gram-positivas têm sido isoladas, tais como, *Lysobacter* sp. NCIMB 9497 (ALLPRESS et al., 2002), *Kocuria rosea* (BERNAL et al., 2006),

Kytococcus sedentarius (LONGSHAW et al., 2002), *Clostridium sporogenes* bv. pennavorans bv. nov. (IONATA et al., 2008), *Microbacterium* sp. kr10 (THYS et al., 2004) e *Nesterenkonia* sp. AL-20 (GESSESSE et al., 2003).

O grupo dos Actinomicetos também possui representantes queratinolíticos, particularmente aqueles pertencentes ao gênero *Streptomyces* (MABROUK, 2008; JAOUADI et al., 2010; XIE et al., 2010) como *S. pactum* (BÖCKLE et al., 1995), *S. albidoflavus* (BRESSOLLIER et al., 1999), *S. thermoviolaceus* (CHITTE et al., 1999), *S. graminofaciens* (SZABO et al., 2000), *S. flavis* (GUSHTEROVA et al., 2005) e *S. gulbargensis* (SYED et al. 2009). Ainda, outros isolados deste grupo vêm sendo descritos, como os actinomicetos *Nocardiopsis* sp. TOA-1 (MITSUIKI et al., 2002) e *Microbispora aerata* (GUSHTEROVA et al., 2005).

Em comparação à abundância de bactérias Gram-positivas, poucas bactérias queratinolíticas Gram-negativas têm sido descritas, entre elas, *Aeromonas* sp. (BACH et al., 2011), *Vibrio* sp. kr2 (SANGALI & BRANDELLI, 2000), *Chryseobacterium* spp. (RIFFEL et al., 2003a; WANG et al., 2008a; BACH et al., 2011), *Serratia* spp. (KHARDENAVIS et al., 2009; BACH et al., 2011), *Stenotrophomonas* (*Xanthomonas*) spp. (DE TONI et al., 2002; YAMAMURA et al., 2002a; CAO et al., 2009), e *Paracoccus* sp. (LEE et al., 2004), incluindo o termofílico *Meiothermus ruber* (MATSUI et al., 2009), e os termofílicos anaeróbios *Fervidobacterium islandicum* (NAM et al., 2002) e *Fervidobacterium pennavorans* (FRIEDRICH & ANTRANIKIAN, 1996). O potencial queratinolítico de bactérias representantes do domínio Archaea também vem sendo demonstrado (KUBLANOV et al., 2009a).

O interesse inicial em fungos queratinolíticos surgiu da importância médica e veterinária de representantes dermatófitos, como *Microsporum* e *Trichophyton* (FILIPELLO-MARCHISIO, 2000). Embora estudos relatem aplicações biotecnológicas para queratinases destes fungos (ANBU et al., 2006, 2008), sua utilização é limitada pelo potencial patogênico (KAUL & SUMBALI, 1997; BLYSKAL, 2009). Assim, atenção especial tem sido destinada ao potencial queratinolítico de representantes não-dermatofíticos, como *Aspergillus* (FARAG & HASSAN, 2004), *Trichoderma* (CAO et al., 2008), *Doratomyces*, *Paecilomyces* (GRADISAR et al., 2005), *Myrothecium* (MOREIRA-GASPARIN et al., 2009), *Scopulariopsis* (ANBU, et al. 2005), *Acremonium*, *Alternaria*, *Beauveria*, *Curvularia* e *Penicillium*, entre outros (MARCONDES et al. 2008; ELÍADES et al., 2010).

A maioria das queratinases, independentemente do microrganismo produtor, é extracelular, com massa molecular geralmente inferior a 50 kDa, sendo predominantemente classificadas como serino ou metaloproteases. Queratinases produzidas por microrganismos do gênero *Bacillus*, por exemplo, são comumente classificadas como serino proteases semelhantes à subtilisina (GUPTA & RAMNANI, 2006).

Estas enzimas usualmente apresentam atividade tanto sobre proteínas insolúveis (como queratinas, colágeno, elastina) quanto sobre proteínas solúveis (caseína, albumina, entre outras), normalmente hidrolisando as últimas mais eficientemente que as primeiras. Particularmente, a ineficiência de queratinases purificadas na hidrólise de queratina relaciona-se à presença de pontes dissulfeto no substrato e à incapacidade das queratinases em reduzir

estas ligações. É importante notar que diferenças estruturais entre as queratinas (α ou β; 'rígidas' ou 'leves') podem definir a capacidade de diferentes microrganismos e queratinases em degradar estes substratos. Sua atividade ótima é normalmente observada em temperaturas de 40-60 °C, e em valores de pH entre 7,5 e 9,0 (BRANDELLI, 2008; BRANDELLI et al., 2010).

2.4.1 Mecanismo de queratinólise microbiana

O mecanismo de queratinólise parece não envolver apenas a clivagem de ligações peptídicas pelas queratinases, mas também sistemas sulfitolíticos. Nas queratinas, o elevado índice de ligações dissulfeto confere às queratinas sua configuração rígida, representando a principal fonte de estabilidade e resistência à proteólise. O processo de sulfitólise compreende a redução das ligações dissulfeto, e evidências da ocorrência deste processo surgem do fato de que níveis elevados de queratinólise ocorrem somente com a clivagem destas ligações (BRANDELLI et al., 2010).

A hidrólise de ligações dissulfeto pode ocorrer pela ação de enzimas denominadas dissulfeto redutases, pela produção de sulfito e tiosulfato pelo microrganismo, ou mesmo através do potencial redutor das células microbianas (BÖCKLE & MÜLLER, 1997; YAMAMURA et al., 2002b; RAMNANI et al., 2005; RAMNANI & GUPTA, 2007). De forma análoga, a hidrólise de queratinas por queratinases purificadas é usualmente mais extensa na presença de agentes redutores que auxiliam na sulfitólise, indicando que a redução destas ligações não é normalmente realizada pelas queratinases (BÖCKLE et al., 1995; GRADISAR et al., 2005; RAMNANI & GUPTA, 2007; CAO et al., 2008). Na

ausência destes agentes, a degradação de queratinas atinge, tipicamente, apenas 10% (GUPTA & RAMNANI, 2006). Apesar da descrição de queratinases 'ativadas' por agentes redutores, o principal efeito destes agentes parece estar relacionado à modificação estrutural do substrato, permitindo a maior acessibilidade da enzima às moléculas de queratina (BRANDELLI et al., 2010). Contudo, Prakash et al. (2010b) relataram a purificação de serino proteases queratinolíticas, produzidas por *Bacillus halodurans* PPKS-2, capazes de reduzir pontes dissulfeto.

A proteólise da queratina é semelhante à hidrólise de outras proteínas, envolvendo a hidrólise das ligações peptídicas na proteína já parcialmente desestruturada pelo processo de sulfitólise (KUMAR & TAKAGI, 1999; BRANDELLI, 2008). Além disso, uma etapa denominada queratinólise mecânica se aplica a fungos e outros microrganismos produtores de micélio. Por este conceito, a degradação da queratina ocorre, inicialmente, como resultado da pressão exercida pela massa micelial e/ou pela penetração do micélio no substrato queratinoso. Esta etapa provavelmente precede a hidrólise enzimática, uma vez que o micélio é o produtor das proteases extracelulares (ONIFADE et al., 1998; MITOLA et al., 2002; BLYSKAL, 2009).

2.5 Aplicações de microrganismos queratinolíticos e queratinases

O bioprocessamento de materiais ricos em queratina é a principal aplicação postulada para microrganismos queratinolíticos e queratinases microbianas. Esta tecnologia oferece a possibilidade de manejo dos resíduos

queratinosos com a concomitante obtenção de produtos com valor agregado, através de processos de baixo custo e de baixo impacto ambiental. Queratinases microbianas também são consideradas como promissores biocatalisadores para diversos propósitos, incluindo aplicações em indústrias de rações, fertilizantes, detergentes, couros e têxtil, bem como aplicações nas áreas farmacêutica e biomédica (BRANDELLI et al., 2010).

A conversão de resíduos de origem animal (como penas) em ração animal pode ser considerada como uma das formas biologicamente mais eficientes para a reciclagem de nutrientes (FREEMAN et al., 2009). As desvantagens de processos hidrotérmicos na produção de rações aumentam o interesse na bioconversão de penas utilizando queratinases e/ou microrganismos queratinolíticos para utilização como suplemento alimentar na produção animal. Através da conversão microbiana, proteínas, aminoácidos e a própria biomassa microbiana são acrescidos aos hidrolisados protéicos produzidos a partir de resíduos queratinosos, originando produtos de elevado valor nutricional e de maior digestibilidade para utilização em rações (BERTSCH & COELLO, 2005; GOUSTEROVA et al., 2005; GRAZZIOTIN et al., 2006, 2007; JEONG et al., 2010a,b). Particularmente, hidrolisado protéico obtido a partir da degradação de penas por *Vibrio* sp. kr2, suplementado com metionina, foi capaz de substituir em até 20% a proteína de soja utilizada em experimentos de alimentação com ratos Wistar (GRAZZIOTIN et al., 2008).

Devido à sua ampla disponibilidade, baixo custo e elevado teor de nitrogênio (aproximadamente 15%) (ICHIDA et al., 2001; TIQUIA et al., 2005), resíduos queratinosos podem ser convertidos em hidrolisados protéicos para o

preparo de fertilizantes nitrogenados, originando produtos com valor agronômico e de fácil produção por processos economicamente viáveis (GOUSTEROVA et al., 2005; VASILEVA-TONKOVA et al., 2009; JEONG et al., 2010a,b). Hidrolisados obtidos a partir da degradação de penas por *Bacillus pumilis* HKS-1 demonstraram desempenho similar ao fertilizante referência em experimentos de crescimento vegetal com cenouras e couve chinesa (KIM et al., 2005). Produtos da degradação de queratina (de cascos e chifres bovinos) por uma queratinase de *Paecilomyces marquandii* apresentaram potencial para utilização como fertilizante foliar (VESELÁ & FRIEDRICH, 2009).

Proteases queratinolíticas podem ser adicionadas diretamente à dieta de animais monogástricos com o intuito de aumentar a digestibilidade de componentes protéicos em rações. A adição de queratinases microbianas como suplementos à dieta pode resultar no incremento do desenvolvimento animal. Neste sentido, a suplementação de rações com queratinase apresentou efeitos positivos no crescimento, digestibilidade de componentes da dieta, desenvolvimento intestinal, e rendimento de carne em frangos de corte (ODETALLAH et al., 2003, 2005; WANG et al., 2006, 2008b).

O processamento de couros envolve uma série de operações, e a etapa de pré-curtimento, que inclui a depilação do couro, contribui com aproximadamente 70% da poluição total do processo, representando um elevado risco ambiental. No processo de depilação tradicional, a utilização de sulfeto de sódio e cal resulta na completa gelatinização dos pêlos, aumentando as demandas biológica e química de oxigênio dos efluentes gerados (THANIKAIVELAN et al. 2004; PILLAI & ARCHANA, 2008). Alternativamente,

proteases queratinolíticas deficientes em atividade colagenolítica, e com atividade elastinolítica moderada, podem ser utilizadas na etapa de depilação de couros (FRIEDRICH & KERN, 2003; RIFFEL et al., 2003b; ANBU et al., 2005; MACEDO et al., 2005; GIONGO et al., 2007; PRAKASH et al., 2010a). Estas enzimas aceleram a depilação através do ataque enzimático às proteínas dos folículos pilosos, permitindo a fácil remoção dos pêlos intactos e, assim, reduzindo a carga poluente em relação aos processos tradicionais (FRIEDRICH et al., 2005; PILLAI & ARCHANA, 2008; JAOUADI et al., 2010; PILLAI et al., 2011; SHRINIVAS & NAIK, 2011).

Assim como outras proteases, queratinases podem ser empregadas como componentes de detergentes, visando a remoção de sujidades de origem protéica (PRAKASH et al., 2010a; RAI et al., 2009; XIE et al., 2010). A atividade e estabilidade em temperaturas e valores de pH prevalentes em condições de utilização, bem como a compatibilidade com ingredientes como surfactantes e agentes oxidantes, são pré-requisitos para a utilização de proteases em detergentes (VENUGOPAL & SARAMMA, 2006). Para tanto, experimentos de compatibilidade entre queratinases e detergentes vêm sendo realizados. Usualmente, queratinases alcalinas demonstram maior potencial para este tipo de aplicação, devido ao pH alcalino dos detergentes comerciais (JAOUADI et al., 2009; RAI et al., 2009; PRAKASH et al., 2010a; RAI & MUKHERJEE, 2011).

Queratinases microbianas também vêm sendo postuladas em outras aplicações, como utilização em cosméticos, medicamentos e produtos farmacêuticos para o tratamento de acne e psoríase, eliminação de calos e cicatrizes e regeneração de epitélios (VIGNARDET et al., 2001; GRADISAR et

al., 2005; MOHORCIC et al., 2007), na potencial degradação de príons, para a descontaminação de instrumentos médicos e equipamentos laboratoriais (LANGEVELD et al., 2003; MITSUIKI et al., 2006; YOSHIOKA et al., 2007), na modificação de fibras, como a lã, para aplicação na indústria têxtil (CAO et al., 2009; LV et al., 2010; CAI et al., 2011), modificação de propriedades funcionais de proteínas na indústria de alimentos, produção de peptídeos bioativos a partir da hidrólise de proteínas alimentares (CASARIN et al., 2008; CORRÊA et al., *no prelo*), remoção de obstruções em ralos e sistemas de tratamento de esgoto (TAKAMI et al., 1992; CHITTE et al., 1999), controle de nematódeos parasitas de plantas (YUE et al., *no prelo*), e síntese de peptídeos (JAOUADI et al., 2010). Ainda, queratina pode ser utilizada na produção de filmes, revestimentos e colas biodegradáveis (FRIEDRICH & ANTRANIKIAN, 1996; SCHROOYEN et al., 2001; MARTELLI et al., 2006), e produtos da queratinólise podem ser utilizados como substratos na produção de combustíveis como metano e biohidrogênio (ICHIDA et al., 2001; BÁLINT et al., 2005).

Em grande parte, as queratinases são utilizadas na forma livre (não-imobilizada); contudo, estudos apresentam a possibilidade da aplicação de enzimas imobilizadas em diversos suportes visando a hidrólise de queratinas e outras proteínas (LIN et al., 1996; WANG et al., 2003; FARAG & HASSAN, 2004) e a depilação de couros (KONWARH et al., 2009).

2.6 Produção de queratinases microbianas

A aplicação biotecnológica de proteases queratinolíticas requer a produção destas enzimas em grande quantidade para utilização comercial. O

primeiro estágio no desenvolvimento de qualquer processo de produção industrial envolve o isolamento de linhagens capazes de produzir a enzima desejada com rendimento superior, e mesmo com novas propriedades (KUMAR & TAKAGI, 1999). Microrganismos representam uma excelente fonte de enzimas para uso comercial devido à sua ampla diversidade bioquímica e susceptibilidade à manipulação genética. Tais organismos podem ser cultivados em grandes quantidades, apresentando rápido crescimento em período curto e em espaço limitado, originando o produto desejado de forma abundante e regular através de métodos de cultivo estabelecidos (RAO et al., 1998; GUPTA et al., 2002a,b; SANCHEZ & DEMAIN, 2011).

Microrganismos são responsáveis por dois terços da produção comercial mundial de proteases (GUPTA et al., 2002a). Contudo, o custo da produção (e também do processamento posterior) destas enzimas é um dos principais obstáculos para sua aplicação industrial (IYER & ANANTHANARAYAN, 2008). Particularmente, o meio de cultivo responde por 30-40% do custo total de produção de enzimas industriais (JOO & CHANG, 2005). Com isso, a busca por substratos alternativos, de baixo custo e de alta disponibilidade, pode contribuir para a diminuição dos custos de produção. Neste contexto surgem os resíduos agroindustriais, gerados em grandes quantidades, e de baixo valor comercial. Estes fatores têm atraído interesse na utilização destes resíduos como substratos em processos de produção de produtos microbianos de elevado valor agregado, como enzimas, pigmentos, bacteriocinas, entre outros (CLADERA-OLIVERA et al., 2004; DAROIT et al., 2007; SILVEIRA et al., 2008).

A produção de queratinases extracelulares é usualmente considerada indutível pela presença de materiais queratinosos no meio de cultivo (GUPTA & RAMNANI, 2006). O gene *kerA* de *Bacillus licheniformis* PWD-1, por exemplo, é expresso especificamente para a hidrólise de queratina, indicando que a presença de queratina como a única fonte de carbono e nitrogênio no meio de cultivo resulta na expressão preferencial de proteases queratinolíticas (LIN et al., 1995). Portanto, penas e outros resíduos queratinosos apresentam-se como substratos abundantes e de baixo custo para a obtenção de queratinases microbianas, resultando na redução dos custos de produção e representando uma forma alternativa de manejo destes resíduos (BRANDELLI et al., 2010).

Contudo, a produção de queratinases pode ocorrer também na ausência de queratina. Substratos como farinha de soja, gelatina, caseína, soro de queijo, amido, entre outros, podem estimular a produção de queratinases (GRADISAR et al., 2005; CASARIN et al., 2008; PILLAI et al., 2011; RAI & MUKHERJEE, 2011). A adição de fontes adicionais de carbono e/ou nitrogênio ao meio de cultivo apresenta efeito variável na produção de queratinases, dependendo do microrganismo, do substrato, do tipo e concentração do suplemento (BRANDELLI et al., 2010). Em alguns casos, a produção de queratinases é constitutiva (MANCZINGER et al. 2003; SON et al. 2008).

A produção de proteases geralmente exibe relação com o estágio do crescimento microbiano, sendo a produção máxima comumente atingida a partir da porção final da fase exponencial de crescimento e/ou fase estacionária (KUMAR & TAKAGI, 1999). O mesmo padrão é usualmente observado na

produção de queratinases por espécies de *Bacillus* (WERLANG & BRANDELLI, 2005; KOJIMA et al., 2006; KUMAR et al., 2008; PARK & SON, 2009; ZHANG et al., 2009), entre outros microrganismos (GUPTA & RAMNANI, 2006). Estes fatores indicam que a expressão de genes que codificam proteases pode ser regulada através de estresses nutricionais (KUMAR & TAKAGI, 1999). Assim, sugere-se que a produção de proteases extracelulares é uma manifestação da limitação de nutrientes no meio quando da chegada à fase estacionária (GUPTA et al., 2002b; VOIGT et al., 2007). Diversos mecanismos de regulação da expressão de proteases são relatados, como o repressor transcricional CodY (SONENSHEIN, 2005), o sistema DegS-DegU (MÄDER et al., 2002), entre outros (KLIER et al., 1992; JAN et al., 2000; VOIGT et al., 2007).

Pelo seu caráter macromolecular e insolúvel, a queratina é incapaz de entrar nas células. Portanto, substratos queratinosos podem atuar apenas indiretamente como indutores da produção de queratinases. Adiguzel et al. (2009) observaram, através do cultivo de duas linhagens de *Bacillus cereus* em meio com limitação de peptídeos (peptona) e suficiência de carbono (maltose), a produção sequencial de colagenases, elastases e queratinases, ou seja, proteases direcionadas a substratos com recalcitrância crescente, em resposta à alteração da disponibilidade nutricional no meio.

Mecanismos de repressão catabólica parecem também estar relacionados ao controle da produção de proteases/queratinases, uma vez que glicose e outros mono e dissacarídeos (frutose, maltose, sacarose), bem como fontes orgânicas de nitrogênio facilmente assimiláveis (peptona, aminoácidos) podem atuar na inibição da produção de queratinases (BRANDELLI et al.,

2010). A atuação positiva destes nutrientes no estado energético das células permite a ativação de proteínas/fatores capazes de ligar-se a operadores de genes responsáveis pela produção de proteases, impedindo sua transcrição (SCHURE et al., 1998; MORENO et al., 2001; GÖRKE & STÜLKE, 2008).

A produção de múltiplas proteases é comumente observada durante o cultivo de microrganismos queratinolíticos (GIONGO et al., 2007; MAZOTTO et al., 2009; RIFFEL et al., 2011), indicando que mecanismos complexos de indução da expressão podem atuar ao mesmo tempo no controle da produção de diferentes enzimas visando o melhor aproveitamento dos substratos para a nutrição microbiana. Esta alta variabilidade implica que a composição do meio de cultivo deve ser determinada para cada situação específica (CAI & ZHENG, 2009). Da mesma forma, condições de temperatura, pH, agitação e inóculo podem afetar potencialmente a produção de queratinases (FAKHFAKH-ZOUARI et al., 2010b).

2.6.1 Produção heteróloga de queratinases microbianas

Técnicas moleculares de clonagem e expressão heteróloga vêm sendo empregadas no intuito de aumentar a produção de queratinases (LIN et al., 1997; RADHA & GUNASEKARAN, 2007, 2009). Sequências codificantes para queratinases de *Streptomyces fradiae* var. 11 (LI et al., 2007), *Bacillus licheniformis* MKU03 (RADHA & GUNASEKARAN, 2009), *Bacillus licheniformis* PWD-1 (PORRES et al., 2002) e *Pseudomonas aeruginosa* (LIN et al., 2009a) foram clonadas e expressas em *Pichia pastoris*. A expressão de queratinase de *Bacillus licheniformis* MKU03 em *Bacillus megaterium* (RADHA &

GUNASEKARAN, 2008), da queratinase de *Bacillus licheniformis* ER-15 em *Escherichia coli* (TIWARY & GUPTA, 2010), e de queratinase de *Pseudomonas aeruginosa* em *Escherichia coli* (LIN et al., 2009b) e *Bacillus subtilis* (LIN et al., 2009b) também são reportadas. Técnicas de integração de múltiplas cópias de genes de queratinases em cromossomos (WANG et al., 2004) ou plasmídeos (ZAGHLOUL, 1998) também vêm sendo investigadas. Tais técnicas contribuem para a utilização do potencial de queratinases produzidas por microrganismos patogênicos, como *Aspergillus fumigatus* (NORONHA et al., 2002) e *Pseudomonas aeruginosa* (LIN et al., 2009a; SHARMA & GUPTA, 2010a).

Um dos inconvenientes da produção heteróloga é a necessidade de processamento pós-traducional observado para algumas queratinases. No caso da queratinase de *Bacillus licheniformis* PWD-1 clonada em *Pichia pastoris*, a expressão não foi observada nos transformantes contendo apenas a sequência codificante para a queratinase madura, destacando a importância do pro-peptídeo na produção de enzima (PORRES et al., 2002). Logo, a maioria dos estudos sobre a produção heteróloga de queratinases realiza a clonagem de sequências que codifiquem a pro-enzima (LIN et al., 1997; LI et al., 2007; RADHA & GUNASEKARAN, 2008), uma vez que a presença do pro-peptídeo está relacionada ao processamento correto do arranjo tridimensional (conformação) da enzima ativa (LIN et al., 2009a,c).

2.7 Estabilidade enzimática

Enzimas são catalisadores altamente seletivos e eficientes; contudo, sua complexa estrutura molecular as torna inerentemente instáveis. O interesse

industrial na estabilidade enzimática deve-se ao caráter dos bioprocessos, que normalmente são realizados em condições diferentes das fisiológicas. Desta forma, além da atividade, a estabilidade de enzimas está diretamente relacionada à aplicação destes biocatalisadores, representando assim um tema crítico para a biotecnologia (IYER & ANANTHANARAYAN, 2008).

Diversos fenômenos podem levar à inativação enzimática, incluindo autólise, agregação e desnaturação devido à exposição da enzima a solventes, extremos de temperatura e pH, entre outros (LADERO et al., 2006). Processos de desnaturação envolvem a desorganização da estrutura terciária da enzima, onde resíduos de aminoácidos não estão mais próximos o suficiente para participação em interações funcionais ou estruturais (Ó'FÁGÁIN, 1995). Particularmente, a temperatura apresenta efeitos opostos sobre a atividade e estabilidade enzimáticas. A temperatura ocasiona o aumento da atividade enzimática até um ponto máximo, a partir do qual o incremento na temperatura provoca a desnaturação protéica. Logo, a temperatura é variável crucial em qualquer processo biocatalítico (THOMAS & SCOPES, 1998; ILLANES, 1999).

O termo *estabilidade* refere-se à resistência da proteína frente a influências adversas, isto é, a persistência de sua integridade molecular ou funcão biológica quando em contato com altas temperaturas ou outros agentes. Neste ponto, podem ser diferenciadas a estabilidade termodinâmica (conformacional) e a estabilidade cinética. A primeira trata da presença de pequenas quantidades da enzima em um estado reversível de (parcial) desnaturação, em equilíbrio com a conformação nativa; enquanto que a última mensura a barreira energética (resistência) que separa a enzima nativa da

inativação irreversível, ou seja, a manutenção da atividade observável (SANCHEZ-RUIZ, 2010). Ambas as definições podem ser representadas pela relação:

$$N \overset{K}{\longleftrightarrow} U \xrightarrow{k} I$$

onde N representa a conformação nativa, U o estágio de desnaturação reversível e I a forma da enzima inativada de forma irreversível. *K* indica a constante de equilíbrio da transição N↔U (estabilidade termodinâmica), enquanto que *k* é a constante da taxa da reação irreversível U→I (estabilidade cinética; Ó'FÁGÁIN, 2003).

A atividade residual das enzimas é o parâmetro fundamental para a aplicação industrial de enzimas. Assim, do ponto de vista biotecnológico, a maior preocupação reside na estabilidade cinética, ou seja,

$$N \xrightarrow{k} I$$

pois implica diretamente na perda da atividade enzimática (EIJSINK et al., 2004; SANCHEZ-RUIZ, 2010). De fato, a estabilidade operacional do biocatalisador determina a viabilidade do processo, sendo razoáveis, portanto, investigações sobre a estabilidade enzimática (ILLANES, 1999). Normalmente, este tipo de inativação enzimática segue uma cinética de primeira-ordem, que propõe a transformação da enzima nativa ativa em uma forma completamente inativa em uma única etapa, indicando que a perda de atividade é controlada por um fenômeno, que define a constante geral de inativação. Este mecanismo é representado pelo decaimento exponencial na atividade enzimática. Contudo,

caso diferentes fenômenos apresentem constantes similares, rotas de inativação previamente ocultas pelo mecanismo anterior podem ser avaliadas, sendo necessários modelos mais complexos, que descrevem a inativação enzimática, por exemplo, através de mecanismos paralelos ou em série (AYMARD & BELARBI, 2000; LADERO et al., 2006 IYER & ANANTHANARAYAN, 2008).

A predição da inativação cinética é essencial na caracterização da enzima. Neste sentido, modelos matemáticos, equações que fornecem um resultado a partir de uma entrada de dados, são ferramentas importantes que permitem a descrição do comportamento de biomoléculas durante a inativação sob a forma de termos matemáticos (VAN BOEKEL, 2008). Esta abordagem matemática permite avaliar o efeito de diversos agentes sobre a estabilidade do biocatalisador, de forma a modelar a atividade residual em relação ao tempo de inativação e à concentração/intensidade dos respectivos agentes (LADERO et al., 2006). Logo, um modelo cinético que defina de forma satisfatória a inativação enzimática pode atuar na otimização do processo, que por sua vez pode ser refletida na diminuição de custos operacionais.

Diversas técnicas de estabilização de enzimas vêm sendo investigadas para minimizar os efeitos deletérios dos mecanismos de inativação. Entre os principais métodos para a obtenção de enzimas com aumentada estabilidade, podem ser citados: o isolamento de novas enzimas produzidas por microrganismos extremófilos; produção heteróloga destas enzimas em microrganismos mesófilos; engenharia de proteínas; estabilização

de biocatalisarores conhecidos através de modificações físicas (imobilização), químicas, uso de aditivos (Ó'FÁGÁIN, 1995, 2003).

As condições do meio reacional são importantes para a minimização da instabilidade enzimática. Neste contexto, um dos métodos mais empregados na estabilização de enzimas, por sua simplicidade e eficácia, é a utilização de aditivos, como é o caso da adição de sais (ILLANES, 1999; IYER & ANANTHANARAYAN, 2008). Particularmente, cátions podem atuar na estabilização de proteases microbianas através da ligação a sítios específicos na estrutura terciária destas enzimas (BRYAN, 2000; ALEXANDER et al., 2001; SILVEIRA et al., 2010).

Enzimas estáveis/estabilizadas permitem sua aplicação em temperaturas elevadas, que podem apresentar efeitos benéficos nas taxas de reação e solubilidade de reagentes, diminuição da viscosidade do meio reacional e do risco de contaminação microbiana (AYMARD & BELARBI, 2000; EIJSINK et al., 2004). Portanto, a investigação de parâmetros de estabilização cinética representa um mecanismo para a otimização das propriedades funcionais dos biocatalisadores, refletindo diretamente nos processos enzimáticos (IYER & ANANTHANARAYAN, 2008; SANCHEZ-RUIZ, 2010).

3 RESULTADOS E DISCUSSÃO

Os resultados deste estudo estão apresentados sob a forma de quatro artigos científicos. Os referidos artigos são apresentados nas Seções 3.1 a 3.4, e identificados como Resultados I a Resultados IV, respectivamente.

O primeiro artigo (Seção 3.1), que versa sobre o potencial queratinolítico de *Bacillus* sp. P45, tem como título “Keratinolytic potential of a novel *Bacillus* sp. P45 isolated from the Amazon basin fish *Piaractus mesopotamicus*”, e foi publicado no periódico *International Biodeterioration & Biodegradation*, v. 63, p. 358-363, 2009.

No segundo artigo (Seção 3.2), “Production of keratinolytic proteases through bioconversion of feather meal by the Amazonian bacterium *Bacillus* sp. P45”, é apresentada a produção de queratinases extracelulares pela referida linhagem em diferentes substratos através de cultivos submersos. Ainda, a otimização da produção de queratinases no meio de cultivo selecionado é relatada. Este artigo foi publicado no periódico *International Biodeterioration & Biodegradation*, v. 65, p. 45-52, 2011.

O terceiro artigo (Seção 3.3) trata da purificação e caracterização de uma queratinase extracelular produzida por *Bacillus* sp. P45. Este artigo, “Characterization of a keratinolytic protease produced by the feather-degrading

Amazonian bacterium *Bacillus* sp. P45", foi publicado no periódico *Biocatalysis and Biotransformation*, v. 28, p. 370-379, 2010.

No quarto artigo (Seção 3.4) são avaliadas a atividade e a estabilidade térmica da queratinase purificada, incubada em diferentes condições de temperatura e na ausência/presença de íons metálicos. Com base nos dados obtidos, e utilizando diferentes equações, a modelagem da cinética de inativação da enzima foi realizada. Este artigo, "Kinetic stability modeling of keratinolytic protease P45: effects of temperature and metal ions", foi recentemente submetido ao periódico *Applied Biochemistry and Biotechnology* para avaliação.

3.1 Resultados I

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Short communication

Keratinolytic potential of a novel *Bacillus* sp. P45 isolated from the Amazon basin fish *Piaractus mesopotamicus*☆

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ABSTRACT

Bacillus sp. P45, isolated from the intestine of the Amazon basin fish *Piaractus mesopotamicus*, showed proteolytic activity when grown on skimmed milk and feather meal agar plates. The keratinolytic potential of this strain was evaluated on whole feather broth and human hair broth. *Bacillus* sp. P45 degraded almost 90% of chicken feathers after 72 h of submerged cultivation on whole feather broth, and the production of extracellular proteases was observed. The formation of thiol groups was also detected during growth, indicating the contribution of sulphitolysis to the efficient hydrolysis of feather keratin. Nevertheless, *Bacillus* sp. P45 was unable to degrade hair keratin, possibly due to the conformational diversity of this substrate in comparison to feather keratin. Additionally, preliminary results demonstrated that this strain might be utilized in the degradation of recalcitrant collagen-containing wastes. The keratinolytic character of *Bacillus* sp. P45 might be utilized in environmental-friendly processes such as bioconversion of waste feathers, representing an alternative way of waste management that could lead to the production of value-added products such as microbial biomass, protein hydrolysates and proteolytic enzymes.

1. Introduction

Keratins are stable and insoluble structural proteins found in the epidermis of vertebrates and its appendages, such as feathers and hair. The α-helix (α-keratin) or β-sheet (β-keratin) keratin chains are tightly packed into a supercoiled polypeptide chain extensively cross-linked with disulfide bridges, hydrogen bonds and hydrophobic interactions, resulting in the mechanical stability of keratin and its recalcitrance to common proteolytic enzymes such as pepsin, trypsin and papain (Onifade et al., 1998).

Keratin-rich wastes, mainly in the form of feathers and hair, are generated in high amounts as byproducts of agroindustrial processing, and the accumulation of such residues could lead to environmental problems (Gupta and Ramnani, 2006). However, despite its recalcitrance, keratin can be efficiently hydrolyzed by keratinolytic enzymes produced by a multitude of bacteria and fungi (Friedrich et al., 1999; Lucas et al., 2003) and, particularly, representatives of the bacterial genus *Bacillus* appear as prominent keratinolytic microorganisms (Manczinger et al., 2003; Macedo et al., 2005; Kumar et al., 2008). The microbial degradation of the insoluble keratin macromolecules depends on the secretion of enzymes with the ability to act on the surface of these substrates. In this sense, keratinases are reported to be mainly extracellular, and the production of such enzymes is generally induced by keratinous substrates such as feathers (Böckle et al., 1995; Gupta and Ramnani, 2006).

Therefore, the bioconversion of keratinous residues is attracting increasing biotechnological interest since it might represent an alternative way of waste management that could be coupled with the production of valuable products (Brandelli, 2008). The physicochemical diversity of habitats has challenged nature to develop equally numerous molecular adaptations in the microbial world; thus, microbial diversity is a major resource for biotechnological products and processes (Gupta et al., 2002a). Although microorganisms capable to degrade keratinous substrates are generally isolated from soil and poultry wastes (Riffel and Brandelli, 2002, 2006; Brandelli, 2008), these microorganisms are almost ubiquitous in nature, thriving under diverse ecological and environmental

☆ Scientific relevance: This manuscript describes a novel feather degrading bacterium, which presents potential application in biotechnological processes involving the biodegradation of keratinous material. This microorganism appears to cause reduction of disulfide bonds during growth on feathers, which is required for extensive keratinolysis. This new keratinolytic bacterium shows high feather degradation at room temperature. Such microorganism will be less energy consuming than the thermophilic strains usually used in feather processing.

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conditions (Onifade et al., 1998). The Amazonian environment is a source of enormous biodiversity and, in this context, the aim of the present study was to investigate the keratinolytic potential of *Bacillus* sp. P45, previously isolated from the intestine of the Amazon basin fish *Piaractus mesopotamicus*, which was already reported to produce an antimicrobial peptide (Sirtori et al., 2006).

2. Materials and methods

2.1. Microorganism

Bacillus sp. P45 was utilized in this study. This strain was assigned the number LBM 1055 and deposited at the culture collection of Food Science and Technology Institute (Porto Alegre, Brazil). Cultures were routinely maintained at 4 °C on brain-heart agar (BHA) plates, and subcultured periodically.

2.2. Qualitative evaluation of protease production

Protease production was qualitatively detected by inoculating *Bacillus* sp. P45 on skim milk agar (SMA) plates (Riffel and Brandelli, 2006). SMA contained peptone (5 g/L), yeast extract (3 g/L), UHT skim milk (100 mL/L) and agar (12 g/L). After incubation at 30 °C for 24 h, the presence of clear haloes around *Bacillus* sp. P45 colonies was evaluated, indicating the production of proteolytic enzymes.

The ability of *Bacillus* sp. P45 to grow on feather meal was evaluated on feather meal agar (FMA), prepared as described elsewhere (Riffel and Brandelli, 2002). The isolate was streaked on FMA plates and incubated at 30 °C for up to 3 days.

2.3. Submerged cultivation on keratinous wastes

The submerged cultivation of *Bacillus* sp. P45 was performed on feather broth (FB) and human hair broth (HHB). FB contained NaCl (0.5 g/L), K_2HPO_4 (0.3 g/L), KH_2PO_4 (0.4 g/L) and whole feathers (10 g/L), whereas HHB contained NaCl (0.5 g/L), K_2HPO_4 (0.3 g/L), KH_2PO_4 (0.4 g/L) and human hair (10 g/L). Feathers and hair were washed with 0.1% (v/v) Triton X-100, and then abundantly with distilled water prior to being used. Initial pH of FB and HBB was adjusted to 7.0 before sterilization by autoclaving at 121 °C, 105 kPa for 15 min. Erlenmeyer flasks (250 mL) containing 50 mL of FB or HHB were inoculated with 500 μL (1%, v/v) of a bacterial suspension ($O.D._{600}$ of 0.5) and incubated at 30 °C in an orbital shaker (125 rpm) for up to 7 days.

2.3.1. Degradation of keratinous wastes

The degradation of feathers and human hair was evaluated by the dry weight of the keratinous wastes remaining on culture broths during incubation. The culture broths were filtered through pre-weighed Whatman no. 1 filter paper and dried at 60 °C until constant weight. Results were expressed as percentage of the initial weight (considered as 100%) of the respective keratinous waste. Additionally, alterations on feather structure during incubation were accompanied by scanning electron microscopy.

2.3.2. Determination of soluble protein

Culture broth filtrates were centrifuged (10,000 × *g* for 10 min) and supernatants were utilized for determining the soluble protein concentration by the method of Lowry et al. (1951).

2.3.3. Enzyme assays

Culture supernatants were utilized for all the enzyme assays. Proteolytic activity was assayed using azocasein as substrate. The reaction mixture (500 μL) contained 100 μL Tris–HCl buffer (100 mM, pH 8.0), 300 μL of 1% (m/v) azocasein (in Tris–HCl buffer) and 100 μL of conveniently diluted enzyme. After incubation at 40 °C for 30 min the reaction was stopped by adding 600 μL of 10% (m/v) trichloroacetic acid (TCA), and the reaction mixture was further incubated at 4 °C for 10 min. After centrifugation (10,000 × *g* for 5 min) of the reaction mixture, 800 μL of the supernatant was mixed with 200 μL of 1.8 N NaOH, and the absorbance at 420 nm was measured. One unit (U) of protease activity was defined as the amount of enzyme that caused an increase of 0.1 absorbance unit at the defined assay conditions. Keratinolytic activity was assayed by a similar protocol utilizing azokeratin as substrate.

Collagenolytic activity was also evaluated using insoluble hide powder azure as substrate. Aliquots of 500 μL of the culture supernatants were incubated in a solution of 5 mg of hide powder azure in 500 μL of 100 mM Tris–HCl buffer, pH 8.0 for 15 min at 40 °C. After centrifugation (10,000 × *g* for 5 min) the absorbance of the supernatants was measured at 595 nm. One unit (U) of enzyme activity was defined as the amount of enzyme that resulted in an increase of 0.1 absorbance unit at the assay conditions.

2.3.4. Determination of thiol formation

Determination of free thiol groups was performed as described by Sangali and Brandelli (2000). Briefly, 200 μL of NH_4OH, 1 mL of 0.5 g/L NaCN and 1 mL distilled water were added to 1 mL of culture supernatant. This mixture was incubated at 25 °C for 20 min and then 200 μL of 0.5 g/L sodium nitroprusside was added. Absorbance at 530 nm was determined within 2 min.

2.3.5. Zymography

Zymogram analysis was performed as previously described (Giongo et al., 2007), with modifications. Culture supernatants were electrophoresed at room temperature on 16% polyacrylamide gels containing gelatin (1 mg/mL) or collagen (1 mg/mL). After electrophoresis, the gels were washed twice with 20 mM Tris–HCl buffer (pH 8.0) containing 2.5% (v/v) Triton X-100 for 30 min, and then with the same buffer without detergent for 60 min. After 12 h of incubation at 30 °C in Tris–HCl buffer, the gels were stained with Coomassie Brilliant Blue R-250. Protease bands appeared as clear zones on a blue background due to gelatin/collagen hydrolysis.

3. Results and discussion

Protein agar plate assays are commonly utilized for the initial qualitative screening of proteolytic activity (Gupta et al., 2002b). Therefore, protease production by *Bacillus* sp. P45 was evaluated employing the SMA assay, and clear proteolysis zones were observed around the colonies of this strain after 24 h of incubation at 30 °C. This isolate was also capable of growing on FMA plates, suggesting that *Bacillus* sp. P45 could use feather keratin as growth substrate (results not shown).

Bacillus sp. P45 was inoculated on feather broth to evaluate its keratinolytic potential. This strain showed to degrade whole feathers during the submerged cultivations, demonstrating that it could utilize feathers as the only source of carbon, nitrogen, sulfur and energy for growth. It was observed that only 12% of the initial feather weight remained after 3 days of cultivation (Fig. 1). Feather degradation in the current study was higher than that reported for *Bacillus* sp. P-001A, which hydrolyzed 60% of feather after 5 days (Atalo and Gashe, 1993), and that for *Bacillus pumilus* F3-4 which degraded 75% of chicken feathers after 7 days of cultivation (Son et al., 2008).

Scanning electron microscopy was utilized to examine structural changes during feather degradation. Controls are shown in Fig. 2(A and B). The partial degradation of feather barbules after 24 h of incubation with *Bacillus* sp. P45 was observed (Fig. 2C).

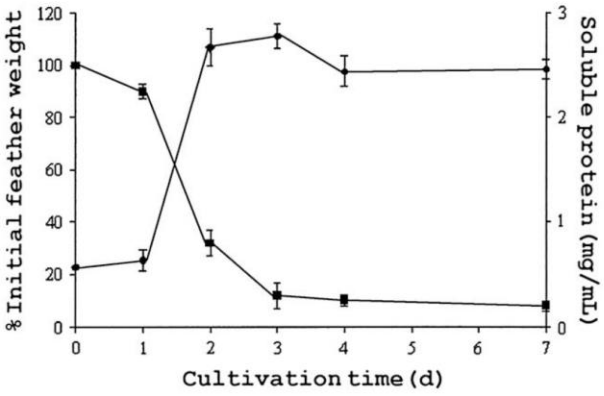

Fig. 1. Feather degradation during growth of *Bacillus* sp. P45 on whole feather broth (■); concentration of soluble protein on culture supernatants during cultivation (◆).

Barbules were almost completely degraded after a 48 h incubation period, and the disintegration of the feather rachis structure was observed (Fig. 2D).

Feather disintegration was concomitant with the increase in soluble protein concentration on culture supernatants (Fig. 1). Although it should be noted that part of the solubilized feather keratin was converted into microbial biomass, and that part of the protein in the culture supernatant has origin on the secretion of proteins/enzymes by the microorganism, the steep increase on soluble protein observed at day 2 is primarily attributed to feather keratin degradation. Accordingly, the increase in soluble protein concentration in fermentation broth is generally employed as a measurement of feather digestion (Szabó et al., 2000).

Feathers are generated in large amounts by the commercial poultry processing and its deposition into the environment could have adverse effects. For instance, during the uncontrolled keratin decomposition, especially by anaerobic bacteria, large quantities of toxic substances such as hydrogen sulfide and ammonia are released (Singh, 2002). However, since this biomass is composed of approximately 90% keratin, it represents a potential source of proteins for the production of value-added products. Currently, waste feathers are converted through steam pressure cooking into feather meal, which is utilized as an ingredient in animal feed; nevertheless, this process is expensive and negatively affects both protein quality and digestibility of the final product (Onifade et al., 1998). In this sense, the microbial/enzymatic hydrolysis may be a viable alternative to enhance the nutritional value of feathers as an ingredient in animal feed or as an organic fertilizer (Grazziotin et al., 2006, 2007).

Extracellular protease and keratinase were produced by *Bacillus* sp. P45 during growth on FB, with both enzyme activities reaching maximum values at 2 days of cultivation, decreasing thereafter (Fig. 3). Since poultry feathers are inexpensive, abundant and readily available, the utilization of such byproducts as substrates by keratin-degrading microorganisms offers a feasible microbial technology for obtaining proteolytic enzymes (Bernal et al., 2003). Although azocaseinolytic activity was much higher when compared to the azokeratinolytic activity, both activities showed to be related (Fig. 3). Thus, *Bacillus* sp. P45 keratinase activity could be monitored by using soluble azocasein rather than the insoluble azokeratin. Similarly, Cheng et al. (1995) reported that the protease productivities of *Bacillus licheniformis* PWD-1, estimated by caseinolytic or keratinolytic activity assays, were well correlated. In fact, caseinolytic activity assays provided accurate results in comparison with keratinolytic activity assays using insoluble keratin or feather powder (Cheng et al., 1995).

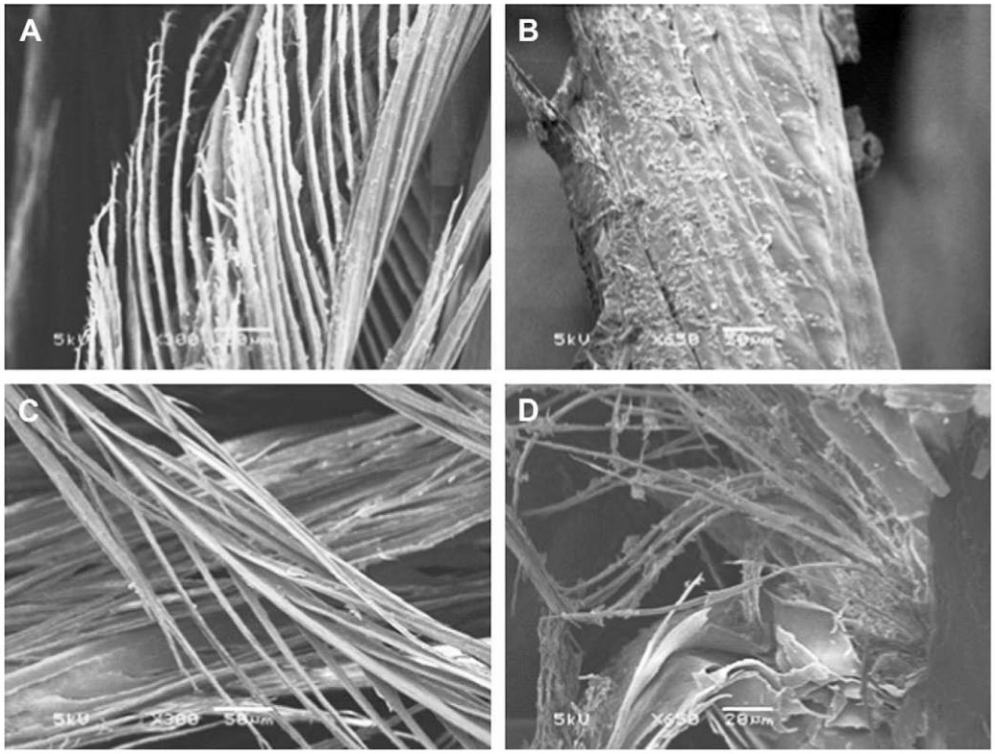

Fig. 2. Changes in feather structure during growth of *Bacillus* sp. P45. (A) and (B) Undegraded feathers. (C) Partial degradation of feather barbules after 24 h of incubation. (D) Disintegration of feather rachis after 48 h of incubation.

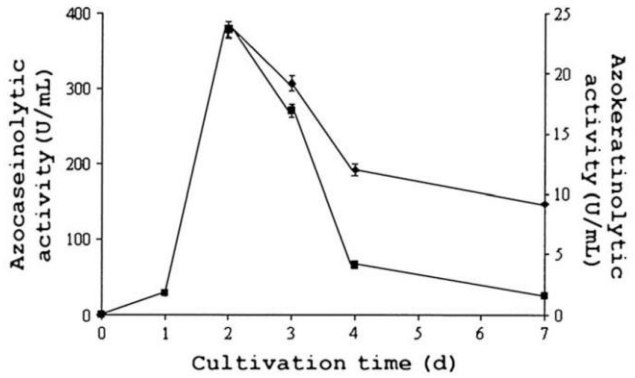

Fig. 3. Azocaseinase (■) and azokeratinase (◆) activities produced by *Bacillus* sp. P45 during growth on whole feather broth.

It is interesting to note that even though fermentations leading to keratinase production are accompanied by subsequent degradation of the keratin substrate, the kinetics of keratinase production usually do not overlap with that of keratin degradation (Gupta and Ramnani, 2006). For instance, in the present study, while highest enzyme production was achieved at the 2nd day of cultivation (Fig. 3), maximum feather degradation was observed at days 3–4 (Fig. 1), suggesting that a complex mechanism controls keratinolysis in *Bacillus* sp. P45.

Feather broth pH increased from 7.1 to 8.6 after 3 days of cultivation with *Bacillus* sp. P45 (Fig. 4). Similarly, Sangali and Brandelli (2000) reported that the growth of *Vibrio* sp. strain kr2 on whole feathers increased the medium pH from 7.0 (or 6.0) to 8.0 after 72 h of cultivation. The increase in culture medium pH during cultivation is an important characteristic accompanying keratin hydrolysis and the keratinolytic potential of microorganisms. In fact, microorganisms with strong keratinolytic ability render the culture medium more alkaline than those that were less keratinolytic (Kaul and Sumbali, 1997). This tendency to increase the pH of the culture medium results from the production of ammonia by means of the deamination of peptides and amino acids originating from keratin degradation; the resulting pH increase is typical of microorganisms growing on protein substrates (Kumar et al., 2008). Therefore, the pH increase during cultivation indicates the remarkable keratinolytic feature of *Bacillus* sp. P45.

The formation of thiol groups was observed to increase during cultivation on FB, reaching its maximum after 3 days (Fig. 4). Thiol

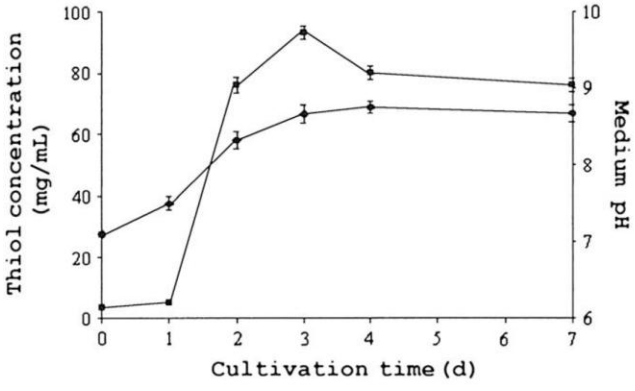

Fig. 4. Formation of thiol groups (■) and variation of medium pH (◆) during cultivation of *Bacillus* sp. P45 on whole feather broth.

formation suggests the presence of disulphide reductase activity along with keratinase activity (Sangali and Brandelli, 2000; Kumar et al., 2008). In this context, Yamamura et al. (2002) proposed a mechanism for extracellular enzymatic keratin degradation by bacteria (or possibly even fungi) in which the disulfide bonds in keratin are attacked by disulfide reductase-like proteins first, yielding a partially chopped protein that is a good substrate for the subsequent action of proteases. In this perspective, Ramnani and Gupta (2007) emphasized the need of a suitable redox environment during keratinolysis by keratinases. Accordingly, the efficiency of feather hydrolysis by filter-sterilized culture supernatants from *Bacillus subtilis* P13 was lower in the absence of β-mercaptoethanol; however, unfiltered enzyme preparations were able to disintegrate feathers in the absence of the reducing agent, suggesting the role of a cell-bound redox system for disulfide bond reduction (Pillai and Archana, 2008). The important role of the reduction of disulfide bonds (sulfitolysis) on keratin hydrolysis is further supported by the fact that keratin degradation by purified keratinases *in vitro* is only accomplished by the addition of reducing agents that help in sulfitolysis (Gupta and Ramnani, 2006).

Production of extracellular proteases by *Bacillus* sp. P45 during growth on FB was also evaluated by gelatin (Fig. 5a) and collagen zymograms (Fig. 5b). It could be observed that various proteolytic enzymes were produced by this strain. Although identification of specific keratinase band(s) was impractical on gels, each protease may contribute to some extent to keratin degradation, since the initial attack by keratinases and disulfide reductases allows other less specific proteases to act, resulting in an extensive keratinolysis (Brandelli, 2008).

Besides utilization on animal feed and fertilizer industries, various other commercial applications are proposed for microbial keratinases. These enzymes are potentially valuable for the detergent, leather, cosmetic, biopolymer and pharmaceutical industries (Brandelli, 2008). The exploitation of proteases/keratinases by the leather industry, particularly in de-hairing processes, is a promising and environmental-friendly alternative to replace the current chemical methods that employ sodium sulfide; however, the risk of collagen degradation hinders its commercial utilization (Thanikaivelan et al., 2004). Collagen degradation during the de-hairing process may result from the substrate specificity of the enzyme, as some protease may act on collagenic proteins; and from the production of more than one extracellular protease by the isolate, as some of these enzymes may lead to the damage of collagen (Wang et al., 2007). The activity bands detected on collagen zymograms (Fig. 5B), although collagen might be partially denatured by electrophoresis conditions, suggest that *Bacillus* sp. P45 produces collagenolytic proteases. Additionally, hydrolysis of insoluble hide powder azure was observed (result not shown). Such dye-impregnated hide powders are utilized as collagenase substrates (Wolf and Wirth, 1996; Hülsmann et al., 2003; Tsuruoka et al., 2003), reinforcing the collagenolytic character of *Bacillus* sp. P45 proteases, which might impair its utilization in de-hairing processes. Contrarily, Macedo et al. (2005) reported that a keratinase from *B. subtilis* S14 was unable to degrade collagen in both enzyme assays and depilation tests. Although the collagen-degrading activity of *Bacillus* sp. P45 needs to be further investigated, these preliminary results indicate that this strain might have potential applications on the bioconversion of collagen-containing wastes, since these residues are generated in enormous quantities by the meat industry and its disposal is extremely difficult (Suzuki et al., 2006).

Bacillus sp. P45 was unable to significantly degrade human hair during a 7-day cultivation period on liquid media (result not shown). Contrarily, *B. pumilus* was showed to solubilize nearly 60% of bovine hair after 16 days of cultivation (Kumar et al., 2008);

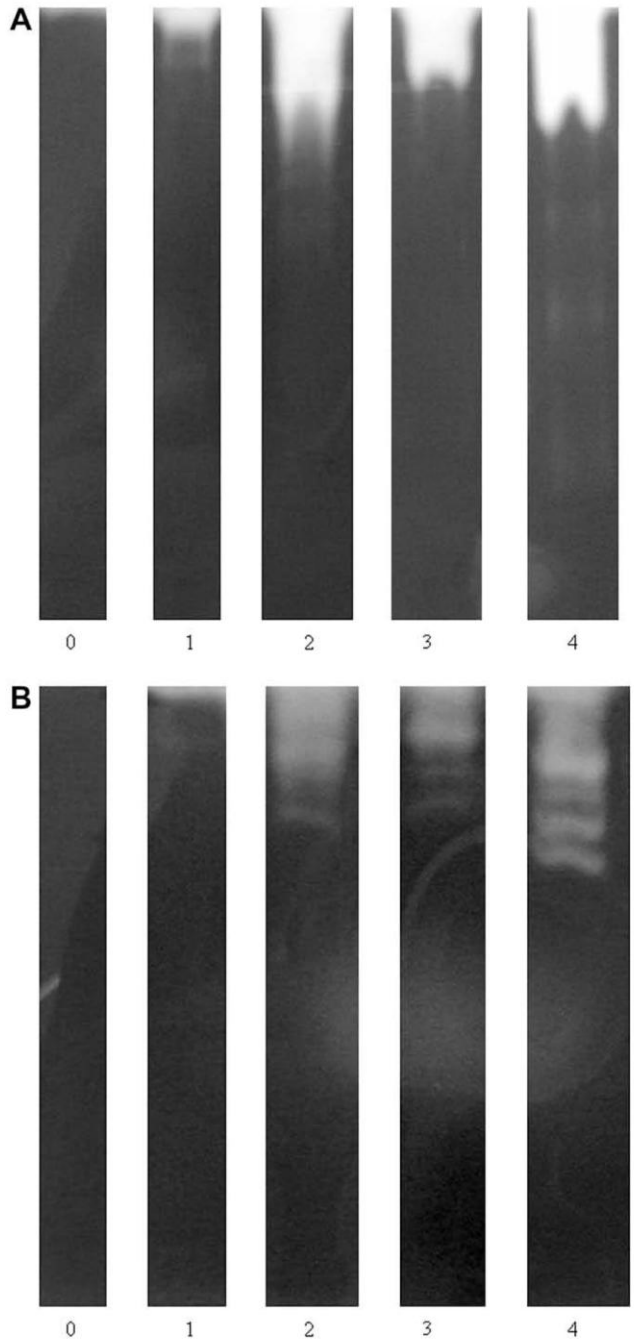

Fig. 5. Zymogram analysis of extracellular protease produced by *Bacillus* sp. P45 on whole feather broth. (A) Gelatin zymogram; (B) Collagen zymogram. Lanes 0–4 correspond to cultivation periods of 0–4 days, respectively.

whereas *B. pumilus* F3-4 degraded 9% of human hair after 7 days of cultivation (Son et al., 2008). Usually, the rate of hair keratin degradation is inferior to that of feather keratin. On the basis of secondary structural conformation, keratins are classified into α-keratin (α-helix) and β-keratin (β-sheet); the former is the major component of hair and wool, whereas the latter is the main constituent of feathers (Onifade et al., 1998). These structural features might explain the differences between hair and feather degradation by *Bacillus* sp. P45.

4. Conclusion

A novel *Bacillus* sp. P45, isolated from the intestine of an Amazonian fish, produced extracellular proteases and efficiently degraded feather keratin during submerged cultivations with whole feathers as the only source of carbon, nitrogen, sulfur and energy. The formation of thiol groups was also observed, suggesting that the production of disulphide reductase(s) contributes to efficient keratin hydrolysis. However, this isolate was not able to degrade hair keratin, probably due to structural differences when compared to feather keratin. Preliminary results showed that the crude protease produced by this strain might degrade collagen, suggesting its utilization in the degradation of collagen-rich wastes. The keratinolytic feature of this mesophilic strain could be potentially employed in the biodegradation of keratinous wastes generated by agroindustrial processing (especially waste feathers), producing value-added products such as protein hydrolysates for utilization as animal feeds or fertilizers, microbial biomass and proteolytic enzymes through an energy-saving process. Studies on the characterization of the proteolytic enzymes produced by *Bacillus* sp. P45 will be performed.

Acknowledgments

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3.2 Resultados II

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Production of keratinolytic proteases through bioconversion of feather meal by the Amazonian bacterium *Bacillus* sp. P45

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ABSTRACT

Extracellular keratinase production by the feather-degrading Amazonian isolate *Bacillus* sp. P45 was evaluated with various growth substrates. Higher enzyme production occurred with feather meal (FM) in comparison to casein, gelatin, and cheese whey, suggesting the specificity of this strain for the utilization of keratinous substrates. Supplementation of FM medium with carbohydrates reduced enzyme production, probably due to catabolite repression. Increased keratinase yield was achieved when NH_4Cl was added to FM medium. The effects of FM and NH_4Cl concentrations on enzyme production were investigated using a 2^2 central composite design. Feather meal was the most significant parameter, while NH_4Cl concentrations resulted in slight differences in enzyme yield. In the range studied, optimal concentrations of FM and NH_4Cl were 43–50 g l^{-1} and 1.8–8.6 g l^{-1}, respectively, resulting in an effective low-cost medium for the production of keratinolytic protease. Crude keratinase showed maximum activity at 50 °C and pH 7.0, and was strongly inhibited by EDTA, indicating the importance of metal ions for activity/stability. The crude keratinase from mesophilic *Bacillus* sp. P45 could potentially be used in the bioconversion of recalcitrant keratinous wastes through an environmentally friendly and energy-saving process, producing protein hydrolysates with commercial value for utilization as animal feed and fertilizers.

1. Introduction

Enzymes have been increasingly utilized in various industrial fields. Consequently, the demand for efficient and robust enzymes, suitable for application at the commercial level, is rapidly expanding. Proteases are among the important hydrolytic enzymes; they are employed mainly in the detergent, food, and leather industries, and account for approximately 40% of world-wide enzyme sales. Microorganisms represent attractive sources of proteases, as they can be cultured in large quantities by established methods in a relatively short time, producing an abundant and regular supply of the enzyme. Among microbial sources, representatives of the bacterial genus *Bacillus* are recognized as good protease producers and, on the commercial scale, proteases are generally produced by submerged fermentations (Rao et al. 1998; Gupta et al. 2002). Keratinolytic proteases in particular are attracting increasing interest due to their potential for the bioconversion of recalcitrant keratinous wastes into valuable products, such as feeds and fertilizers (Brandelli et al. 2010).

Agro-industrial wastes, which are generated in high amounts globally, have low commercial value. The disposal of such wastes often results in additional costs to the producers, and their accumulation can lead to environmental problems. Such factors are resulting in growing interest in the utilization of agro-industrial wastes in biotechnological processes, for instance, as low-cost substrates for the production of enzymes and other value-added microbial products (Ko et al. 2010; Lateef et al. 2010; Siqueira et al. 2010). Specifically, approximately 5 million tons of chicken feathers are produced annually as a waste stream from the production of chicken meat (Poole et al. 2009).

For biotechnological applications, enzymes need to be produced in sufficient amounts for commercial purposes. In this context, the composition of culture media plays a crucial role in obtaining high enzyme yields (Anbu et al. 2008). One of the approaches employed to optimize culture media and cultivation conditions involves statistical techniques (Bernal et al. 2006; Thys et al. 2006; Anbu et al. 2007), which generally produce advantageous results when compared to classical methods that typically modify one factor at a time, allowing for the analysis of multiple variables per trial,

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reduction in the number of experiments, and showing the effects of interactions between parameters (Myers and Montgomery 2002).

Protease production is an inherent capacity of microorganisms (Gupta et al. 2002), and the exploitation of biodiversity is considered a promising way to provide microorganisms and proteases well suited for diverse applications (Rao et al. 1998). The first stage in the development of industrial fermentation processes is to isolate strain(s) capable of producing the target product in commercial yields (Kumar and Takagi 1999). In particular, fish intestinal bacteria are considered an untouched bio-resource for enzyme production (Esakkiraj et al. 2009). From this perspective, the Amazonian isolate *Bacillus* sp. P45 was able to grow on and efficiently degrade whole feathers, producing keratinolytic proteases (Daroit et al. 2009). This article describes the screening of growth substrates suitable for production of extracellular keratinase by *Bacillus* sp. P45. The influence of selected substrates on enzyme production was investigated employing a central-composite design (CCD). Additionally, partial characterization of the crude keratinase was performed.

2. Materials and methods

2.1. Microorganism and inoculum preparation

Bacillus sp. P45, routinely maintained at 4 °C on brain-heart agar (BHA; Hi Media, India) plates, was utilized in this study. This strain was assigned the number LBM 1055 and deposited in the culture collection of the Food Science and Technology Institute, Porto Alegre, Brazil. Previous characterization based on 16S rDNA sequencing showed that this strain clustered with the *Bacillus subtilis* group (Sirtori et al. 2006). For inoculum preparation, *Bacillus* sp. P45 was inoculated on BHA plates and incubated at 30 °C for 24 h. The cultures were gently scraped from the agar surface, added to a sterile NaCl solution (8.5 g l^{-1}), and mixed until a homogeneous suspension with $O.D._{600}$ of 0.5 was obtained.

2.2. Screening of growth substrates for production of keratinolytic proteases

Casein (Synth, Brazil), gelatin (Kraft Foods, Brazil), cheese whey powder (CWP; Elegê, Brazil) and feather meal (FM; Bunge, Brazil) were screened as growth substrates (10 g l^{-1}) for production of keratinolytic proteases in mineral medium (Table 1). Mineral medium contained NaCl (0.5 g l^{-1}), K_2HPO_4 (0.3 g l^{-1}), and KH_2PO_4 (0.4 g l^{-1}). The initial pH of the medium was adjusted to 7.0. Erlenmeyer flasks (250 ml) containing 50 ml of medium were inoculated with 500 μl (1%, v/v) of a bacterial suspension ($O.D._{600}$ of 0.5) and incubated at 30 °C in a rotary shaker (125 rpm) for up to 96 h.

After selection of the best substrate for enzyme production, the effect of various medium supplements, such as carbohydrates (5 g l^{-1}), CWP (5 g l^{-1}), NH_4Cl (2.5 g l^{-1}), $CaCl_2$ (10 mM), $MgCl_2$ (10 mM), and $MnSO_4$ (10 mM), was studied (Table 1). Glucose, maltose, saccharose, and soluble starch were from Sigma (St. Louis, USA), whereas all salts utilized throughout this study were from Merck (Darmstadt, Germany).

2.3. Enzyme assay

Production of extracellular keratinolytic protease by *Bacillus* sp. P45 was estimated in culture supernatants, obtained by centrifugation (10,000 × g for 15 min) of culture media at defined cultivation periods. Enzyme assays were performed as described elsewhere, using azocasein as substrate (Daroit et al. 2009). This substrate was utilized since the proteolytic and keratinolytic activities of *Bacillus* sp. P45, evaluated using azocasein and azokeratin as substrates, respectively, were shown to be related (Daroit et al. 2009).

Table 1
Effect of different growth substrates on the production of keratinolytic protease by *Bacillus* sp. P45.

Substrate (g l^{-1})	Enzyme activity (U ml^{-1})
Growth substrate	
Feather meal (FM; 10)	195 ± 9.1
Whole feathers (10)	28 ± 0.9[a]
Gelatin (10)	2 ± 0.3
Casein (10)	3 ± 0.5
Cheese whey powder (CWP; 10)	9 ± 0.7
Glucose (10) + NH_4Cl (2.5)	1 ± 0.6
Selected substrate plus supplement(s)	
FM (10) + CWP (5)	208 ± 8.7
FM (10) + glucose (5)	60 ± 5.0
FM (10) + maltose (5)	4 ± 0.5
FM (10) + saccharose (5)	71 ± 4.2
FM (10) + soluble starch (5)	76 ± 5.0
FM (10) + NH_4Cl (2.5)	265 ± 8.5
FM (10) + CWP (5) + NH_4Cl (2.5)	244 ± 9.0
FM (10) + NH_4Cl (2.5) + $CaCl_2$ (10 mM)	135 ± 5.1
FM (10) + NH_4Cl (2.5) + $MgCl_2$ (10 mM)	270 ± 8.2
FM (10) + NH_4Cl (2.5) + $MnCl_2$ (10 mM)	194 ± 8.3

[a] As previously reported by Daroit et al. (2009).

2.4. Medium optimization for production of keratinolytic proteases

The medium composition resulting in the higher protease production was further analyzed. The influence of the concentrations of the selected medium components (FM and NH_4Cl) was evaluated using a 2^2 CCD with star configuration (Myers and Montgomery 2002). In the CCD, five levels were set for each of the two independent variables, with four replicates in the central points, resulting in 12 experiments (Table 2). Erlenmeyer flasks (125 ml) containing 25 ml of the respective medium were inoculated and incubated at 30 °C in an orbital shaker (125 rpm). The proteolytic activity (U ml^{-1}) was taken as the dependent variable. The results obtained were analyzed using the software Statistica 7.0 (Statsoft, Tulsa, OK, USA). Coefficients were generated by regression analysis, and the fit of the model was evaluated by the determination coefficient (R^2) and analysis of variance (ANOVA).

2.5. Bacterial growth determination

At defined periods, aliquots of the culture medium were withdrawn and serially diluted up to 10^{-8} in sterile NaCl solution

Table 2
Production of keratinolytic proteases by *Bacillus* sp. P45 on different runs of a 2^2 CCD.

Run	Independent variables[a]		Enzyme activity (U ml^{-1})[b]
	Feather meal (X_1)	NH_4Cl (X_2)	
1	43.02 (+1)	8.62 (+1)	630.8
2	43.02 (+1)	1.88 (−1)	641.2
3	8.98 (−1)	8.62 (+1)	245.3
4	8.98 (−1)	1.88 (−1)	223.2
5	50.00 (+1.41)	5.25 (0)	611.9
6	2.00 (−1.41)	5.25 (0)	110.6
7	26.00 (0)	10.00 (+1.41)	370.9
8	26.00 (0)	0.50 (−1.41)	331.8
9	26.00 (0)	5.25 (0)	345.6
10	26.00 (0)	5.25 (0)	346.3
11	26.00 (0)	5.25 (0)	351.6
12	26.00 (0)	5.25 (0)	350.5

[a] Codified values are in parentheses; real values are expressed as g l^{-1}.
[b] Dependent variable.

(8.5 g l^{-1}), and 20-μL samples were loaded in triplicate onto plate count agar (PCA) plates. After incubation at 30 °C for 24 h, counts were performed on plates having 20–100 colonies. Results were expressed as colony forming units per milliliter (CFU ml^{-1}).

2.6. Zymography

Zymogram analysis was performed as previously described (Giongo et al. 2007). Crude protease was electrophoresed at room temperature on 16% polyacrylamide gels containing gelatin (10 g l^{-1}). After electrophoresis, the gels were washed twice with 20 mM Tris–HCl buffer (pH 8.0) containing 2.5% (v/v) Triton X-100 for 30 min, and then with the same buffer without detergent for 60 min. After 12 h of incubation at 30 °C in the last buffer, the gels were stained with Coomassie Brillant Blue R-250 and then destained. Protease bands appeared as clear zones on a blue background due to gelatin hydrolysis. In parallel, EDTA (5 mM) or PMSF (1 mM) were added during gel incubation, and the effect of these protease inhibitors on the proteolytic banding pattern of the gels was evaluated.

2.7. Partial characterization of the extracellular crude keratinase

Bacillus sp. P45 was grown in optimized medium for 48 h and, after centrifugation (10,000 × g for 10 min), the culture supernatant was used as the enzyme source (crude keratinase). To evaluate the effect of pH on crude enzyme, the proteolytic assay was performed in pH values ranging from 5.0 to 10.0. The effect of temperature was evaluated by performing the enzyme assay at temperatures from 20 to 80 °C.

The influence of various reagents on proteolytic activity was investigated by pre-incubating (30 °C for 20 min) the conveniently diluted enzyme with the respective chemical. After pre-incubation, the azocaseinolytic enzyme assay was performed at optimal conditions of pH and temperature. Salts ($CaCl_2$, $CoCl_2$, $CuSO_4$, $MgCl_2$, $MnSO_4$, and $ZnSO_4$) were tested at 5 mM; phenylmethylsulfonyl fluoride (PMSF), EDTA, and 1,10-phenanthroline were evaluated as protease inhibitors at 1 and 5 mM; and the effects of detergents Triton X-100 (1%, v/v) and SDS (1%, w/v), as well as the reducing agent 2-mercaptoethanol (1% v/v), were also evaluated. The enzyme activity in the presence of chemicals was expressed as a percentage of relative activity of the control assay (no additions), which was considered as 100%.

3. Results and discussion

3.1. Screening of growth substrates for keratinase production

Production of extracellular keratinase by *Bacillus* sp. P45 in submerged cultivations was initially investigated using different growth substrates (Table 1). Among the organic nitrogen sources examined, feather meal (FM) was shown to be the best substrate for enzyme production after 24 h of submerged cultivation and was selected for further studies. The production of extracellular keratinases by this strain on whole feathers was previously reported (Daroit et al. 2009) as being lower than that produced on FM (Table 1). Similarly, keratinase production by *Bacillus* sp. P7 (Corrêa et al. 2010) and *Bacillus pumilis* F3-4 (Son et al. 2008) was observed to be higher on FM than on feathers, which could be the result of the improved accessibility of the former in comparison to the latter. Casein, gelatin, and whey-based media resulted in low protease production at 24 h (Table 1). Although protease production in these media increased for up to 96 h, enzyme yields were always inferior to that observed in FM at 24 h, a behavior that appeared to be related with the capability of *Bacillus* sp. P45 to utilize these substrates (data not shown). Additionally, the combination of glucose and NH_4Cl yielded the lowest proteolytic activity (Table 1). These results indicate that keratinase production by *Bacillus* sp. P45 is not constitutive, and that specific organic nitrogen sources, particularly keratinous substrates, are required for higher induction of enzyme production. Accordingly, the production of microbial keratinases is usually reported to be induced by keratins (Anbu et al. 2008; Lateef et al. 2010); however, other growth substrates, such as soy flour and gelatin, might act as inducers of keratinase production (Brandelli et al. 2010). Furthermore, the constitutive production of keratinases has also been reported (Son et al. 2008).

Feathers, composed of approximately 90% keratin, represent up to 10% of the total chicken weight, and the huge increase of the poultry industry has generated a large amount of feathers as byproduct. This fact, combined with the increased need for energy conservation and recycling, has strongly stimulated the search for alternatives to manage these recalcitrant keratinous wastes (Brandelli 2008). Since approximately 30–40% of the production costs of industrial enzymes have been associated with the growth medium (Joo and Chang 2005), feathers and FM represent inexpensive and abundant growth substrates (Thys et al. 2006) that could be employed for protease production by the feather-degrading *Bacillus* sp. P45, resulting in the reduction of enzyme production costs and also representing an alternative way to manage waste.

Usually, feathers are converted to feather meal through hydrothermal degradation, a process that yields a product with poor animal digestibility, and also results in the degradation of essential amino acids. Owing to these drawbacks, the microbial/enzymatic bioconversion of raw feathers and feather meal seems to be an advantageous alternative in terms of improving nutritional value as protein-rich feeds for livestock and also as nitrogen fertilizers (Brandelli 2008; Mabrouk 2008). Considering that *Bacillus* sp. P45 was isolated from fish intestine, the bioconversion of FM and native feathers by this strain might result in the improvement of the nutritional value of FM for fish diets, as in the case of the bioconversion of fish wastes by a *Bacillus cereus* strain isolated from the gut of the estuarine fish *Mugil cephalus* (Esakkiraj et al. 2009).

Supplementation of FM medium with carbohydrates such as glucose, lactose, saccharose, and soluble starch resulted in lower protease production (Table 1), probably due to carbon catabolite repression, a common mechanism controlling the biosynthesis of bacterial proteases (Gupta and Ramnani 2006). Nevertheless, in some cases, the addition of glucose (and/or other mono or disaccharides) to the culture medium might result in higher enzyme yields (Son et al. 2008; Park and Son 2009). For instance, glucose and maltose diminished keratinase production by *Bacillus* sp. KD-N2 during growth on human hair medium, whereas sucrose showed a positive effect on enzyme yield (Cai and Zheng 2009). The presence of starch resulted in lower keratinase production in *Streptomyces* sp. MS-2 (Mabrouk 2008); however, starch supplementation to feather meal medium was shown to increase keratinase production by *Myrothecium verrucaria* (Gioppo et al. 2009). Also, the concentration of the supplementary carbon sources seems to affect the enzyme yield. Anbu et al. (2008) reported that although 1% glucose increased keratinase production by *Trichophyton* sp. HA-2, higher glucose concentrations repressed enzyme production.

The supplementation of NH_4Cl to FM medium resulted in significantly higher protease yield, whereas CWP showed only a slightly positive effect on protease production in FM medium (Table 1). Increased keratinase production was also observed by other authors when specific inorganic nitrogen sources were added to culture media containing organic nitrogen sources (Bernal et al. 2006; Anbu et al. 2007). On the other hand, the presence of

inorganic nitrogen sources was reported to inhibit keratinase production by, for instance, *Myrothecium verrucaria* (Gioppo et al. 2009) and *B. megaterium* F7-1 (Park and Son 2009). Therefore, the effects of extra carbon and nitrogen sources on keratinase production vary according to the species, substrate, and carbon or nitrogen concentration; thus, the optimization of medium composition for keratinase production should be performed on a case-by-case basis (Cai and Zheng 2009).

Addition of divalent ions to culture media was previously shown to enhance protease/keratinase production (Anbu et al. 2007; Cai and Zheng 2009). However, the addition of $MgCl_2$ to NH_4Cl-supplemented FM medium did not increase the protease yield after 24 h of cultivation, whereas $CaCl_2$ and $MnSO_4$ inhibited protease production (Table 1). Similarly, protease production by a haloalkaliphilic bacterium was shown to be slightly stimulated by $MgCl_2$, but highly inhibited by $CaCl_2$ and $MnCl_2$ (Joshi et al. 2008).

In summary, the best substrate combination for protease production in mineral medium was observed between FM and NH_4Cl. In this context, the effect and contribution of different FM and NH_4Cl concentrations on production of keratinolytic proteases were evaluated using a CCD approach.

3.2. Effect of medium components on keratinase production

The concentrations of the independent variables (FM and NH_4Cl), as well as the response evaluated (proteolytic activity) at 48 h of cultivation, are presented in Table 2. The 48-h culture period was selected because the best enzyme activity values were observed at this point. Statistical analysis of the obtained results showed that, in the range studied, all variables were significant at the 95% confidence level (Table 3). Feather meal concentration had a strongest effect on keratinolytic protease production when compared to NH_4Cl, and a significant interaction between these two variables was observed. In particular, the quadratic effect of FM was lower than the linear effect, indicating that when the lowest or highest FM concentrations are approached a decrease on enzyme productivity should be expected, as observed in runs 5 and 6 (Table 2). High feather concentrations were previously reported to cause substrate inhibition or repression of keratinase production (Suntornsuk and Suntornsuk 2003); alternatively, high substrate concentration might increase the medium viscosity, which possibly results in oxygen limitation for bacterial growth (Park and Son 2009).

The regression analysis of the obtained data was performed, and the model described by Eq. (1) was generated:

$$P = 348.26 + 189.35.X_1 + 8.38.X_2 + 26.34.X_1^2 + 21.36.X_2^2 - 8.125.X_1.X_2 \quad (1)$$

where P is the enzymatic activity ($U\,ml^{-1}$), X_1 is feather meal concentration, and X_2 is NH_4Cl concentration as codified values.

Table 3
Main effects and interaction analysis for the production of keratinolytic protease by *Bacillus* sp. P45 in the 2^2 CCD.

Factor[a]	Effect ($U\,ml^{-1}$)	Std. err.	*t*-value	*p*-value
Mean	348.265	1.496	232.783	0.000[b]
X_1	378.710	2.119	178.723	0.000[b]
$X_1.X_1$	52.685	2.375	22.179	0.000[b]
X_2	16.757	2.119	7.908	0.004[b]
$X_2.X_2$	42.726	2.375	17.986	0.000[b]
$X_1.X_2$	−16.250	2.992	−5.431	0.012[b]

[a] X_1: feather meal; X_2: NH_4Cl.
[b] Significant factor at 95% confidence level ($p < 0.05$).

The significance of the generated model was analyzed by ANOVA through Fischer's *F*-test (Table 4). The *F*-value was calculated as 25.82, which is 5.8-fold higher than the tabulated *F* ($F_{0.95,5;6} = 4.39$), indicating that the model is predictive at a 95% confidence level (Myers and Montgomery 2002). The coefficient of determination R^2 was observed to be 0.95, indicating that 95% of the variability could be explained by the model. Also, a good correlation was observed between predicted and observed values.

In the range tested, the increase in FM concentration generally resulted in enhanced enzyme production. However, variations on NH_4Cl concentration appeared to present a smaller effect on the response evaluated at fixed FM contents, indicating that the utilization of low-to-intermediate NH_4Cl concentrations could be satisfactory for the production of keratinolytic proteases by *Bacillus* sp. P45.

3.3. Bacterial growth and enzyme production in optimized medium

Submerged cultivations with *Bacillus* sp. P45 were carried out (30 °C for 72 h in a orbital shaker) in optimized medium (43 g l^{-1} of FM and 1.88 g l^{-1} of NH_4Cl) and also in FM medium (10 g l^{-1} of FM). Enzyme production (U ml^{-1}) and bacterial growth (CFU ml^{-1}) were determined at defined culture periods.

In FM medium, *Bacillus* sp. P45 reached stationary phase at 24 h and maximum protease activity (245 U ml^{-1}) was observed at 32 h of incubation, after which a marked decline in enzyme activity was detected (Fig. 1). In optimized medium, however, both the stationary phase and maximum protease production (652 U ml^{-1}) appeared to be reached at 48 h of cultivation (Fig. 1). These results are in agreement with other investigations with *Bacillus* species, where maximum protease production is generally reached at the late exponential or at the stationary growth phases (Werlang and Brandelli 2005; Kojima et al. 2006; Park and Son 2009). In the current study, protease production at 32 h culture period was 2.3-fold higher in optimized medium than in FM medium, while the enzyme yield was 3.5-fold higher in optimized conditions at 48 h of cultivation. From the analysis of the protease activity peaks, a 2.6-fold increase in protease production was observed in the optimized medium when compared to FM medium.

3.4. Characterization of the crude keratinase

The effect of pH (5.0–10.0) and temperature (20–80 °C) on crude keratinase produced by *Bacillus* sp. P45 was investigated. Maximum activity was observed at pH 7.0, while acidic and alkaline conditions decreased enzyme activity; however, relative activities above 50% were observed at pH values between 6.0 and 10.0 (Fig. 2A). Conversely, keratinolytic proteases produced by other *Bacillus* species isolated from the Amazon basin were reported to be optimally active at pH 9.0 (Giongo et al. 2007; Corrêa et al. 2010), and most studies on *Bacillus* species report the production of alkaline proteases, with particular emphasis on their utilization as detergent additives or in the leather industry (Joo and Chang 2005; Giongo et al. 2007). Actually, only a few *Bacillus* keratinases are

Table 4
Analysis of variance (ANOVA) for the regression model.

Sources of variation	SS[a]	DF[b]	MS[c]	*F*-value
Regression	294114.1	5	58822.82	25.82
Residual	13668.2	6	2278.03	
Total	307782.3	11		

[a] Sum of squares.
[b] Degrees of freedom.
[c] Mean square.

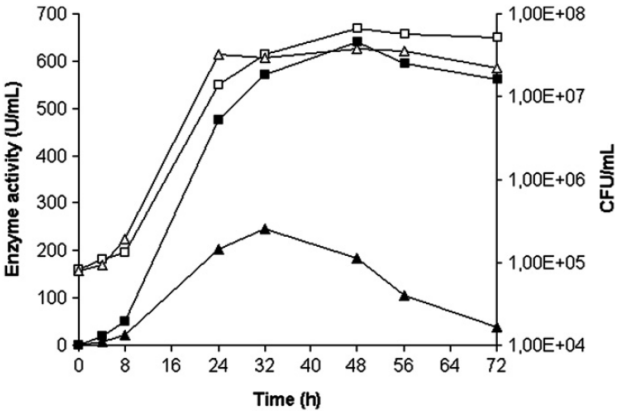

Fig. 1. Profile of growth (open symbols) and production of keratinolytic proteases (solid symbols) by *Bacillus* sp. P45 in optimized medium (squares) and FM medium (triangles).

optimally active at neutral pH (Brandelli et al. 2010). Bioprocesses employing enzymes at nearly neutral conditions might be advantageous in comparison to the utilization of alkaline enzymes since the generation (and subsequent treatment) of highly alkaline effluents is potentially diminished/avoided.

The crude keratinolytic protease had an optimum temperature of 50 °C, which is in the range usually observed for *Bacillus* spp. proteases and keratinases (Gupta et al. 2002; Gupta and Ramnani 2006), and above this value a marked decline in proteolytic activity was observed (Fig. 2B). A crude keratinase from *Bacillus cereus* LAU 08 showed identical temperature (50 °C) and pH (7.0) values for optimal activity (Lateef et al. 2010). Typically, bacterial neutral proteases present relatively low thermotolerance and intermediate rates of reaction, which are advantageous properties for controlling their reactivity during the production of protein hydrolysates (Rao et al. 1998). Most important, since enzyme activity was observed at moderate temperatures (30–40 °C, Fig. 2B), this crude keratinase preparation might be valuable for the bioconversion of agro-industrial residues in processes requiring lower energy inputs when compared to thermophilic enzymes.

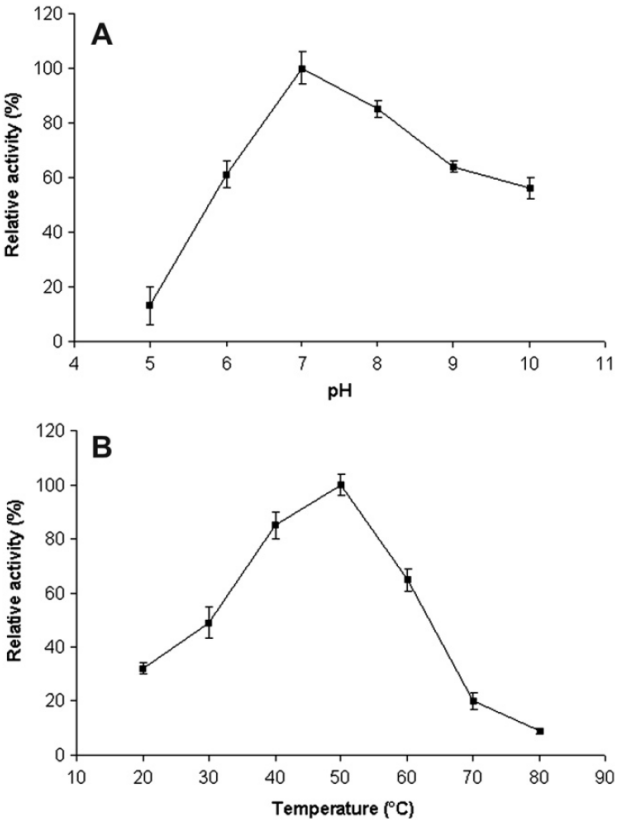

Fig. 2. Effect of pH (A) and temperature (B) on the crude keratinase produced by *Bacillus* sp. P45.

The proteolytic activity was slightly stimulated by $CaCl_2$ and $MgCl_2$ and strongly inhibited by $CuSO_4$ and $ZnSO_4$, whereas $CoCl_2$ and $MnSO_4$ caused a moderate inhibition (Table 5). Similarly, the activity of crude extracellular keratinase from *Serratia* sp. HPC 1383 was stimulated by Mg^{2+} and Ca^{2+}, and inhibited by Zn^{2+}; however, only a slight decline in keratinase activity was observed in the presence of Cu^{2+}, Co^{2+}, and Mn^{2+} metal ions (Khardenavis et al. 2009). Microbial keratinases are often stimulated by Ca^{2+} and Mg^{2+}, whereas transition and heavy metals usually inhibit keratinolytic enzymes (Gupta and Ramnani 2006). However, the effect of different metal ions on microbial keratinases is usually highly variable, depending both on their nature and concentration (Werlang and Brandelli 2005). In particular, excess Zn^{2+} may be inhibitory to some metalloproteases due to the formation of bridges between zinc monohydroxide ($ZnOH^+$) and catalytic zinc ions at the active site (Riffel et al. 2007). The stimulation by metal ions, particularly Ca^{2+} and Mg^{2+}, may be due to the formation of salt or ion bridges, which stabilizes the enzyme in its active conformation, and could protect the enzyme against thermal denaturation (Bressollier et al. 1999; Farag and Hassan 2004).

Triton X-100, a non-ionic detergent, caused minor activity loss; nevertheless, the enzyme was inactivated by the addition of the anionic detergent SDS, possibly through the destabilization of the enzyme structure (unfolding). The reducing agent 2-mercaptoethanol caused a moderate inhibition of the proteolytic activity (Table 5), suggesting that disulfide bonds might be important in maintaining the molecular structure of the enzymes (Thys and Brandelli 2006; Riffel et al. 2007).

Most of the *Bacillus* spp. keratinases have been found to be serine proteases; however, reports on keratinolytic metalloproteases have been increased (Brandelli et al. 2010). In the current study, the chelating agent EDTA inhibited almost completely the crude enzyme preparation, whereas the serine protease inhibitor PMSF showed only a moderate negative effect on proteolytic activity (Table 5). These results might indicate that the

Table 5
Effect of various chemicals on crude keratinase activity.

Chemical	Concentration	Relative activity (%)
None	–	100
$CaCl_2$	5 mM	110
$CoCl_2$	5 mM	56
$CuSO_4$	5 mM	14
$MgCl_2$	5 mM	106
$MnSO_4$	5 mM	70
$ZnSO_4$	5 mM	28
PMSF	1 mM	75
	5 mM	68
EDTA	1 mM	9
	5 mM	3
1,10-phenanthroline	1 mM	42
	5 mM	39
Triton X-100	1% (v/v)	76
SDS	1% (w/v)	4
2-mercaptoethanol	1% (v/v)	40

proteolytic enzymes produced by *Bacillus* sp. P45 have a prominent neutral metalloprotease character. However, EDTA is also observed to inhibit serine proteases due to the chelation of metal ions, particularly Ca^{2+}, which is responsible for enzyme stabilization (Gupta and Ramnani 2006). The moderate effect of 1,10-phenanthroline suggests the presence of Zn-metalloprotease(s) (Thys and Brandelli 2006). From these inhibition assays, it could be inferred that *Bacillus* sp. P45 secretes diverse proteolytic enzymes during growth on feather meal optimized medium, as previously suggested for *Bacillus* sp. kr16 (Werlang and Brandelli 2005). This was confirmed by zymogram analysis of *Bacillus* sp. P45 culture supernatants (Fig. 3). Large clear zones were visible at the top of the gels, and at least six major proteolytic bands were discerned. In agreement with the inhibition assays (Table 5), the proteolytic bands tended to disappear or to become fainter in the presence of EDTA, and the effect of PMSF was less pronounced (Fig. 3). Accordingly, various proteolytic bands were detected in culture supernatants of *Bacillus* spp. (Giongo et al. 2007) and *Chryseobacterium* sp. kr6 (Riffel et al. 2007). Similar analysis of *Streptomyces albidoflavus* showed that it secretes at least six extracellular proteases during growth on feather meal (Bressollier et al. 1999). Although these bands could indicate true different extracellular proteases, some of the observed bands might represent active forms resulting from the processing of proteases by non-enzymatic or autoproteolytic mechanisms (Kumar and Takagi 1999). Nevertheless, since extracellular protease production by *Bacillus* sp. P45 was shown to be inducible, the different proteolytic enzymes probably contribute to the complex process of keratin degradation, yielding the peptides and amino acids utilized for microbial nutrition.

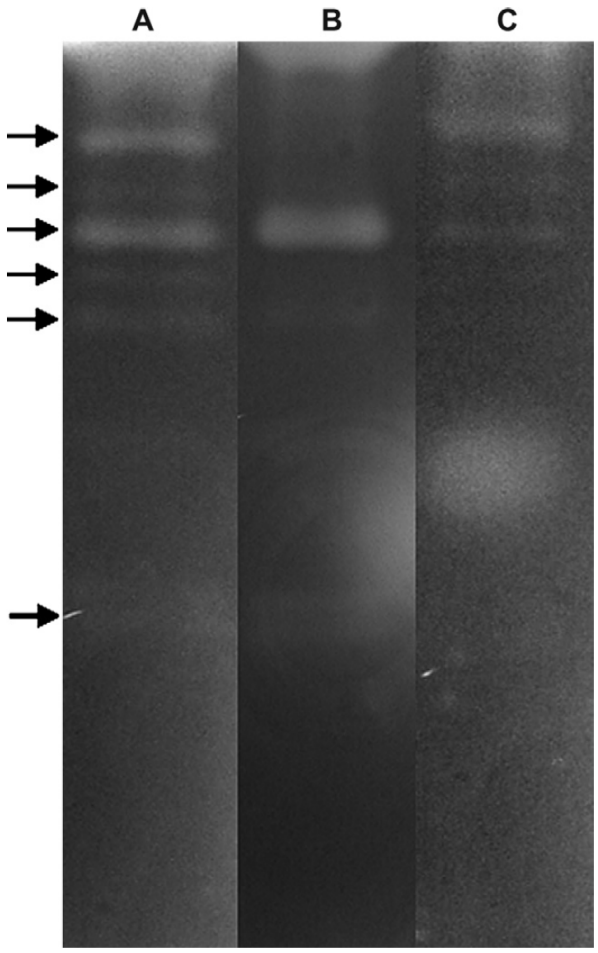

Fig. 3. Zymogram analysis of proteases produced by *Bacillus* sp. P45 in optimized medium. Lane A: crude protease; lane B: crude protease plus EDTA (5 mM); lane C: crude protease plus PMSF (1 mM). Arrows indicate major proteolytic bands.

4. Conclusion

Feather meal (FM) was the best growth substrate for production of extracellular keratinolytic proteases by the feather-degrading *Bacillus* sp. P45, and addition of NH_4Cl increased keratinase yield, resulting in a low-cost medium for keratinase production. *Bacillus* sp. P45 and its crude keratinase might potentially be employed in the bioprocessing of keratinous wastes generated by the poultry industry, and the resulting protein hydrolysates could be utilized as animal feed or nitrogen fertilizers. Such microbial/enzymatic technology represents a valuable approach with two intrinsic advantages, namely, the recycling of agro-industrial residues and the concomitant aggregation of value to these inexpensive raw materials. Further efforts are now focused on the purification and characterization of extracellular keratinase(s).

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3.3 Resultados III

Abaixo pode ser observado o volume e páginas do periódico no qual o manuscrito foi publicado.

Characterization of a keratinolytic protease produced by the feather-degrading Amazonian bacterium *Bacillus* sp. P45

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Summary | Full Text | PDF (234 KB) | PDF Plus (237 KB)

Characterization of a keratinolytic protease produced by the feather-degrading Amazonian bacterium *Bacillus* sp. P45

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Keywords: *Bacillus*, keratinase, protease, characterization, serine protease, substrate specificity

Abstract

An extracellular keratinolytic protease produced by *Bacillus* sp. P45 was purified and characterized. The keratinase had a molecular weight of approximately 26 kDa and was active at wide pH and temperature ranges, presenting optimal activity at 55°C and pH 8.0. This enzyme displayed low thermostability, being completely inactivated after 10 min at 50°C. Keratinase activity increased with Ca^{2+}, Mg^{2+}, Triton X-100, ethanol and DMSO, was stable with the reducing agent 2-mercaptoethanol, and inactivated by SDS. Phenylmethylsulfonyl fluoride (PMSF) completely inactivated, and ethylenediaminetetraacetic acid (EDTA) strongly inhibited the enzyme, indicating that the keratinase is a serine protease depending on metal ions for optimal activity and/or stability. Accordingly, analysis of tryptic peptides revealed sequence homologies which characterize the keratinase as a subtilisin-like serine protease. The purified enzyme was able to hydrolyze azokeratin and keratin azure. Casein was hydrolyzed in higher rates than keratinous substrates, and 2-mercaptoethanol tended to enhance keratin hydrolysis. With synthetic substrates, the keratinase showed preference for aromatic and hydrophobic residues at the P1 position of tetrapeptides; the enzyme was not active, or the activity was drastically diminished, towards shorter peptides. Keratinase from *Bacillus* sp. P45 might be potentially employed in the production of protein hydrolysates at moderate temperatures, being suitable for the bioconversion of protein rich wastes through an environmental-friendly process requiring low energy inputs.

Introduction

Keratins are stable and insoluble proteins, resistant to hydrolysis by proteases such as trypsin, papain and pepsin. These recalcitrant proteins are produced in enormous amounts as byproducts of agroindustrial activities (Brandelli 2008). Particularly, poultry processing contributes with a significant portion of keratinous wastes, mainly in the form of feathers, reaching approximately 5 million tonnes per year (Poole et al. 2009). As a result, keratinous wastes are increasingly accumulating in the environment, creating a demand for the development of biotechnological alternatives for recycling such wastes (Gupta & Ramnani 2006). Hence, there is a growing interest in keratinolytic microorganisms and keratinolytic proteases due to its potential to hydrolyze such refractory proteins.

The production of keratinases is widespread among microorganisms. Within fungi, keratinolytic proteases were isolated, for instance, from *Trichophyton* sp. (Anbu et al. 2008), *Paecilomyces marquandii* and *Doratomyces microsporus* (Gradisar et al. 2005), and *Trichoderma atroviride* (Cao et al. 2008). Among bacteria, keratinases from *Chryseobacterium* sp. (Riffel et al. 2007), *Microbacterium* sp. (Thys & Brandelli 2006), *Streptomyces* sp. (Syed et al. 2009) and *Kocuria rosea* (Bernal et al. 2006), for example, were characterized. Specifically, prominent keratinolytic activity is reported within the genus *Bacillus*, such as *Bacillus subtilis* (Suh & Lee 2001), *B. licheniformis* (Lin et al. 1992), *B. cereus* (Ghosh et al. 2008), and *B. pseudofirmus* (Kojima et al. 2006).

Keratin bioconversion is not only an alternative way of waste management, but also represents a method to obtain valuable products such as animal feeds, fertilizers, and biopolymers (Brandelli 2008). Additionally, microbial keratinases might be employed as feed supplements, in pharmaceutical and biomedical applications, prion hydrolysis, and also in the detergent, textile, and leather industries (Brandelli et al. 2010). Therefore, the purification and characterization of keratinases is fundamental to better understand the mechanism of keratin degradation, and also to evaluate the potential industrial uses of such enzymes (Bernal et al. 2006).

Fish intestinal bacteria are considered as an untouched bioresource for enzyme production (Esakkiraj et al. 2009). In this sense, *Bacillus* sp. P45 was isolated from the intestine of an Amazonian fish and reported to efficiently degrade whole feathers, producing keratinolytic proteases and also being capable of reducing disulfide bonds (Daroit et al. 2009). Subsequently, keratinase production by this strain in submerged cultivations was investigated, and higher enzyme yields were observed using feather meal and NH4Cl as growth substrates, representing a low-cost medium feasible for keratinase production (unpublished results). Thus, aiming to further investigate the keratinolytic potential of *Bacillus* sp. P45, the present study describes the purification and characterization of a keratinolytic protease produced by this strain.

Materials and Methods

Microorganism and keratinase production

Bacillus sp. P45 (GenBank accession number AY962474) was utilized in the present study. Previous characterization based on 16S rDNA sequencing showed that this strain clustered with the *Bacillus subtilis* group (Sirtori et al. 2006). For keratinase production, the cultivation medium contained feather meal (FM; 43 g L^{-1}) and NH_4Cl (1.9 g L^{-1}) in mineral medium (0.5 g NaCl L^{-1}, 0.3 g K_2HPO_4 L^{-1}, and 0.4 g KH_2PO_4 L^{-1}), with the initial pH adjusted to 7.0. Erlenmeyer flasks (500 mL) containing 100 mL of medium were inoculated and incubated at 30°C for 48 h.

Enzyme assays

Proteolytic activity was determined by a method described elsewhere using azocasein as substrate (Daroit et al. 2009), with slight modifications. The reaction mixture (500 µL) contained 100 µL of Tris-HCl buffer (100 mM, pH 7.0), 300 µL of 1% (w/v) azocasein (in Tris-HCl buffer) and 100 µL of conveniently diluted enzyme. Incubation was carried out at 50°C for 30 min and the reaction was stopped by adding 600 µL of 10% (w/v) trichloroacetic acid (TCA). After centrifugation (10,000 × *g* for 5 min) of the reaction mixture, 800 µL of the supernatant was mixed with 200 µL of 1.8 M NaOH, and the absorbance at 420 nm was measured. One unit (U) of protease activity was defined as the amount of enzyme that caused an increase of 0.1 absorbance unit at the defined assay conditions. Keratinolytic activity was assayed by a similar protocol utilizing azokeratin as substrate. Azokeratin was synthesized as described elsewhere (Riffel et al. 2003).

Enzyme activity was also evaluated using insoluble keratin azure and hide powder azure as substrates. Aliquots of 100 µL of the purified enzyme were incubated in a solution of 5 mg of keratin azure (Sigma Co., St. Louis, USA) or hide powder azure (Sigma Co., St. Louis, USA) in 400 µL of 100 mM Tris-HCl buffer, pH 8.0 for 1 h at 50°C. After centrifugation (10,000 × *g* for 5 min) the absorbance of the supernatants was measured at 595 nm. One unit (U) of enzyme activity was defined as the amount of enzyme that resulted in an increase of 0.1 absorbance unit at the assay conditions.

For comparison with commercial proteases, pronase (Sigma Co., St. Louis, USA), proteinase K (Merck, Darmstadt, Germany) and Alcalase (Novozymes, Bagsvaerd, Denmark) were tested. Enzymes were dissolved at 1 mg mL^{-1} in 50 mM Tris pH 8.0 and assayed for azoproteins as described above.

Keratinase purification

After the cultivation period, the medium (100 mL) was centrifuged (10,000 × *g* for 20 min) and the supernatant was utilized as enzyme source for the purification protocol (Table I). Ammonium sulfate precipitation was carried out in an ice bath to achieve 30% saturation. This mixture was centrifuged (10,000 × *g* for 20 min), the pellet was discarded, and ammonium sulfate was added to the supernatant in order to achieve 60% saturation. After centrifugation (10,000 × *g* for 20 min), the pellet was resuspended in Tris-HCl buffer (20 mM, pH 8.0) and this material was applied into a Sephadex G-75 (GE Healthcare, Sweden) gel filtration column (37×0.9 cm), with a flow rate of 0.3 mL min^{-1}. The column was equilibrated and eluted with Tris-HCl buffer (20 mM, pH 8.0), and a total of 40

fractions of 1 mL were collected. Each fraction was assayed for protease activity and protein estimation at 280 nm. Fractions with enzyme activity were pooled and applied to a DEAE-Sepharose Fast Flow (GE Healthcare, Sweden) ion-exchange column (9×0.6 cm) equilibrated with Tris-HCl buffer (20 mM, pH 8.0). A NaCl step-gradient (0, 200, 500, 1000 and 2000 mM) was utilized to elute the column at a flow rate of 0.4 mL min^{-1}, and a total of 25 fractions of 1 mL were collected. Collected fractions were assayed for protease activity and protein estimation at 280 nm. The fractions showing proteolytic activity were pooled and submitted to the protease activity assay, and to protein determination following the Lowry method (Lowry et al. 1951). This pool was utilized for protease characterization.

Characterization of the purified enzyme

Electrophoresis

Samples of the purification protocol were submitted to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which was carried out on 10% polyacrylamide gels according to standard conditions (Laemmli 1970). After electrophoresis, protein bands were revealed by silver staining; alternatively, the gels were stained with Coomassie Brilliant Blue R-250, and then destained. The relative molecular mass of the keratinase was determined by comparing with a commercial molecular weight marker (BenchMark™ Protein Ladder, Invitrogen, USA).

Temperature and pH optima

For determination of optimum pH, azocaseinolytic activity was assayed at 50°C in a pH range from 6.0 to 10.0 using the following buffers (100 mM): sodium phosphate (pH 6.0, 6.5) and Tris-HCl (pH 7.0-10.0). To determine optimum temperature, azocasein hydrolysis was assessed at optimum pH in temperatures ranging from 30 to 70°C. All assays were done in triplicate.

Thermostability

The thermal stability of the purified keratinase was evaluated by pre-incubating the enzyme without substrate at different temperatures (between 30 and 70°C) for 10 min. After this procedure the azocasein hydrolysis assay was performed, and residual enzyme activities were expressed as percentages of the activity at time zero, considered as 100%. All assays were done in triplicate.

Effect of reagents on keratinase activity

The effect of salts ($CaCl_2$, $CoCl_2$, $CuSO_4$, $HgCl_2$, $MgSO_4$, $MnSO_4$ and $ZnSO_4$), detergents (SDS and Triton X-100), solvents [dimethyl sulfoxide (DMSO), and ethanol], reducing agent (2-mercaptoethanol), and protease inhibitors PMSF, EDTA, and 1,10-phenanthroline on enzyme activity was evaluated by pre-incubating the enzyme with the respective chemical at 25°C for 10 min before the enzyme assay with azocasein was performed. Concentrations of different reagents are presented in Tables II and III. Enzyme activity determined without additions was considered as 100%. All assays were done in triplicate.

Identification of the enzyme

The keratinase band was excised from a SDS-PAGE gel and digested with trypsin. The peptide mixture was concentrated and applied to a nanoAcquity UPLC Column BHE130 C18 (100 µm×100 mm, 1.7 µm particle size, Waters). The UPLC system was coupled to a Q-TOF micro (Waters) tandem mass spectrometer with nano-ESI. Generated data were processed with MASCOT Distiller (Matrix Science, UK), and peptide homologues were searched in the NCBI database (www.ncbi.nlm.nih.gov) using the MASCOT software v. 2.2 (Matrix Science, UK).

Hydrolysis of protein substrates

The hydrolysis of albumin (Sigma Co.), casein (Synth, Brazil), feather meal (Bunge, Brazil), fish meal (Bunge, Brazil), native feather, chicken nail, and human hair by the purified keratinase was investigated. Native feathers and human hair were chopped into small fragments (approx. 0.5 cm), and chicken nails were sheared (0.1 cm) before utilization in the assays. In the case of native feathers, chicken nails and human hair, the effect of 2-mercaptoethanol (1 mM final concentration) was also assessed. An aliquot (45 U; 100 µL) of the enzyme was added to a suspension of the respective substrate (400 µL; 5 mg mL^{-1}) and this mixture was incubated at 55°C for 1 h. The reaction was stopped with 500 µL of 10% (w/v) TCA solution, this mixture was centrifuged (10,000 × *g* for 15 min), and the supernatant was utilized to measure the absorbance at 280 nm (Ionata et al. 2008). Results were presented as percentage relative to the substrate showing higher absorbance at 280 nm, considered as 100%. All assays were done in triplicate.

Hydrolysis of synthetic peptides

Activity of the purified keratinase was evaluated towards the following *p*-nitroanilide (*p*NA) peptide substrates: succinyl(Suc)-L-Phe-*p*NA, Suc-Gly-Gly-Phe-*p*NA, Suc-Ala-Ala-Val-*p*NA, Suc-Ala-Ala-Pro-Phe-*p*NA, Suc-Ala-Ala-Pro-Leu-*p*NA, Suc-Ala-Ala-Val-Ala-*p*NA, methoxy(MeO)-Suc-Ala-Ala-Pro-Val-*p*NA, MeO-Suc-Ala-Ala-Pro-Met-*p*NA, benzoyl(Bz)-L-Arg-*p*NA, Bz-Phe-Val-Arg-*p*NA, Bz-Val-Gly-Arg-*p*NA, Gly-Pro-*p*NA, Gly-Arg-pNA, L-Ala-*p*NA, Ala-Ala-*p*NA, Ala-Ala-Ala-*p*NA, and Ala-Ala-Phe-*p*NA (Sigma). Stock solutions (50 mM) of these substrates were prepared in DMSO. The reaction mixture contained 930 µL of 100 mM Tris-HCl buffer (pH 8.0), 20 µL of substrate solution (for 1 mM final concentration) and 50 µL of the purified enzyme. After incubation for 10 min at 55°C, *p*NA release was measured at 410 nm. Results were presented as percentage relative to the substrate showing the highest *p*Na release (i.e, higher absorbance), taken as 100%. All assays were done in triplicate.

Kinetic constants

The influence of substrate concentration on the keratinase reaction velocities was investigated with azocasein (0.3-30.0 mg mL^{-1}) and Suc-Ala-Ala-Pro-Phe-*p*NA (0.2-5.0 mM). The molar absorption coefficient of *p*NA at 410 nm was taken to be 8,480 M^{-1} cm^{-1} (Bakhtiar et al. 2005). The enzymatic activity was assayed at temperature and pH optima. The Michaelis constant (K_m) and maximum velocity (V_{max}) were determined from Lineweaver-Burk plots. All assays were done in triplicate.

Results and discussion

Bacillus sp. P45 produces multiple extracellular proteolytic enzymes during growth on whole feather or feather meal media (Daroit et al. 2009). To isolate one of these proteases, *Bacillus* sp. P45 was cultivated on FM-NH4Cl medium for 48 h and, subsequently, the culture supernatant was submitted to a purification protocol involving (i) ammonium sulphate precipitation, (ii) gel-filtration and (iii) ion-exchange chromatography. A summary of the purification process is presented in Table I.

The sample from protein concentration was applied into a gel-filtration column, and two protein peaks and two activity peaks were observed (Figure 1a). The second activity peak (fraction 14), displaying the higher proteolytic activity, was eluted between the two protein peaks. Fractions with enzyme activity (12-18) were pooled and submitted to anion-exchange chromatography. In this stage, the first five fractions were eluted with Tris-HCl buffer (20 mM; pH 8.0), and the subsequent elution was performed with a NaCl step-gradient (Figure 1b). The higher peak of enzyme activity was eluted without NaCl, whereas a minor activity peak was eluted with 200 mM NaCl; in the remaining steps of the NaCl gradient, only protein elution was observed. Thus, the protease did not interacted with the column, indicating that it had no net charge under the conditions investigated. Fractions 3-5 were pooled and employed in enzyme characterization. The extracellular keratinase present in culture supernatants of *Bacillus* sp. P45 was purified about 20-fold, with a final yield of approximately 18%. Ion-exchange chromatography is usually utilized in

purification protocols where the molecule of interest binds to the resin. Although the keratinase have not associated with the column, the purification was efficient due to the binding of contaminant proteins to the ion-exchanger (Suntornsuk et al. 2005; Wang & Yeh 2006).

The molecular mass of the purified *Bacillus* sp. P45 extracellular keratinase was estimated to be approximately 26 kDa by SDS-PAGE (Figure 2). The majority of keratinolytic proteases present molecular weights of less than 50 kDa (Brandelli et al. 2010). For instance, *Bacillus subtilis* KS-1 produced a 25.4 kDa keratinase (Suh & Lee 2001), a 32 kDa keratinase was produced by *Bacillus licheniformis* RPk (Fakhfakh et al. 2009), and *Chryseobacterium* sp. kr6 showed to produce two keratinases with 64 kDa (Riffel et al. 2007) and 20 kDa (Silveira et al. 2010). Also, a 28.7 kDa keratinase was purified from submerged cultures of *Clostridium sporogenes* (Ionata et al. 2008), and the molecular mass of keratinases from *Streptomyces gulbargensis* (Syed et al. 2009) and from the fungus *Trichophyton* sp. HA-2 (Anbu et al. 2008) were estimated to be 46 kDa and 34 kDa, respectively.

Bacillus sp. P45 keratinase was active in pH values between 6.0 and 10.0 (Figure 3a) and in temperatures ranging from 30 to 70°C (Figure 3b). Maximum activity was observed at pH 8.0 and 55°C, as reported for an alkaline protease from *Vibrio fluvialis* strain VM10 (Venugopal & Saramma 2006), and for a keratinase from *Clostridium sporogenes* bv. pennavorans bv. nov. (Ionata et al. 2008). Similarly, the optimal activity of *Chryseobacterium* sp. kr6 keratinase (Riffel et al. 2007) and *Bacillus cereus* DCUW protease (Ghosh et al. 2008) were observed at pH 8.5 and 50°C; and *Bacillus licheniformis* PWD-1 (Lin

et al. 1992) and *Microbacterium* sp. kr10 (Thys & Brandelli 2006) displayed optimal activity at pH 7.5 and 50°C. A keratinase from *Bacillus* sp. P7 showed identical optimum temperature (55°C), but optimal pH at 9.0-10.0 (Corrêa et al. 2010). Thus, optimal conditions of pH and temperature for *Bacillus* sp. P45 keratinase activity are in the range usually reported for microbial keratinases.

The purified enzyme showed to be completely stable after 10 min of incubation at 30 or 35°C; however, only 49% of the initial activity was observed after 10 min at 45°C, and the enzyme was inactivated after pre-incubation at 50°C (Figure 3b). Likewise, a keratinase from *Bacillus subtilis* S14 was completely inactivated after incubation at 50 and 60°C for less than 10 min (Macedo et al. 2008). A keratinolytic protease from *Bacillus* sp. SCB-3 retained more than 90% of its initial activity up to 40°C, but it was gradually inactivated at higher temperatures (Lee et al. 2002); and a keratinase from *Trichophyton* sp. HA-2 was stable at temperatures from 20 to 45°C (Anbu et al. 2008). On the other hand, a thermostable keratinase from *Aspergillus oryzae* retained 58% of its initial activity after 60 min at 60°C (Farag & Hassan 2004); a *Streptomyces* sp. S7 keratinase maintained 80% of its activity after 60 min at 50°C (Tatineni et al. 2008); and a keratinase from *Clostridium sporogenes* bv. pennavorans bv. nov. showed a half-life of 36 h at 55°C (Ionata et al. 2008). It could be concluded that the purified keratinolytic protease from *Bacillus* sp. P45 has low thermostability. The enzyme inactivation observed during pre-incubation (without substrate) at temperatures lower than enzyme optimum might suggest the role of substrate in stabilizing the enzyme (Bombara et al. 2004), or

protecting against enzyme autolysis (Gradisar et al. 2005; Suntornsuk et al. 2005).

Enzyme activity was slightly stimulated in the presence of Ca^{2+} and Mg^{2+}, whereas Co^{2+}, Cu^{2+}, Mn^{2+} and Hg^{2+} inhibited enzyme activity at both concentrations investigated (Table II). At 1 mM, Zn^{2+} has not affected enzyme activity; however, a negative effect was observed at 5 mM. The effect of metal ions on different keratinases is highly variable. For instance, Ca^{2+} and Mg^{2+} (5 mM) stimulated a keratinase from *Bacillus subtilis* S14 (Macedo et al. 2008), whereas the same metal ions (5 mM) inhibited a *Bacillus subtilis* KD-N2 keratinase (Cai et al. 2008). The increased activities imply that some metal ions could maintain the active conformation of the enzyme, or act to stabilize the binding of enzyme-substrate complex (Lee et al. 2002; Farag & Hassan 2004; Cao et al. 2009). Additionally, metal ions might protect the enzyme against thermal denaturation (Bressollier et al. 1999; Fakhfakh et al. 2009).

SDS stability is usually regarded as an interesting property for application of proteases in detergent formulations (Gupta et al. 2002). However, the anionic detergent SDS caused a prominent inhibition of P45 keratinase, whereas the non-ionic detergent Triton X-100 tended to stimulate enzyme activity (Table III). The effect of Triton X-100 could result from the increased substrate accessibility to the enzyme; conversely, SDS might have disturbed the active enzyme conformation. SDS (0.1%, w/v) also inhibited keratinases from *Paecilomyces marquandii* and *Doratomyces microsporus* (Gradisar et al. 2005), and was showed to completely inactivate a keratinase from *Bacillus subtilis* S14 (Macedo et al. 2008). The activity of a *Streptomyces albidoflavus*

K_{1-02} keratinase was slightly increased by SDS and Triton X-100 (Bressollier et al. 1999); and keratinases from *Streptomyces* sp. P7 (Tatineni et al. 2008) and *Nocardiopsis* sp. TOA-1 (Mitsuiki et al. 2004) were inhibited by SDS and stable towards Triton X-100.

Ethanol and DMSO slightly stimulated the keratinase activity (Table III). DMSO was previously shown to not strongly affect (increase or decrease) the activity of microbial keratinases (Riffel et al. 2007; Corrêa et al. 2010). Nevertheless, DMSO (1%, v/v) and ethanol (1%, v/v) inhibited a *Bacillus* sp. KD-N2 keratinase in 48% and 38%, respectively (Cai et al. 2008). Contrarily, a serine protease from *Bacillus subtilis* TKU007 maintained more than 80% of its initial activity in the presence of 25% (v/v) ethanol (Wang & Yeh 2006). The stability towards organic solvents suggests the utilization of proteases in peptide synthesis (Gupta et al. 2002).

The reducing agent 2-mercaptoethanol stimulated keratinase activity at 1 mM, whereas a minor negative effect was observed at 5 mM (Table III). Keratinase from *Streptomyces* sp. S7 was also stimulated by 5 mM dithiothreitol (Tatineni et al. 2008); and reducing agents had no significant effect on keratinases from *Nocardiopsis* sp. TOA-1 (Mitsuiki et al. 2004) and *Streptomyces albidoflavus* K1-02 (Bressollier et al. 1999). However, keratinases from *Chryseobacterium* sp. kr6 (Riffel et al. 2007), *Bacillus pseudofirmus* FA30-01 (Kojima et al. 2006) and *Bacillus subtilis* S14 (Macedo et al. 2008) were strongly inhibited, or completely inactivated, by reducing agents, indicating that disulfide bonds are important to maintain the active conformation of the enzyme. However, the negative effect of Hg^{2+} and the stability towards reducing

agents might suggest that disulfide bonds are not essential for catalytic activity (Corrêa et al. 2010).

Purified keratinase was almost completely inhibited by the serine protease inhibitor PMSF; the metalloprotease inhibitor EDTA also caused a pronounced inhibition, but to a lesser extent than did PMSF (Table III). Additionally, 1,10-phenanthroline, which is a more ideal metalloprotease inhibitor, presented only a slight inhibitory effect (Table III). The inhibition of keratinolytic proteases by both PMSF and EDTA was previously demonstrated, for instance, in *Bacillus* sp. P7 (Corrêa et al. 2010), *B. licheniformis* FK 14 (Suntornsuk et al. 2005), *B. licheniformis* RPk (Fakhfakh et al. 2009), and *Myrothecium verrucaria* (Moreira-Gasparin et al. 2009). Inhibition of serine proteases by EDTA might be attributed to the chelation of metal ions acting on enzyme stabilization (Gupta & Ramnani 2006). Therefore, these results indicate that P45 keratinase belongs to the serine protease group, depending on metal ions for its optimal activity and/or stability.

Peptides generated by trypsin digestion of the keratinase band excised from SDS-PAGE, numbered from 1 to 8, are presented in Table IV. Exact matches were observed among the amino acid sequences of the eight keratinase P45 peptides and, for instance, subtilisins from *Bacillus amyloliquefaciens* strain Ba-32732C (GenBank accession number ACT33949.1) and *Bacillus* sp. SJ (ABU93241.1), a serine alkaline protease (subtilisin E) coded by *AprE* gene of a plant growth-promoting *Bacillus amyloliquefaciens* FZB42 (ABS73414.1), and fibrinolytic subtilisin-like proteases from *Bacillus* sp. DJ-4 (AAT45900.1), *Bacillus amyloliquefaciens* CH51 (ACA34903.1), and

Bacillus vallismortis Ace02 (ABI35684.1). The majority of mature subtilisins and keratinases from *Bacillus* species range from 268 to 310 aa residues (Siezen & Leunissen 1997; Gupta & Ramnani 2006). Thus, the tryptic peptides obtained from P45 keratinase covered approximately 50% of the mature sequences from the subtilisins cited above. The amino acid residues Asp (D; peptide 1) and Ser (S; peptide 5), belonging to the catalytic triad of subtilisins and some keratinases (Siezen & Leunissen 1997; Gupta & Ramnani 2006), are conserved in *Bacillus* sp. P45 keratinase (Table IV). Additionally, a conserved sequence corresponding to the region around the Asn residue of the oxyanion hole in subtilisins and keratinases was observed (Table IV, peptide 2). Altogether, these results indicate that keratinase P45 is a subtilisin-like serine protease, as observed for several microbial keratinases (Brandelli et al. 2010).

Keratinase from *Bacillus* sp. P45 was capable to hydrolyze different proteins. Among non-chromogenic substrates, casein was the most hydrolyzed, followed by fish meal and feather meal (Table V). Hydrolysis of fish meal suggests the potential use of *Bacillus* sp. P45 keratinase in the bioconversion of fish proteins, or its utilization as a feed supplement, aiming the improvement of nutrient digestibilities for aquaculture feeds (Esakkiraj et al. 2009). The other protein substrates were less hydrolyzed and, specifically, native keratins (feathers, hair, nails) were only slightly attacked during the assay (Table V).

Soluble proteins, such as casein, are typically reported to be hydrolyzed more effectively by keratinases than insoluble ones, for instance keratin (Lin et al. 1992; Farag & Hassan 2004; Brandelli 2005; Suntornsuk et al. 2005; Syed et al. 2009). Indeed, purified keratinolytic enzymes are generally

observed to not efficiently hydrolyze keratin (Gupta & Ramnani 2006). For example, a keratinase from *Chryseobacterium* sp. kr6 was not capable to solubilize feather keratin, even though it was able to hydrolyze keratin azure (Riffel et al. 2007). Although the keratinolysis mechanism is not completely understood, sulfitolysis appears to be essential for keratin degradation since disulfide bonds are critical structural attributes of the keratin molecules (Brandelli 2008). In this sense, a keratinase from *Bacillus licheniformis* FK 14 showed partial digestion of feathers and hair, indicating that the enzyme could not cleave disulfide bonds, and may hydrolyze only peptide bonds in the substrate (Suntornsuk et al. 2005). Accordingly, a keratinase from the feather-degrading fungus *Trichoderma atroviride* F6 did not degraded feather keratin completely, suggesting that some components needed for reduction of disulfide bridges were removed during purification (Cao et al. 2008). Although a keratinase from *Myrothecium verrucaria* was capable to efficiently degrade native feathers (80.3%) in a 12-h hydrolysis assay, it was probably not able to attack the portions of the keratin chain richer in disulfide bonds (Moreira-Gasparin et al. 2009). Keratinase P45 was observed to hydrolyze native feathers less efficiently than feather meal (Table V). Feather meal is obtained by high pressure cooking and milling of feathers, resulting in the decharacterization of the native feathers and destabilization of keratin structure due to the breakdown of, for instance, disulfide bridges. Thus, the combination of lower recalcitrance and enhanced accessibility might be responsible for the higher hydrolysis of feather meal when compared to native feathers. Furthermore, such result indicates that *Bacillus* sp. P45 keratinase is not

capable to hydrolyze disulfide bridges, as showed for keratinolytic proteases (Brandelli et al. 2010). In this perspective, the addition of reducing agents tended to stimulate keratin hydrolysis by *Bacillus* sp. P45 keratinase (Table V), probably through the disruption of disulfide bonds, in agreement with previous reports (Suh & Lee 2001; Lee et al. 2002; Thys & Brandelli 2006; Cai et al. 2008; Cao et al. 2008), corroborating that the mechanism of keratin hydrolysis involves at least sulfitolysis and proteolysis (Brandelli et al. 2010). Therefore, the stability of keratinase P45 toward reducing agents (Table III) is a significant and desirable feature for its application on industrial processes of keratin hydrolysis, as reducing agents could be utilized to weaken and destabilize the keratin structure, facilitating keratinase action.

The hydrolysis of β-keratins (native feathers) was higher than the hydrolysis of α-keratins (chicken nails and human hair) (Table V). This trend is typically observed for microbial keratinases and is related to differences in the structure and in the content of disulfide bridges among β-keratins (lower cysteine content) and α-keratins (higher cysteine content), which makes the former a usually more accessible substrate for keratinases (Brandelli et al. 2010). On the other hand, keratinases from *Doratomyces microsporus* and *Paecilomyces marquandii* hydrolyzed α-keratins from skin, nail, and hair, but not β-keratins from chicken feathers (Gradisar et al. 2005). Insoluble substrates keratin azure, hide powder azure and azokeratin were also hydrolyzed (Table V). Keratinolytic/caseinolytic activity ratio (K:R) might indicate the enzyme specificity for keratinous substrates. The K:R of azokeratin to azocasein was 0.25, similarly to that obtained for keratinases from *Bacillus subtilis* S14

(Macedo et al. 2008), *Microbacterium* sp. kr10 (Thys & Brandelli 2006). The kinetic parameters K_m and V_{max} using azocasein were 2.85 mg mL^{-1} and 59.2 U min^{-1} mg $protein^{-1}$, respectively. Keratin azure was hydrolyzed at a slower rate than hide powder azure, corresponding to a K:R of 0.20 (Table V). The commercial proteases tested for comparison, namely pronase, alcalase and proteinase K, showed K:R values of 0.27, 0.30 and 0.68, respectively. Similarly, subtilisin presented a K:R of 0.30; however, for comparison purposes, proteinase K, a fungal keratinolytic protease with broad substrate specificity, usually show higher K:R values (0.70-0.89; Gradisar et al. 2005; Li et al. 2007).

Hide powder azure is often utilized as a substrate in collagenase activity assays. Thus, the hydrolysis of this substrate is an unwanted feature of the keratinase if its application as depilatory agent in tannery is concerned, since collagen is the main constituent of leather. However, the true enzyme applicability in dehairing should be tested on appropriate systems at conditions resembling those utilized in industrial processes. On the other hand, this result could indicate the suitability of the enzyme for biodegradation of recalcitrant collagen-containing wastes, generated in high amounts by the meat industry (Suzuki et al. 2006).

Among the synthetic peptides tested as enzyme substrates, the keratinase P45 showed the highest hydrolysis towards Suc-Ala-Ala-Pro-Phe-*p*NA, followed by Suc-Ala-Ala-Pro-Leu-*p*NA (Table VI), whereas MeO-Suc-Ala-Ala-Pro-Met-*p*NA, Suc-Ala-Ala-Val-Ala-*p*NA were hydrolyzed to a much lesser extent. The kinetic parameters for Suc-Ala-Ala-Pro-Phe-*p*NA were K_m = 1.69 mM and V_{max} = 54.34 mmol *p*Na min^{-1} mg $protein^{-1}$. Analogously to the present

results, microbial keratinases usually show preference for aromatic and hydrophobic residues at P1 position (Bakhtiar et al. 2005; Brandelli et al. 2010). As keratins contain about 50% of hydrophobic and aromatic residues, the specificity of keratinases towards keratinous materials may arise from the amino acid composition of keratins (Gradisar et al. 2005). Substrates with Arg at P1 were only slightly or not hydrolyzed at all (Table VI) and, in this sense, only few microbial keratinases show preference for these substrates (Macedo et al. 2008; Silveira et al. 2009). Suc-L-Phe-*p*NA was not hydrolyzed under the assay conditions, as well as di- and tripeptides containing Phe at P1, demonstrating the preference for longer peptides and indicating the presence of an extended active site (Bressollier et al. 1999). Similarly, the release of *p*NA from Suc-Ala-Ala-Val-*p*NA and Suc-Ala-Ala-Ala-*p*NA was not observed under the assay conditions, whereas the hydrolysis of Suc-Ala-Ala-Val-Ala-*p*NA was detected. Usually, the specificity of subtilisin-like proteases appears to be largely determined by interactions of the substrate P4-P1 residue side chains to the S4-S1 enzyme binding sites, respectively (Siezen & Leunissen 1997). Additionally, the higher hydrolysis of Bz-Phe-Val-Arg-*p*NA in comparison to Bz-Val-Gly-Arg-*p*NA (Table VI) point out that amino acid residues at the vicinity (P2, P3) of the cleaved bond might influence the specificity for P1 (Brandelli et al. 2010).

Conclusion

An extracellular keratinolytic subtilisin-like serine protease, produced by *Bacillus* sp. P5 on a simple and inexpensive medium, was purified and characterized. This enzyme has a mesophilic nature and is active at near-neutrality pH values,

which might be advantageous for industrial applications, with the requirement of low energy inputs and potentially avoiding the generation (and posterior treatment) of highly alkaline effluents. Additionally, the low thermostability of keratinase P45 could be an important characteristic for the effective control of enzyme reactivity. According to its features, keratinase P45 might be potentially employed in protein hydrolysis, finding applications in the bioprocessing of protein-rich agroindustrial wastes aiming the obtainment of highly valuable protein hydrolysates. The effects of metal ions (Ca^{2+} and Mg^{2+}) on the thermostability of keratinase P45 are under investigation.

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Table I. Purification of a keratinolytic protease produced by *Bacillus* sp. P45.

Step	Total activity (U)	Activity ($U\ mL^{-1}$)	Protein ($mg\ mL^{-1}$)	Specific activity ($U\ mg^{-1}$)	Purification factor
Crude enzyme	45,900	612	15.93	38.4	1.0
$(NH_4)_2SO_4$ precipitation	28,531	8,916	21.82	408.6	10.6
Sephadex G-75	9,590	274	0.52	526.9	13.7
DEAE-Sepharose	8,184	116	0.15	773.3	20.1

Table II. Influence of various salts on proteolytic activity.

Salt	Concentration	Residual activity (%)
None	-	100.0 ± 2.2
$CaCl_2$	1 mM	118.3 ± 2.2
	5 mM	121.5 ± 1.9
$CoCl_2$	1 mM	61.8 ± 1.4
	5 mM	52.3 ± 0.9
$CuSO_4$	1 mM	86.4 ± 1.2
	5 mM	62.0 ± 1.1
$HgCl_2$	1 mM	89.8 ± 1.3
	5 mM	48.1 ± 0.8
$MgSO_4$	1 mM	113.8 ± 1.8
	5 mM	124.2 ± 1.6
$MnCl_2$	1 mM	82.1 ± 2.2
	5 mM	45.3 ± 1.3
$ZnSO_4$	1 mM	101.4 ± 2.4
	5 mM	68.5 ± 1.7

Table III. Influence of various chemicals on proteolytic activity.

Chemical	Concentration	Residual activity (%)
None	-	100.0 ± 2.1
SDS	0.5% (w/v)	3.8 ± 1.3
	1.0% (w/v)	5.4 ± 0.5
Triton X-100	0.5% (v/v)	102.2 ± 2.4
	1.0% (v/v)	125.0 ± 3.2
Ethanol	0.5% (v/v)	116.1 ± 1.9
	1.0% (v/v)	115.5 ± 2.3
DMSO	0.5% (v/v)	128.3 ± 2.4
	1.0% (v/v)	112.8 ± 2.6
2-mercaptoethanol	1 mM	123.3 ± 2.9
	5 mM	89.4 ± 2.4
EDTA	1 mM	11.6 ± 0.8
	5 mM	14.8 ± 0.9
1,10-phenanthroline	1 mM	88.4 ± 1.2
	5 mM	87.9 ± 1.8
PMSF	1 mM	8.2 ± 0.9
	5 mM	1.9 ± 0.8

Table IV. Amino acid sequence of tryptic peptides from *Bacillus* sp. P45 keratinase.

Peptide	Sequence*
1	VAVI□SGIDSSHPDLK
2	AVAS<u>GVVVVAAAGN</u>EGTSGGSSTVGYPGK
3	YPSVIAVGAVNSSNQR
4	ASFSSVGSELDVMAPGVSIQSTLPGNK
5	YGAYNGT□MASPHVAGAAALILSK
6	HPNWTNTQVR
7	SSLENTTTK
8	LGDAFYYGK

* □ indicate the catalytic amino acid residues conserved among subtilisins and keratinases; underlined amino acids indicate the conserved region around the Asn residue of the oxyanion hole in subtilisins and keratinases (Siezen & Leunissen 1997; Gupta & Ramnani 2006).

Table V. Hydrolysis of different protein substrates by the purified keratinase.

Substrate	Relative activity (%)
Non-chromogenic substrates	Absorbance at 280 nm
Casein	100.0 ± 3.4
Albumin	7.5 ± 0.4
Feather meal	21.7 ± 1.6
Native feather	2.0 ± 0.1
Native feather plus 2-mercaptoethanol*	3.2 ± 0.1
Chicken nails	1.1 ± 0.1
Chicken nails plus 2-mercaptoethanol*	1.7 ± 0.2
Human hair	0.5 ± 0.1
Human hair plus 2-mercaptoethanol*	1.3 ± 0.1
Fish meal	25.3 ± 1.2
Chromogenic substrates	Absorbance at 595 nm
Hide powder azure	100.0 ± 2.8
Keratin azure	20.8 ± 0.5
	Absorbance at 420 nm
Azocasein	100.0 ± 2.9
Azokeratin	25.5 ± 1.8

* Final concentration of 2-mercaptoethanol in the assay was 1 mM.

Table VI. Hydrolysis of different synthetic peptides by the purified keratinase.

Substrate*	Relative activity (%)†
Suc-Ala-Ala-Pro-Phe-*p*NA	100.0 ± 2.3
Suc-Ala-Ala-Pro-Leu-*p*NA	30.6 ± 0.8
Suc-Ala-Ala-Val-Ala-*p*NA	8.9 ± 0.5
MeO-Suc-Ala-Ala-Pro-Met-*p*NA	12.5 ± 0.4
Bz-Phe-Val-Arg-*p*NA	2.3 ± 0.1
Bz-Val-Gly-Arg-*p*NA	1.4 ± 0.1

*Suc, succinyl; *p*NA, *p*-nitroanilide; MeO, methoxy; Bz, benzoyl.

† No activity was detected against Suc-Gly-Gly-Phe-*p*NA, Suc-L-Phe-*p*NA, Suc-Ala-Ala-Val-*p*NA, MeO-Suc-Ala-Ala-Pro-Val-*p*NA, Bz-L-Arg-*p*NA, Gly-Pro-*p*NA, Gly-Arg-pNA, L-Ala-*p*NA, Ala-Ala-*p*NA, Ala-Ala-Ala-*p*NA, and Ala-Ala-Phe-*p*NA under the assay conditions (see Materials and Methods section).

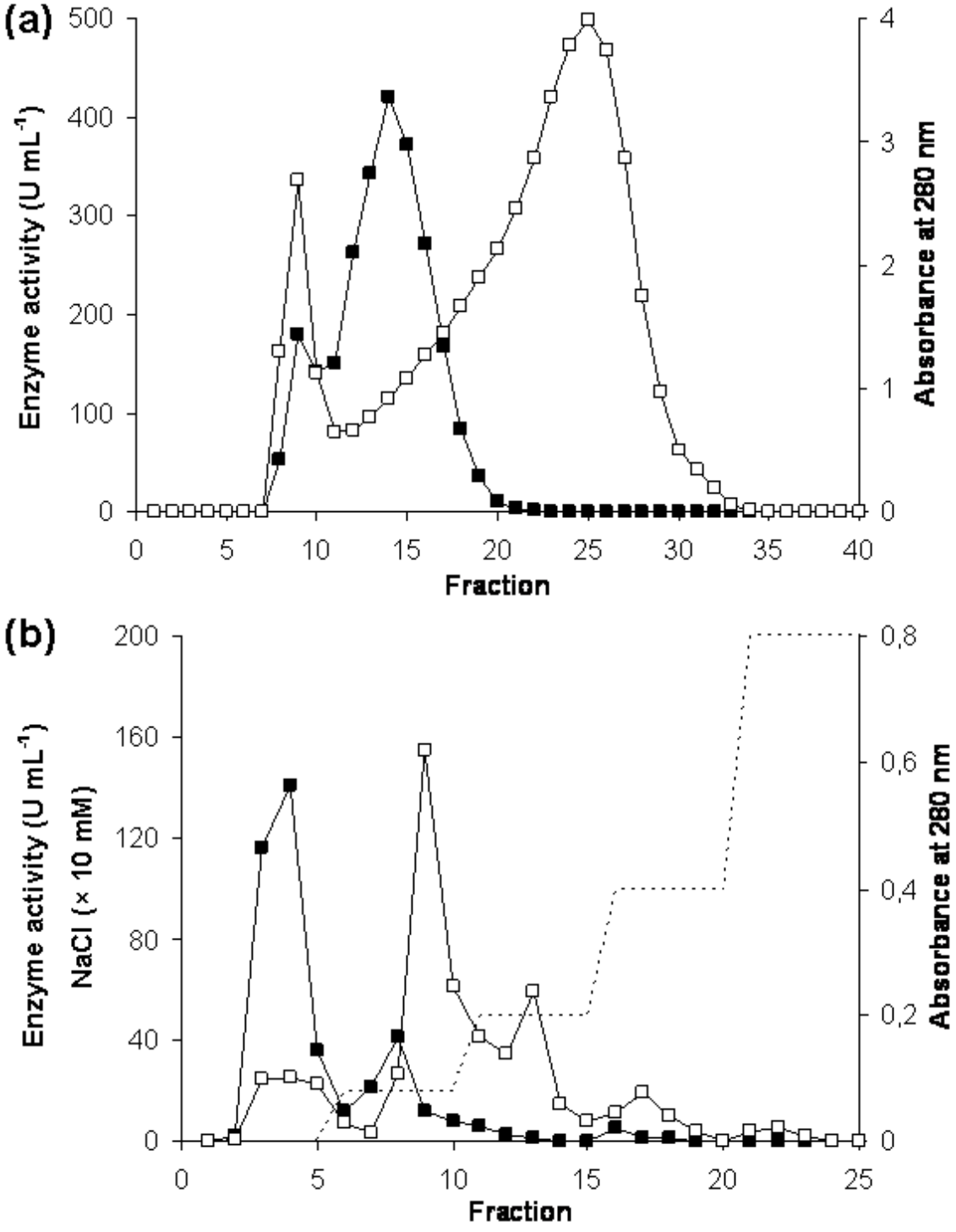

Figure 1. Liquid chromatography steps for purification of *Bacillus* sp. P45 keratinase. (a) Elution profile of ammonium sulphate-concentrated culture supernatant on a Sephadex G-75 column. (b) Elution profile of the pooled active fractions from gel-filtration on a DEAE-Sepharose column. (□) protein elution; (■) proteolytic activity; (---) NaCl concentration (× 10 mM).

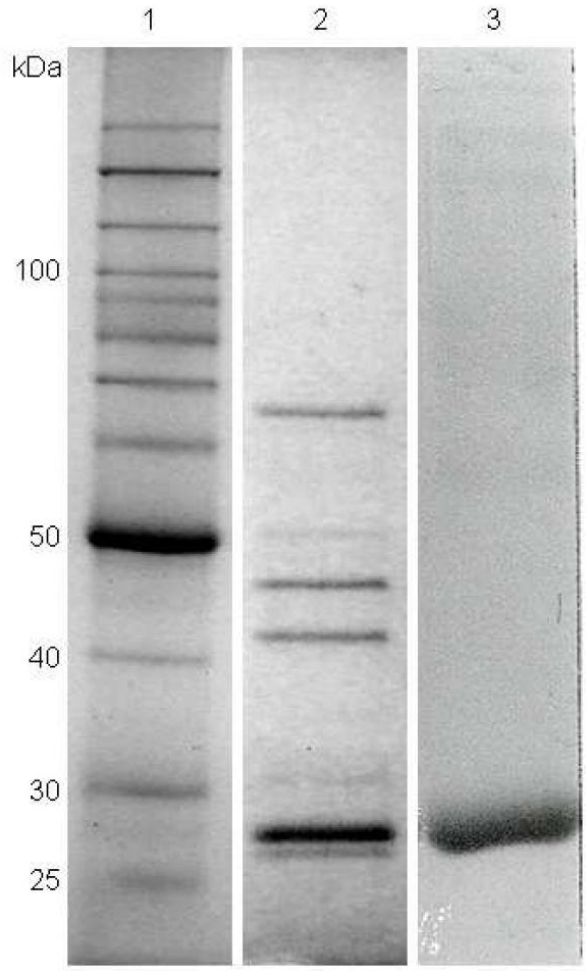

Figure 2. SDS-PAGE of *Bacillus* sp. P45 keratinase. *Lane 1*, molecular weight marker; *lane 2*, sample from Sephadex G-75 column; *lane 3*, sample of purified keratinase after DEAE-Sepharose column. For enzyme samples, 60 µg protein was loaded in lanes 2 and 3.

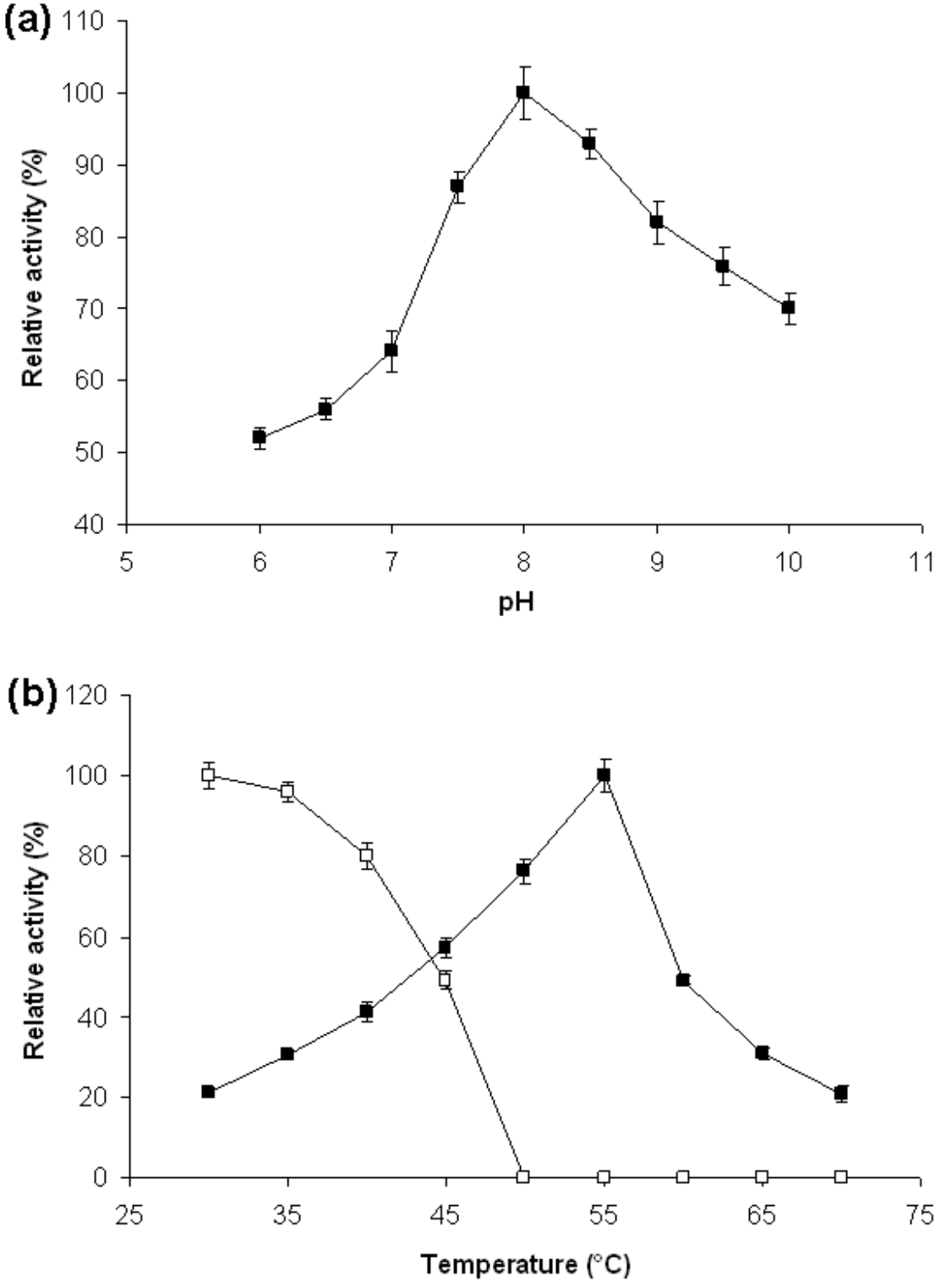

Figure 3. (a) Proteolytic activity at different pH values. (b) Activity (■) and thermal stability (□) of the keratinase at different temperatures. In thermal stability tests, the enzyme was pre-incubated for 10 min at various temperatures before the activity assay on azocasein was performed.

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3.4 Resultados IV

Kinetic stability modeling of keratinolytic protease P45: influence of temperature and metal ions

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Abstract

The activity and kinetic stability of a keratinolytic subtilisin-like protease from *Bacillus* sp. P45 was investigated in 100 mM Tris-HCl buffer (pH 8.0; control) and in buffer with addition of Ca^{2+} or Mg^{2+} (1-10 mM), at different temperatures. Addition of 3 mM Ca^{2+} or 4 mM Mg^{2+} resulted in a 26% increment on enzyme activity towards azocasein when compared to the control (100%; without added Ca^{2+} or Mg^{2+}) at 55°C. Optimal temperature for activity in the control (55°C) was similar with Mg^{2+}; however, temperature optimum was increased to 60°C with 3 mM Ca^{2+}, displaying an enhancement of 42% in comparison to the control at 55°C. Stability of protease P45 in control buffer and with Mg^{2+} addition was assayed at 40-50°C, and at 55-62°C with Ca^{2+} addition. Data were fitted to six kinetic inactivation models, and a first-order equation was accepted as the best model to describe the inactivation of protease P45 with and without metal ions. The kinetic and thermodynamic parameters obtained showed the crucial role of calcium ions for enzyme stability. As biocatalyst stability is fundamental for commercial/industrial purposes, the stabilizing effect of calcium could be exploited aiming the application of protease P45 in protein hydrolysis.

Keywords: protease; kinetic modeling; stability; enzyme inactivation; calcium

Introduction

Proteolytic enzymes are important commercial biocatalysts exploited by the food, detergent, leather, among other industries. Although animal and plant proteases are currently employed in various processes, the higher yields obtained with microorganisms amplify their significance as enzyme sources, which meet the increasing demand for proteases. The vast microbial diversity could provide proteolytic enzymes suitable for diverse applications and, among microorganisms, strains of the bacterial genus *Bacillus* are considered as remarkable producers of potentially valuable proteases. The major and best known proteases produced by *Bacillus* spp. are subtilisins and subtilisin-like proteases, which have been investigated for their structural and catalytic properties, and also to evaluate industrial applications (*1-3*).

Keratins are insoluble and stable proteins found as major components of the skin and its appendages, such as feathers, hair and wool. These recalcitrant proteins are produced in large amounts by agroindustrial processing, and are usually not degraded by common proteases such as pepsin and trypsin. Among microbial proteases, keratinases are able to hydrolyse keratins (*4*). Therefore, increasing interest has been focused on the application of these enzymes for the bioconversion of keratin-rich wastes, producing protein hydrolysates for utilization as nitrogen fertilizers and animal feed. Also, keratinases are investigated in prion degradation, as a component in detergent and feed formulations, and as a dehairing agent in the leather industry (*5*). Production of keratinolytic enzymes is considered to be largely induced by keratinacious substrates, and maximal yields are usually achieved during the late exponential

and/or stationary phase of microbial growth. Keratinases are mainly serine or metalloproteases, optimally active at 40-60°C and at pH values of 7.0-9.0. Particularly, keratinases from *Bacillus* spp. are mainly characterized as subtilisin-like proteases preferring aromatic and hydrophobic amino acid residues at position P1 (*6*).

Enzyme activity and stability are topics directly linked with enzyme application. Various phenomena could lead to enzyme inactivation, including autolysis, aggregation, coagulation, denaturation due to exposure to solvents, surfactants, salts, and extremes of temperature and pH (*7,8*). Enzyme stabilization techniques have been investigated to counteract these deleterious mechanisms, providing biocatalysts with higher efficiency that, in turn, could increase the biotechnological and economical potential of enzyme-based processes (*8*). In this context, predicting enzyme inactivation is essential for enzyme characterization from both scientific and technological perspectives. Mathematical models, consisting of equations that provide an output based on a set input data, represent a powerful and concise way to express physical behavior in mathematical terms (*9*). Therefore, adequate kinetic models and thermodynamic studies may enable a better knowledge of the enzyme behavior during inactivation.

Bacillus sp. P45, an effective feather-degrading strain isolated from the intestine of an Amazonian fish, produces diverse proteolytic enzymes during submerged cultivations on media containing whole feathers or feather meal as the only organic source of carbon and nitrogen (*10,11*). As fish intestinal bacteria are considered as an untouched resource for enzyme production, fish

microbiota might produce enzymes well-suited for different applications. In this sense, a subtilisin-like protease was purified from culture supernatants, showing optimal azocaseinolytic activity at 50°C and pH 8.0 (*12*). This enzyme is completely inhibited by sodium dodecyl sulfate (SDS) and slightly stimulated by Triton X-100, solvents (dimethyl sulfoxide and ethanol), and some metal ions (such as Ca^{2+} and Mg^{2+}). The thermostability properties of this enzyme, in addition to enzyme activity at low/moderate temperatures, might suggest the suitability of P45 protease for utilization as a biocatalyst in energy-saving processes of protein hydrolysis/modification (*12*). Therefore, aiming to further characterize the biotechnological potential of protease P45, the kinetic stability of this enzyme was investigated at different temperatures (40-62ºC), with and without the addition of metal ions (Mg^{2+} or Ca^{2+}) to assay buffer, and the isothermal data was statistically treated using different enzyme inactivation models.

Materials and Methods

Enzyme production and purification

Production of keratinolytic protease by *Bacillus* sp. P45 (GenBank accession number AY962474) was performed in Erlenmeyer flasks (250 ml) containing 100 ml of mineral medium: NaCl (0.5 g l^{-1}), K_2HPO_4 (0.3 g l^{-1}), and KH_2PO_4 (0.4 gl^{-1}) supplemented with feather meal (FM; 43 g l^{-1}) and NH_4Cl (1.9 g l^{-1}) (*11*). After 48 h of cultivation at 30°C in an orbital shaker (125 rpm) the medium was centrifuged (10,000 × *g* for 15 min) and the supernatant was submitted to a purification protocol involving ammonium sulfate concentration (30-60%

saturation) followed by gel-filtration chromatography on Sephadex G-100 and anion-exchange chromatography on DEAE-Sepharose (*12*). Fractions with proteolytic activity were pooled and employed in the following studies.

Enzyme assay

Proteolytic activity was determined by a method described elsewhere using azocasein as substrate (*12*). The reaction contained 100 µl of 100 mM Tris-HCl buffer (pH 8.0), 300 µl of 10 mg ml^{-1} azocasein (in Tris-HCl buffer) and 100 µl of conveniently diluted enzyme. After incubation (55°C for 15 min) the reaction was stopped by adding 600 µl of 10% (w/v) trichloroacetic acid (TCA), and this mixture was centrifuged (10,000 × *g* for 5 min). The supernatant (800 µl) was mixed with 1.8 M NaOH (200 µl) and the absorbance at 420 nm was measured. One unit (U) of protease activity was defined as the amount of enzyme that caused an increase of 0.1 absorbance unit at the defined assay conditions.

Effects of calcium and magnesium on proteolytic activity

The effects of Ca^{2+} and Mg^{2+} were tested by adding different concentrations (0-10 mM final concentration) to the enzyme assay performed at 55°C. Enzyme activity determined without additions was considered as 100%. Subsequently, the enzyme activity was evaluated at different temperatures (40-70°C) in assays containing the Ca^{2+} or Mg^{2+} concentrations which resulted in the higher enzyme activity at 55°C (above experiment). Enzyme activity determined at 55°C without the addition of Ca^{2+} or Mg^{2+} to assay buffer was considered as 100%. All assays were done in triplicate.

Enzyme inactivation at different temperatures

Protease solutions in buffer (0.1 ml; 0.07 mg ml^{-1}) without added metal ions, or containing 3 mM Ca^{2+} or 4 mM Mg^{2+}, were heated in sealed tubes (1 mm of thickness, 7 mm of internal diameter and 3 cm of length) at temperatures ranging from 40 to 62°C, in a thermostatically controlled water bath (Thermomix BM-S, B.Braun Biotech International, Melsungen, Germany). Time exposure varied between 1 and 29 min, and the tubes were immediately immersed in an ice bath, thereby stopping the heat inactivation reaction. The activity after 1 min of heating-up time (t = 0) was considered to be the initial activity, eliminating the effects of heating-up (*13*). Assays were done in duplicate. Residual proteolytic activities with respect to processing time at different temperatures were fitted to several models (Table 1) through non-linear regression using the software Statistica 7.0 (StatSoft Inc., Tulsa, OK).

Kinetic modeling

In the model equations (Table 1), A/A_0 represents the residual proteolytic activity at time t (min), and k (min^{-1}) is the inactivation rate constant at a given temperature. First-order kinetics (Eq. 1) is commonly employed to describe enzyme thermal inactivation (*14*). The utilization of the nth order model (Eq. 2) is not exceptional, where n is the order of the reaction (*15*). Parallel models, indicating the existence a mixture of enzymes (isoforms, isoenzymes) with different heat sensitivities (labile and resistant fractions) and/or catalytic properties (*16*), were checked for protease P45. Mathematical equations which

suggest that residual activity could be described by the summation of such distinct fractions are represented by Eq. 3-5. Residual activities for the 'labile' and 'resistant' isoenzymes are represented by A_L and $A_{R,}$ respectively, k_L and k_R are the correspondent first-order reaction rate constants for each fraction, respectively, and coefficient *a* represents activity fraction of the thermal labile group in relation to the total activity (*14,17*). Fractional conversion, Eq. 5, refers to a first-order inactivation process and takes into account of the non-zero enzyme activity upon prolonged heating because of the presence of an extremely heat resistant enzyme fraction (*18*). The series-type model (Eq. 6) is based on a succession of two irreversible first-order steps (*19*). In the first step the protein unfolds from the native structure (E) to yield an intermediate (E_1) with lower specific activity, which is followed by the conversion of the intermediate into a final enzyme state (E_2) with possible non-zero activity. In this model, k_1 and k_2 are the first-order deactivation coefficients, and α_1 and α_2 are the ratios os specific activities of E/E_1 and E/E_2, respectively.

Comparison of kinetic models

For comparison of fits obtained with nonlinear regression, statistical and physical criteria were considered. Statistical criteria included coefficient of determination (r^2), chi-square (χ^2), and standard error of means (S.E.M.). These criteria have been used succesfully to compare kinetics of thermal inactivation models of several bioactive proteins (*13,20,21*).

Calculation of χ^2 is done by the equation:

$$\chi^2 = \frac{\sum \left(a_{mensured} - a_{predicted}\right)^2}{\left(m - p\right)} \quad (7)$$

S.E.M. is defined as:

$$S.E.M. = \frac{\sum (a_{measured} - a_{predicted})^2}{\sqrt{m}} \quad (8)$$

where *m* is the number of observations and *p* the number of parameters.

Estimation of negative kinetic parameter at a given temperature is a physical criterion for rejection of a model. The model with the lowest χ^2 and S.E.M., and higher r^2 for the residual proteolytic activity is the best choice from a statistical point of view (*13*).

Thermodynamic analysis

The relationship between temperature and rate parameters of the thermal inactivation models showed on Table 1 can be expressed algebraically by Arrhenius' law (Eq. 9):

$$\ln\left(k\right) = \ln(A) - \frac{E_a}{RT} \quad (9)$$

where *A* is the Arrhenius constant, E_a the activation energy, *R* the universal gas constant (8.31 J mol^{-1} K^{-1}), and *T* is the absolute temperature. The E_a can be

estimated by the slope of linear regression analysis of the natural logarithm of rate constant versus the reciprocal of the absolute temperature.

Obtained value of E_a, the activation enthalpy for inactivation ($\Delta H^{\#}$) for each temperature was calculated was by:

$$\Delta H^{\#} = E_a - R.T \qquad (10)$$

The free energy of inactivation ($\Delta G^{\#}$) can be determinated according to the expression:

$$\Delta G^{\#} = -R.T.\ln\left(\frac{k.h}{K_B T}\right) \qquad (11)$$

where h (6.6262 × 10^{-34} J s) is the Planck´s constant, K_B (1.3806 × 10^{-23} J K^{-1}) is the Boltzmann´s constant, and k (s^{-1}) the inactivation rate constant of each temperature.

From Eq. 10 and Eq. 11 it is possible to calculate the activation entropy of inactivation ($\Delta S^{\#}$) by:

$$\Delta S^{\#} = \frac{\Delta H^{\#} - \Delta G^{\#}}{T} \qquad (12)$$

Data analysis

Statistical analysis of the data was performed using the Statistica 7.0 software (Statsoft Inc., Tulsa, OK, USA) and plots using Microsoft Excel 2000 (MapInfo

Corporation, Troy, NY, USA). Obtained *k*-values were compared using Tukey's test, and a $P<0.05$ was considered statistically significant.

Results and Discussion

Protease P45, a 26-kDa subtilisin-like enzyme produced by *Bacillus* sp. P45, was previously reported to act on various protein substrates, including casein, feather meal, azocasein, hide powder azure and keratin azure; and also to preferably hydrolyse synthetic tetrapeptides (*p*-nitroanilide derivatives) containing aromatic and hydrophobic residues at position P1 (*12*). Such features suggest the suitability of protease P45 as a valuable biocatalyst for industrial processes. Therefore, as an essential stage for enzyme characterization, the activity and kinetic stability of protease P45 was investigated in Tris-HCl buffer (100 mM, pH 8.0) with or without the addition of metal ions (Ca^{2+} or Mg^{2+}) at different temperatures.

Initially, the effect of different concentrations (0-10 mM) of calcium and magnesium on P45 activity was assessed at 55°C, which is the temperature optimum previously reported in buffer without added metal ions (*12*). Enzyme activity showed to be slightly enhanced by all metal ion concentrations (Fig. 1). Maximum activity was observed with 3 mM Ca^{2+} (125%) or 4 mM Mg^{2+} (126%) in comparison to the control (100%, without metal ions). Subsequently, Ca^{2+} (3 mM) or Mg^{2+} (4 mM) were added to enzyme assays performed at different temperatures (40-70°C), and the results were compared with enzyme activities observed without the addition of metal ions to the buffer (Fig. 2). At lower temperatures (40-45°C) the effect of metal ions was not apparent, but as the

temperature increased, the presence of Ca^{2+} or Mg^{2+} tended to enhance enzyme activity. With 4 mM Mg^{2+}, the maximum activity was reached at 55°C, although it was 21% higher than that of the control (55°C; 100%); and a similar activity to that of control optimum was observed at 60°C (Fig. 2). The effect of calcium was most pronounced, with maximal activity (142% of control) observed at 60°C; also, enzyme activity at 65°C was higher than that of control optimum (Fig. 2). The positive effect of metal ions, particularly Ca^{2+}, on proteolytic activity is generally reported for alkaline proteases of *Bacillus* spp. (*22-25*), and also for other microbial proteases (*6*). Enhanced activity in the presence of calcium, at temperatures in which the P45 activity without metal ions was highly diminished, suggest that Ca^{2+} might act improving the protein conformational stability at higher temperatures (*22,26*). Also, metal ions might contribute to stabilize the binding of the substrate and enzyme complex (*27*).

The influence of Ca^{2+} and Mg^{2+} ions on isothermal inactivation treatments of protease P45 in temperatures between 40 and 62°C was then evaluated (Fig. 3), and residual activities were fitted for different thermal inactivation models. In assays using buffer without additions and buffer with 4 mM Mg^{2+}, enzyme inactivation was assessed at temperatures of 40 to 50°C; however, with 3 mM Ca^{2+}, protease P45 was stable up to 50°C (data not shown). Due to the increased stability observed with calcium, temperatures employed in enzyme inactivation assays with calcium ranged from 55 to 62°C. As expected, proteolytic activity decreased as the heating time increased as well as at higher temperatures (Fig. 3).

Although the increased thermostability of subtilisin-like proteases in the presence of calcium ions is described, few studies deal with the mathematical modeling of kinetic thermal inactivation of proteases, simply assuming a first-order kinetics from semi-logarithmic plots of activity *versus* time. The choice of the best equation for process modeling is essential from both engineering and economical points of view, minimizing errors and improving the effectiveness of the process, ultimately resulting in lower costs. Several mathematical equations have proposed to explain the behavior of a biocompound during thermal degradation (*9*). Therefore, six inactivation kinetic models were tested to fit the experimental data for heat treatments of protease P45 (Table 1). The performance of these models for the three experiments is summarized in Table 2, and the model which satisfactorily described the inactivation pattern for all experiments (with and without metal ions) was selected. For *n*th order (Eq. 2), two-fraction (Eq. 4), fractional conversion (Eq. 5), and series (Eq. 6) models, negative parameter values were obtained, which is a physical criterion for rejection of the equations. In the distinct isoenzymes model (Eq. 3), equal inactivation rate parameters were calculated, excluding this model. This result, in accordance to that obtained by the two-fraction and fractional conversion models, indicate the absence of isoforms/isoenzymes or different proteases with distinct heat stabilities and/or substrate specificities. Although microbial serine proteases are mainly monomeric enzymes, such parallel models of inactivation (Eqs. 3-5) were evaluated since the occurence of proteolytic enzymes with quaternary structures is reported (*28*) and, particularly, a subtilisin-like protease from *Bacillus intermedius* secreted by a *Bacillus subtilis*

recombinant strain was reported to form dimers, possibly through interactions mediated by calcium ions (*29*).

Rejection of the series model (Eq. 6) suggests that, under the conditions presently evaluated, irreversible kinetic inactivation of protease P45 is sufficiently fast to make negligible the population of intermediate states (*30*). Alternatively, the unfolded or partially unfolded enzyme might not be active, as these intermediates may not refold correctly on cooling, producing thermodynamically stable but inactive molecules (*23,31*). This seems to be particularly true for subtilisins. When the forces leading to enzyme denaturation are reversed, subtilisins are often unable to refold from denatured states. This occurs since these enzymes are produced as precursors that require its N-terminal propeptide to act as an intramolecular chaperone to yield the mature enzyme (*32,33*). Therefore, the thermal inactivation of protease P45 seems to proceed through a single-step mechanism.

First-order model yielded good statistical criteria for the three conditions evaluated. The r^2 values ranged between 0.896 and 0.999; the χ^2 values were low, ranging from 0.00006 to 0.0138, meanwhile S.E.M. varied between 0.007 and 0.107. These values are comparable to those calculated for model acceptance in other investigations (*13,15,21,34*). Hence, among the mathematical models evaluated, first-order was accepted as the best equation to describe the inactivation of protease P45 in the conditions employed. The results of experimental data fitted to first-order model are shown in Fig. 3, and an exponential behavior could be observed. Accordingly, several investigations suggest that the thermal inactivation of proteases with respect to time obey first-

order kinetics (*23,35,36*); however, in those studies, the first-order activity decay is assumed without model testing. An apparent first-order behaviour indicates that one of the processes leading to enzyme denaturation (probably temperature) is the controlling step, fixing the overall inactivation rate (*37*). Previously, subtilisin thermal inactivation rate was reported to be characterized by a single exponential decay curve, determined by the rate of subtilisin unfolding (*38*).

The *k*-values increased significantly ($P<0.05$) and $t_{1/2}$ decreased with increasing temperature (Table 3), indicating a faster inactivation at higher temperatures (*13*). *k*-values for protease P45 are significantly ($P<0.05$) higher without ions than in presence of Mg^{+2} in the 43-50°C range, suggesting a protective effect of this ion against protease inactivation. Thermal inactivation concepts (*D*- and *z*-values) are generally used to represent a first-order reaction. *D*-value is the time needed for a 10-fold reduction of the initial activity at a given temperature, and it is obtained by plotting the activity values on a log scale against the corresponding inactivation times. The *z*-value is the temperature needed to reduce the *D*-value in one log-unit, and it is obtained by plotting the *D*-values on a log scale against the corresponding temperatures (*39*). The higher *D*-values in the presence of metal ions represent the enhanced stability of protease P45 (Table 3). *z*-values were calculated to be 7.16°C (no added ions), 9.18°C (with Mg^{2+}) and 8.04°C (with Ca^{2+}) indicating the slight but significant protective effect of Mg^{2+} against heat inactivation. As the temperatures employed in the heat inactivation assays with Ca^{2+} needed to be

raised, it is suggested that protease P45 achieved a higher stability when compared to that observed with Mg^{2+} or without metal ions.

Subtilases typically contain one or more calcium binding sites, and native subtilisins are thermodynamically unstable in the absence of bound metals (*33*). In subtilisins (from family A), two Ca^{2+} binding sites are usually present: a strong site and a weak site. The former specifically binds calcium, and the later can bind both divalent and monovalvent cations (*26*). Calcium binding serve to tie together surface loops, reducing the flexibility of the polypeptide chain and inhibiting local unfolding, diminishing the rate of thermal inactivation of the enzyme (*40*). Thermal denaturation temperature decreases with the removal of calcium from the strong binding site, and only a slight decrease is observed by removing calcium from the weak binding site; however, the portion of random coil structure increases significantly when calcium is removed from the weak as well as the strong binding sites, indicating that both sites are essential in maintaining a stable enzyme structure (*41*). The effect of Mg^{2+} in protecting protease P45 from thermal inactivation was less pronounced when compared to Ca^{2+}, which could be explained by the preference of calcium to bind to carboxylate and other oxygen ligands, which are the metal binding groups most likely to be presented in external loops (*40*). Mg^{2+} ion has an effective ionic radius considerably lower than Ca^{2+}, which might partly explain the lower degree of stabilization by the former and, additionally, Mg^{2+} has a preference to bind nitrogen rather than oxygen ligands (*42*).

Estimation of thermodynamic parameters is an important issue to determine the industrial potential of bioactive compounds and their structure-

stability relationships. The data fitted well to Arrhenius equation (data not shown), enabling the calculation of thermodynamic parameters (Table 4) by the transition state theory (Eqs. (10-12)). $\Delta G^{\#}$ is a measure of the spontaneity of the inactivation process, depending on $\Delta H^{\#}$ (heat change) and $\Delta S^{\#}$ (entropy change) for inactivation, which provide a measure of the number of non-covalent bonds to be broken during inactivation, and the disorder change of molecules in the system, respectively (*43,44*). Positive $\Delta H^{\#}$ values indicate the endothermic character of the inactivation process (*36*). $\Delta G^{\#}$ and $\Delta H^{\#}$ decreased as the temperature increased (Table 4), suggesting the destabilization of protease P45 at higher temperatures (*39,45*). The $\Delta S^{\#}$ values have not presented a continuous behaviour, which could result from the difficult evaluation of system disorder in such a small temperature variation.

From the analysis of the thermodynamic parameters obtained in buffer with and without metal ion additions, it is observed that values of $\Delta H^{\#}$ and $\Delta S^{\#}$ were lower, and $\Delta G^{\#}$ was higher, in the presence of metal ions (Table 4). Enzyme kinetic inactivation involves the unfolding of the protein tertiary structure to a disordered polypeptide due to the breakage of weak, non-covalent bonds (*30*). As $\Delta G^{\#} = \Delta H^{\#} - T\Delta S^{\#}$, protein stabilization might be improved by higher values of $\Delta H^{\#}$ (more ionic or hydrophobic interactions) or lower values of $\Delta S^{\#}$. However, if large values of $\Delta H^{\#}$ are coupled with a large increase in the values of $\Delta S^{\#}$, a destabilizing effect could be expected. This occurs since the increase of $\Delta S^{\#}$ compensates the high inactivation barrier, causing $\Delta G^{\#}$ to be low enough, resulting in an overall less energy requirement for the inactivation process to proceed relatively fast (*44,45*).

Activation energy (E_a) can be seen as an energy barrier that molecules need to cross in order to be able to react, and the proportion of molecules able to do that usually increases with temperature, qualitatively explaining the effect of temperature on rates (*9*). Therefore, higher values of E_a for enzyme inactivation could indicate an increased stability at higher temperatures. In subtilisins, by binding at specific sites in the enzyme structure, calcium ions contribute their binding energy to the stability of the native state, which was reported to increase the activation energy of unfolding (*38*). Nevertheless, although the value of E_a for inactivation of protease P45 was higher in buffer without added Ca^{2+} or Mg^{2+} than in the presence of added metal ions (Table 4), the enzyme was inactivated at slower rates in the presence of metal ions (*k*-values; Table 3), probably due to the usual dominant role of $\Delta S^{\#}$ in the thermal inactivation of proteins in aqueous solutions (*45*). Therefore, it is suggested that the decrease in the inactivation rate constant (*k*), or the increase in $\Delta G^{\#}$ values, are more reliable criteria to observe the enhancement of enzyme stabilization than the increase in the E_a for inactivation (*46*).

Investigations on thermal inactivation of proteases might become more intrincate due to autolysis (*7*). Although the contribution of autolysis to enzyme inactivation is minimized by employing low protease concentrations (*35*), the analysis of second-order plots of residual enzyme activity with respect to time might indicate if this mechanism is adding to the activity decay (*42*). As the pre-incubation temperature decreased, both in the assays with and without metal ions, second-order plots (1/% relative activity *versus* time) became more linear (data not shown), suggesting that at lower temperatures autolysis might

contribute to some extent to enzyme inactivation (*47*), even without deviation from the apparent first-order kinetics. Particularly, subtilisin BPN' is reported to possess two autoproteolysis sites (Ala48-Ser49 and Serl63-Thr164) in regions of high mobility localized in extended surface loops (*48*), which are present in protease P45 (*12*). As these autolysis sites appear to be involved in calcium binding, or to be close to calcium binding sites (*26,40*), it is suggested that the decrease in thermal denaturation by calcium binding could also cause a decrease in the autolysis rate, since unfolded or partially folded proteins tend to be more susceptible to proteolysis (*42*).

Conclusions

The kinetic inactivation of protease P45 in Tris-HCl buffer (100 mM, pH 8.0) at different temperatures, with or without the addition of Mg^{2+} or Ca^{2+}, was investigated. Although addition of Mg^{2+} showed a slight enhancement in the kinetic stability of this enzyme, a pronounced positive effect was observed with Ca^{2+}, which could be a useful feature of this protease for applications involving protein hydrolysis. Despite a possible effect of autolysis, the kinetic inactivation data of protease P45 showed to be best represented by first-order kinetics when compared to the other models evaluated. As residual enzyme activities were employed in the present analysis, differentiation among distinct inactivation mechanisms is difficult. However, regardless of the specific processe(s) leading to protease inactivation, activity loss and enzyme stability are the fundamental issues from a technological standpoint and, therefore, the

modelisation of protease P45 inactivation is of remarkable importance in evaluating its biotechnological potential.

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Table 1

Kinetic equations used to analyze the inactivation of keratinolytic protease P45.

Model (reference)	**Equation (nº)**
First-order (*14*)	$\frac{A}{Ao} = \exp(-kt)$ (1)
*n*th order (*15*)	$\frac{A}{Ao} = \left\{A_0^{\,1-n} + (n-1kt)\right\}^{1/(1-n)}$ (2)
Distinct isoenzymes (*14*)	$\frac{A}{Ao} = A_L \times \exp(-k_L t) + A_R \exp(-k_R t)$ (3)
Two-fraction (*17*)	$\frac{A}{Ao} = a \times \exp(-k_L t) + (1-a) \times \exp(-k_R t)$ (4)
Fractional conversion (*18*)	$\frac{A}{Ao} = Ar + (A_o - A_r) \times \exp(-kt)$ (5)
Series (*19*)	$\frac{A}{Ao} = \alpha_2 + \left[1 + \frac{\alpha_1 k_1}{k_2 - k_1} - \frac{\alpha_2 k_2}{k_2 - k_1}\right] \exp(-k_1 t) - \left[\frac{\alpha_1 k_1}{k_2 - k_1} - \frac{\alpha_2 k_1}{k_2 - k_1}\right] \exp(-k_2 t)$ (6)

Table 2

Summary of the performance of models selected to describe inactivation of keratinolytic protease P45.

Model (Equation)	r^2	χ^2	S.E.M.	Remarks
First-order (1)	[0.896;0.999]	[0.00006;0.0138]	[0.007;0.011]	High values for r^2 and low for S.E.M. and χ^2; accepted
*n*th order (2)	[0.986;0.999]	[0.000002;0.0009]	[0.001;0.027]	Negative parameters estimate; rejected
Distinct isoenzymes (3)	[0.869;0.995]	[0.0005;0.0371]	[0.005;0.075]	$k_L=k_S$; rejected
Two-fraction (4)	[0.947;0.999]	[0.0008;0.006]	[0.007;0.119]	Negative parameters estimate; rejected
Fractional conversion (5)	[0.983;0.998]	[0.0001;0.002]	[0.006;0.043]	Negative parameters estimate; rejected
Series (6)	[0.989;0.999]	[0.0003;0.0010]	[0.006;0.067]	Negative parameters estimate; rejected

Table 3

Kinetic parameters for first-order thermal inactivation of keratinolytic protease P45.

Metal ion	T (ºC)	r^2	k (min^{-1})	D (min)	$t_{1/2}$ (min)	z (°C)
Buffer with no additions	40	0.984	0.0259±0.003	88.80	26.73	7.16
	43	0.987	0.0728±0.001	31.61	9.52	
	45	0.896	0.1004±0.002	22.93	6.90	
	47	0.992	0.2543±0.012	9.06	2.73	
	50	0.989	0.6556±0.024	3.51	1.06	
Buffer plus 4 mM Mg^{2+}	40	0.968	0.0245±0.005	93.99	28.29	9.18
	43	0.986	0.0494±0.003	46.58	14.02	
	45	0.969	0.0760±0.002	30.28	9.11	
	47	0.978	0.1722±0.013	13.37	4.03	
	50	0.989	0.2728±0.021	8.44	2.54	
Buffer plus 3 mM Ca^{2+}	55	0.995	0.0494±0.010	46.59	14.03	8.04
	56.5	0.999	0.0815±0.004	28.24	8.50	
	58	0.969	0.1083±0.003	21.26	6.40	
	60	0.969	0.2171±0.013	10.61	3.19	
	62	0.974	0.3694±0.007	6.23	1.88	

Table 4

Thermodynamic parameters for first-order thermal inactivation of keratinolytic protease P45.

Metal ion	T (°C)	E_a (kJ mol^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$ (J mol^{-1} K^{-1})
Buffer with no additions	40	270.00	75.60	267.42	612.87
	43		73.63	267.40	613.18
	45		73.27	267.38	610.42
	47		71.28	267.37	612.78
	50		69.43	267.34	612.74
Buffer plus 4 mM Mg^{2+}	40	210.73	75.74	208.13	422.96
	43		74.65	208.11	422.33
	45		74.00	208.09	421.66
	47		72.31	208.07	424.26
	50		71.78	208.05	421.89
Buffer plus 3 mM Ca^{2+}	55	262.00	77.59	258.83	552.57
	56.5		76.59	258.82	553.06
	58		76.17	258.81	551.79
	60		74.72	258.79	552.77
	62		73.70	258.77	552.45

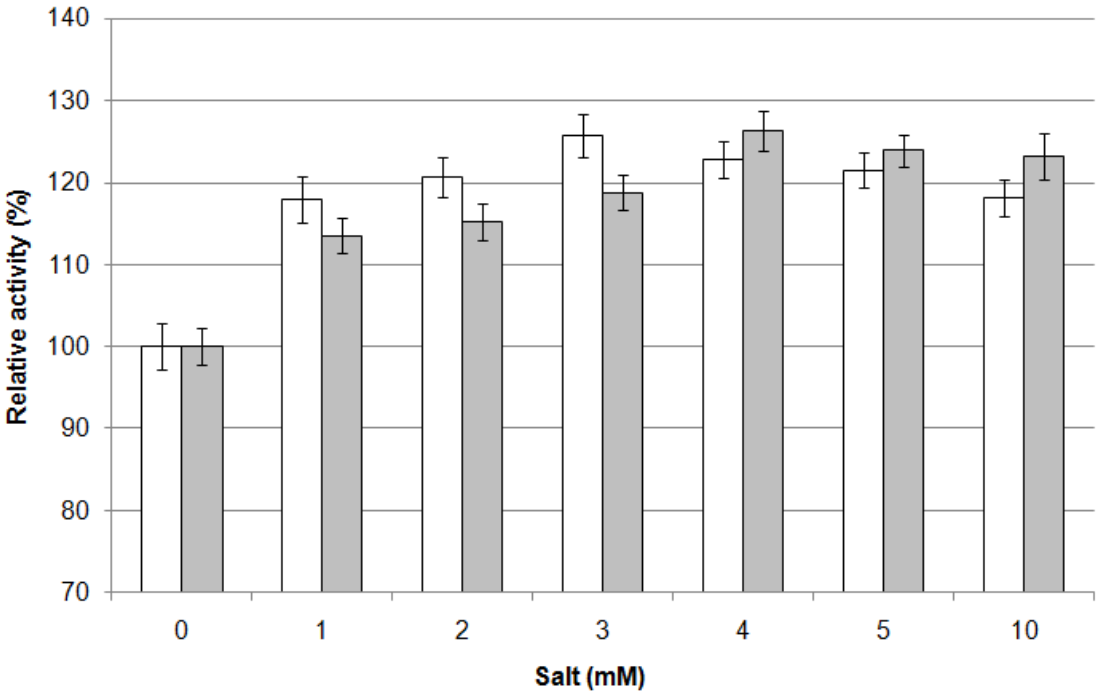

Fig. 1. Effect of different concentrations of calcium (white columns) and magnesium (gray columns) ions on the activity of protease P45 at 55°C.

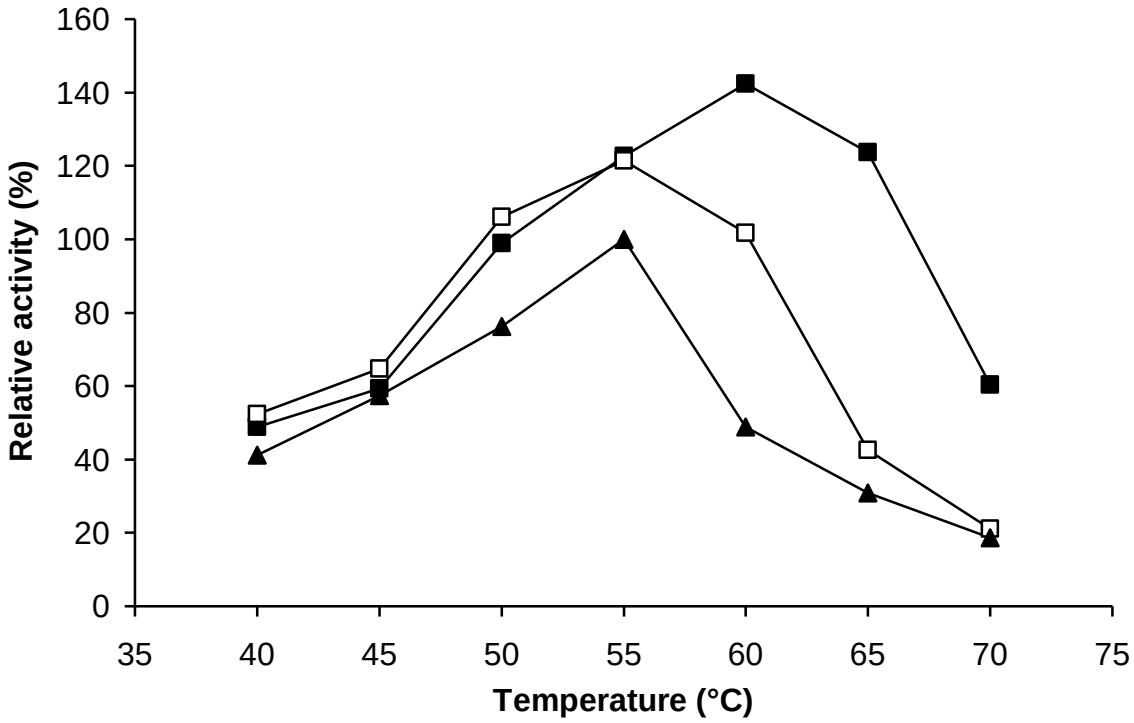

Fig. 2. Effect of temperature on the activity of protease P45 in buffer (triangle), and buffer in the presence of 3 mM calcium (black squares) or 4 mM magnesium (white squares).

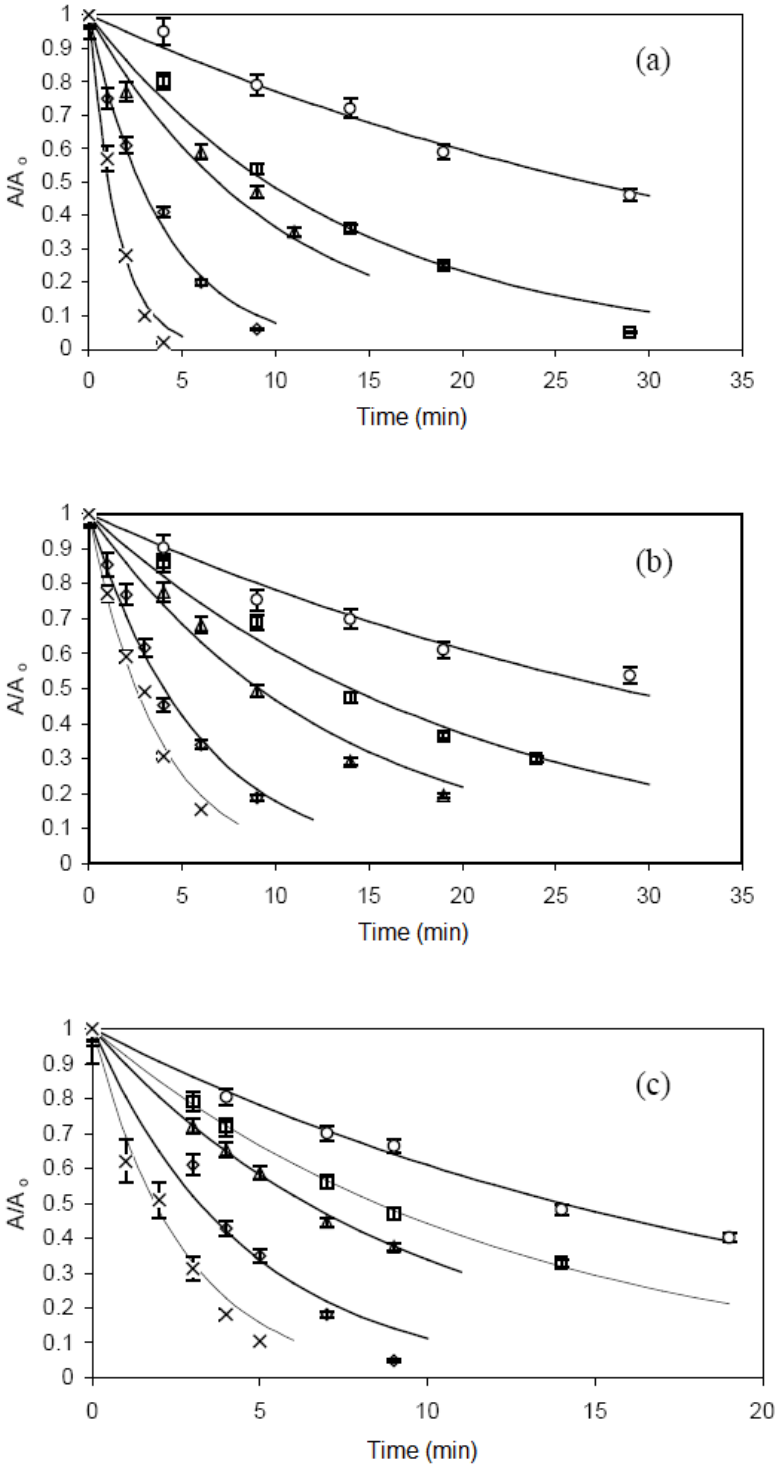

Fig. 3. Residual activity of protease P45 submitted to heating, fitted to first-order model. (a) In 100 mM Tris-HCl buffer (pH 8.0) without added metal ions at 40 (○), 43 (□), 45 (Δ), 47 (◊), and 50°C (×); (b) in buffer with 4 mM Mg^{2+} at 40 (○), 43 (□), 45 (Δ), 47 (◊), and 50 °C (×); and (c) in buffer with 3 mM Ca^{2+} at 55 (○), 56.5 (□), 58 (Δ), 60 (◊), and 62°C (×). Data presented are average values of two independent experiments, and standard deviations were always lower than 4%.

4 DISCUSSÃO GERAL

A biotecnologia industrial consiste na aplicação de células e/ou suas enzimas para a produção sustentável de compostos químicos, combustíveis, metabólitos, entre outros, a partir de fontes renováveis. Seu desenvolvimento e implementação são dirigidos também pela economia, visto que a biotecnologia prenuncia processos altamente eficientes com baixos custos operacionais e de capital. As demandas políticas e sociais por sistemas de produção ecologicamente corretos e sustentáveis, combinados com o aumento da necessidade de matérias-primas e energia, também atuam de forma importante no desenvolvimento da biotecnologia (TANG & ZHAO, 2009; SINGH, 2010).

O Brasil ocupa o terceiro posto na produção mundial de frangos de corte. Dados preliminares indicam que aproximadamente 5 bilhões de frangos foram abatidos no Brasil em 2010 em estabelecimentos sob inspeção federal, estadual ou municipal, totalizando valores próximos a 10,7 milhões de toneladas de carcaças (IBGE, 2011). Assumindo que as penas representam aproximadamente 7% do peso corpóreo das aves, estes números indicam que 0,7 milhões de toneladas de penas foram produzidas como resíduo pela indústria avícola nacional, representando um potencial problema ambiental devido à recalcitrância da queratina, principal componente das penas. Portanto,

tecnologias são necessárias para o descarte/manejo destes resíduos. Neste sentido, processos de bioconversão apresentam-se como alternativas apropriadas para a destinação e agregação de valor a estes resíduos (GOUSTEROVA et al., 2005; TIQUIA et al., 2005; FREEMAN et al., 2009; MATSUI et al., 2009).

Diversos microrganismos capazes de degradar queratina vêm sendo isolados de diferentes ambientes, indicando a ampla distribuição desta capacidade metabólica e a sua importância na ciclagem da queratina. Contudo, predomina o isolamento de microrganismos queratinolíticos eficientes pertencentes ao gênero *Bacillus* (ZAGHLOUL et al., 1998; GHOSH et al., 2007; BACH et al., 2011). No presente estudo procurou-se avaliar o potencial queratinolítico da bactéria *Bacillus* sp. P45 (Seção 3.1), previamente isolada do intestino do peixe Amazônico *Piaractus mesopotamicus*. Esta linhagem é reportada como produtora de peptídeos antimicrobianos e também surfactantes, metabólitos que apresentam potencial biotecnológico para o controle de microrganismos patogênicos e deteriorantes, bem como para aplicações em outras áreas (MOTTA et al., 2004; SIRTORI et al., 2006; VELHO et al., 2011, *no prelo*).

O potencial proteolítico desta linhagem foi inicialmente avaliado através de cultivos em placas de ágar leite. Após 24 h de cultivo, observou-se um halo transparente em torno da colônia, indicando a capacidade de produção de proteases extracelulares (Figura 1). O estágio subseqüente foi a investigação da capacidade de degradar resíduos queratinosos (penas e cabelo humano) em cultivos submersos. Observou-se a solubilização de penas

de frango durante o cultivo com esta linhagem, indicando sua habilidade em utilizar queratina como única fonte de carbonos, nitrogênio e energia. O aumento da concentração de proteínas solúveis e o aumento do pH durante o cultivo corroboram a eficiência na hidrólise de queratina. A produção de proteases/queratinases e o aumento do conteúdo de grupos tiol (-SH) foram detectados nos cultivos, indicando que tanto a proteólise quanto a sulfitólise são importantes mecanismos para a degradação das penas, originando peptídeos e aminoácidos passíveis de absorção para a nutrição microbiana (BRANDELLI, 2008; BRANDELLI et al., 2010).

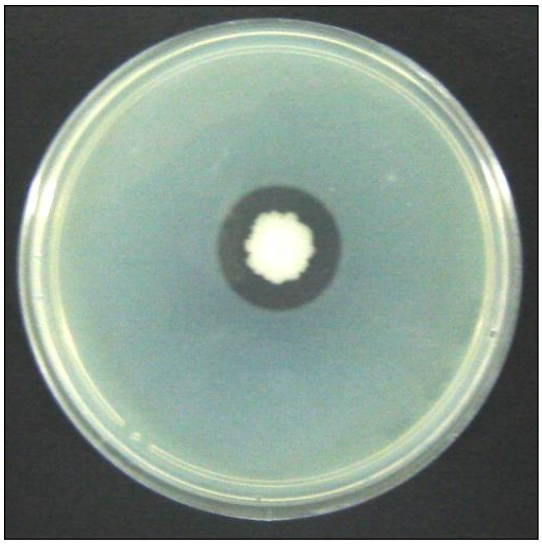

Figura 1. Colônia de *Bacillus* sp. P45 após cultivo em placa de ágar-leite a 30 °C por 24 h. O halo transparente em torno da colônia é representativo de proteólise.

A degradação de penas por diferentes microrganismos queratinolíticos é extremamente variável. Em bactérias queratinolíticas isoladas do solo, por exemplo, o percentual residual de penas após três semanas de

cultivo foi de 15 a 95% (LUCAS et al., 2003). No presente estudo, extensa solubilização das penas (88%) foi observada após 3 dias de cultivo, de forma similar a resultados reportados para diversos representantes do gênero *Bacillus* (SUNTORNUK & SUNTORNSUK, 2003; CORTEZI et al., 2008; SON et al., 2008; CORRÊA et al., 2010). A eficiência de *Bacillus* sp. P45 na degradação de penas sugere sua potencial aplicação na bioconversão destes resíduos. Contudo, não foi observada a degradação significativa de cabelo humano por esta linhagem, fato que pode ser atribuído a diferenças estruturais entre os substratos (Seção 3.1).

Espécies de *Bacillus* são predominantes em cultivos microbianos para a produção de metabólitos em escala industrial. Altas taxas de crescimento, produção de grandes quantidades de enzimas extracelulares e sua diversidade metabólica posicionam estas bactérias como as mais importantes produtoras de enzimas comerciais, incluindo amilases e proteases. Estima-se que enzimas de *Bacillus* contribuam com aproximadamente 50% do mercado mundial de enzimas. Ao conhecimento da bioquímica, fisiologia e genética deste gênero soma-se o fato de diversas linhagens de *Bacillus* serem geralmente consideradas seguras (*generally regarded as safe* - GRAS), o que favorece o desenvolvimento de novos processos (SCHALLMEY et al., 2004). O mercado de enzimas e processos enzimáticos desenvolve-se rapidamente devido à descoberta de novas enzimas, novas áreas de aplicação e à diminuição dos custos de produção de enzimas (VAN BEILEN & LI, 2002).

Neste sentido, a produção de queratinases por *Bacillus* sp. P45 foi avaliada em meios de cultivo contendo diferentes fontes de carbono e

nitrogênio. A maior produção de queratinase foi observada em meio contendo farinha de penas (FP) como substrato orgânico (Seção 3.2). A produção de baixos níveis de enzima em meio contendo caseína, comparada à elevada produção em meio de cultivo contendo penas ou FP, pode indicar a maior adaptação de *Bacillus* sp. P45 para a utilização de queratina como fonte de carbono e nitrogênio, também sugerindo que substratos específicos são requeridos para produção destas proteases (RIFFEL et al., 2011).

A produção de proteases queratinolíticas é comumente induzida pela presença de queratina no meio de cultivo (GUPTA & RAMNANI, 2006). De fato, a maioria das investigações utiliza substratos queratinosos como fontes de carbono e/ou nitrogênio para a obtenção de queratinases microbianas (BRANDELLI et al., 2010). Particularmente, resíduos agroindustriais ricos em queratina gerados pela agroindústria representam substratos abundantes e de baixo custo para a obtenção de proteases queratinolíticas.

A Tabela 1 apresenta as condições de produção de algumas queratinases microbianas utilizando resíduos queratinosos como substratos. Cultivos submersos são geralmente utilizados na produção de queratinases microbianas (Tabela 1). Esta modalidade de cultivo é comumente utilizada na produção de proteases em escala comercial, sendo preferida em relação a cultivos semi-sólidos (GUPTA et al., 2002b). Contudo, a produção de queratinases em cultivos semi-sólidos vem sendo recentemente relatada (GIOPPO et al. 2009; RAI et al. 2009; KUMAR et al., 2010; MORSY & El-GENDY, 2010; AWAD et al., *no prelo*), bem como a obtenção destas enzimas utilizando células microbianas imobilizadas (PRAKASH et al., 2010).

Tabela 1. Condições para produção de queratinases microbianas em cultivos submersos utilizando resíduos queratinosos*

Microrganismo	Substrato queratinoso	Condições de cultivo	Referência
Bactérias			
Bacillus sp. P45	Farinha de penas (4,3-5,0%)	30 °C; pH 7,0; 125 rpm; 48 h	Este estudo
Bacillus amyloliquefaciens	Penas (1%)	40 °C; pH 7,5; 150 rpm; 48 h	CORTEZI et al., 2008
Bacillus cereus DCUW	Penas (1%)	30-37 °C; pH 7,5; 140 rpm; 96 h	GHOSH et al., 2008
Bacillus halodurans JB 99	Penas (0,5%)	55 °C; pH 10,0; 220 rpm; 48 h	SHRINIVAS & NAIK, 2011
Bacillus licheniformis FK 14	Farinha de penas (1%)	37 °C; pH 7,0; 150 rpm (biorreator); 72 h	SUNTORNSUK et al., 2005
Bacillus licheniformis RPk	Penas (0,75%)	37 °C; 200 rpm; 48 h	FAKHFAKH et al., 2009
Bacillus pumilus A1	Farinha de penas (3%)	30 °C; pH 6,0; 250 rpm; 28 h	FAKHFAKH-ZOUARI et al., 2010b
Bacillus pumilis KHS-1	Penas moídas (0,5%)	40 °C; pH 6,0; 110 rpm; 84 h	KIM et al., 2005
Bacillus pumilus	Pelo bovino (1%)	35 °C; pH 8,0; 216 h	KUMAR et al., 2008
Bacillus pumilus FH9	Penas (1,5%)	37 °C; pH 8,0; 200 rpm; 48 h	EL-REFAI et al., 2005
Bacillus subtilis AMR	Cabelo humano (1%)	28 °C; pH 8,0; 300 rpm; 192 h	MAZOTTO et al., 2010
Bacillus subtilis MTCC (9102)	Farinha de chifres (1%)	37 °C; pH 7,0; 120 h	BALAJI et al., 2008
Bacillus subtilis KD-N2	Penas (1%)	23 °C; pH 7,5; 200 rpm; 30 h	CAI et al., 2008
	Cabelo humano (1,6%)	28 °C; pH 6,5; 200 rpm; 36 h	CAI & ZHENG, 2009
Bacillus sp. 50-3	Farinha de penas (1%)	37 °C; pH 7,0; 150 rpm; 36 h	ZHANG et al., 2009
Bacillus sp. P7	Farinha de penas (1%)	30 °C; pH 8,0; 120 rpm; 48 h	CORRÊA et al., 2010
Chryseobacterium sp. kr6	Penas (3%)	23 °C; pH 9,0; 125 rpm; 16 h	CASARIN et al., 2008
Chryseobacterium L99	Penas moídas (4%)	30 °C; 200 rpm; 30 h	LV et al., 2010

(continua)

Continuação - **Tabela 1.** Condições para produção de queratinases microbianas em cultivos submersos utilizando resíduos queratinosos*

Microrganismo	Substrato queratinoso	Condições de cultivo	Referência
Clostridium sporogenes bv. pennavorans bv.nov.	Penas (1%)	42 °C; pH 7,0; anaeróbica; 168 h	IONATA et al., 2008
Microbacterium sp. kr 10	Farinha de penas (1,25%)	25 °C; pH 7,0; 125 rpm; 96 h	THYS et al., 2006
Paracoccus sp. WJ-98	Queratina (1%)	37 °C; pH 7,5; 84 h	LEE et al., 2004
Stenotrophomonas maltophilia R13	Farinha de penas (0,1%)	30 °C; pH 7,0; 24 h; 200 rpm	JEONG et al., 2010b
Streptomyces sp. AB1	Farinha de penas (1%)	30 °C; pH 9,0; 200 rpm; 120 h	JAOUADI et al., 2010
Streptomyces sp. MS-2	Penas (1%)	35 °C; pH 8,0; 150 rpm; 72 h	MABROUK, 2008
Streptomyces sp. BA7	Pelo de cachorro (0,1%)	30 °C; pH 7,5; 250 rpm; 168 h	KORKMAZ et al., 2003
Streptomyces sp7	Farinha de penas (0,7%)	45 °C; pH 11,0; 300 rpm; 96 h	TATINENI et al., 2007
Thermoanaerobacter sp. 1004-09	Pelo suíno (0,2%)	65 °C; pH 6,8; anaerobiose; 96 h	KUBLANOV et al., 2009b
Fungos			
Aspergillus oryzae	Penas (1%)	30 °C; pH 6,0; estática; 120 h	FARAG & HASSAN, 2004
Myceliophthora thermophila	Farinha de penas (2,01%)	37 °C; pH 7,9; 120 rpm; 144 h	LIANG et al., 2011
Myrothecium verrucaria	Farinha de penas (1%)	28 °C; pH 6,0; 120 rpm; 96 h	GIOPPO et al., 2009
Scopulariopsis brevicaulis	Penas (1,0-1,5%)	30 °C; pH 7,5; 100 rpm; 5 semanas	ANBU et al., 2006, 2007
Trichoderma atroviride F6	Penas (1%)	30 °C; 150 rpm; 120 h	CAO et al., 2008
Trichophyton mentagrophytes	Penas (1%)	30 °C; pH 7,5; 100 rpm; 5 semanas	ANBU et al., 2006

* Em alguns estudos, fontes adicionais de carbono e/ou nitrogênio foram utilizadas além dos substratos queratinosos.

A suplementação do meio FP resultou em diferentes níveis de produção de queratinase. O efeito de diferentes fontes suplementares de carbono/nitrogênio sobre a produção de proteases microbianas é altamente variável, dependendo do microrganismo e substratos utilizados (SON et al., 2008; RIFFEL et al., 2011). No entanto, a presença de carboidratos prontamente disponíveis, por exemplo, atuou de forma negativa no rendimento, provavelmente através de repressão catabólica (KUMAR & TAKAGI, 1999; GUPTA & RAMNANI, 2006), mecanismo geralmente observado no controle da síntese de proteases em *Bacillus* (ÇALIK & ÖZDAMAR, 2001; PATEL et al., 2005). Contudo, a adição de NH_4Cl resultou em aumento da produção de enzima. Assim, o efeito da concentração de FP e NH_4Cl sobre a produção de queratinases foi avaliado em um experimento fatorial 2^2 completo. Nos intervalos investigados, 43-50 g/L de FP e 1,8-8,6 g/L de NH_4Cl resultaram no rendimento máximo de enzima. Concentrações elevadas de FP apresentaram tendência negativa na produção de queratinase (Seção 3.2). O modelo gerado a partir da análise de regressão dos dados demonstrou ser significativo e preditivo para a produção de queratinases. A superfície de resposta e a superfície de contorno, produzidas com base no modelo matemático gerado, são apresentadas nas Figuras 2 e 3, respectivamente.

Métodos convencionais de otimização da produção de enzimas usualmente modificam uma variável por experimento, mantendo as demais em níveis constantes. Tais experimentos não permitem a análise de possíveis interações entre variáveis. Assim, técnicas de experimento fatorial e superfície de resposta apresentam-se como eficientes estratégias para a obtenção de

condições ótimas em sistemas de múltiplas variáveis (KALIL et al., 2002; MANDENIUS & BRUNDIN, 2008). Neste sentido, diversos estudos utilizam estas técnicas para a otimização das condições de cultivo visando o incremento da produção de queratinases (ANBU et al., 2006, 2007; THYS et al., 2006; TATINENI et al., 2007; CASARIN et al., 2008; CAI & ZHENG, 2009; FAKHFAKH-ZOUARI et al., 2010b; PILLAI et al., 2011; LIANG et al., 2011).

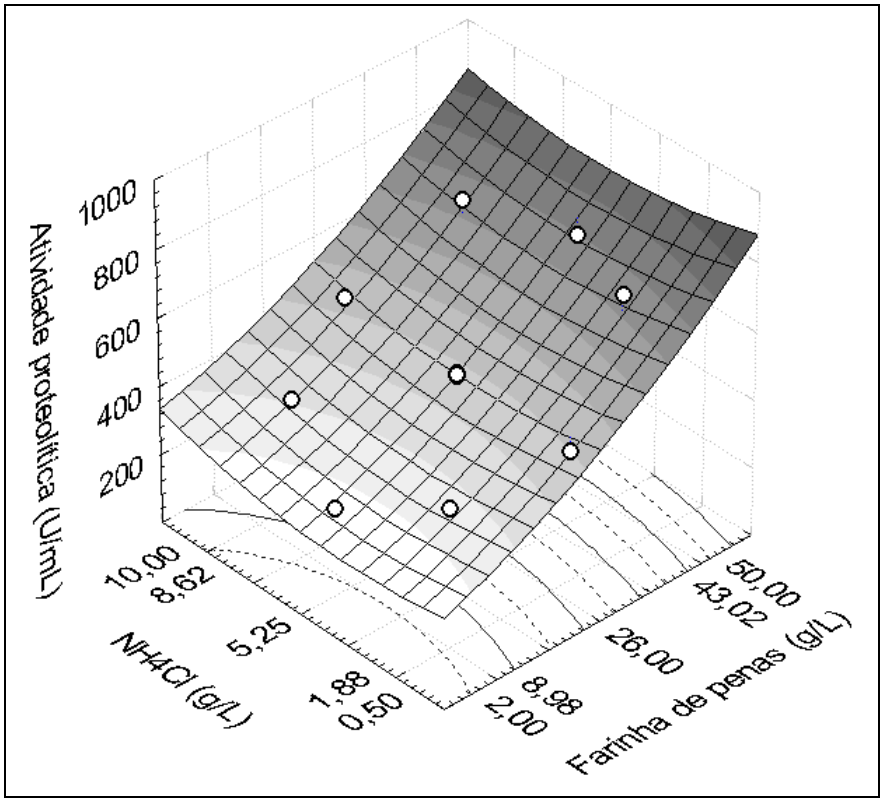

Figura 2. Superfície de resposta para os efeitos da concentração de farinha de penas (FP) e NH_4Cl sobre a produção de queratinases por *Bacillus* sp. P45 em cultivos submersos (30 °C, 48 h, 125 rpm).

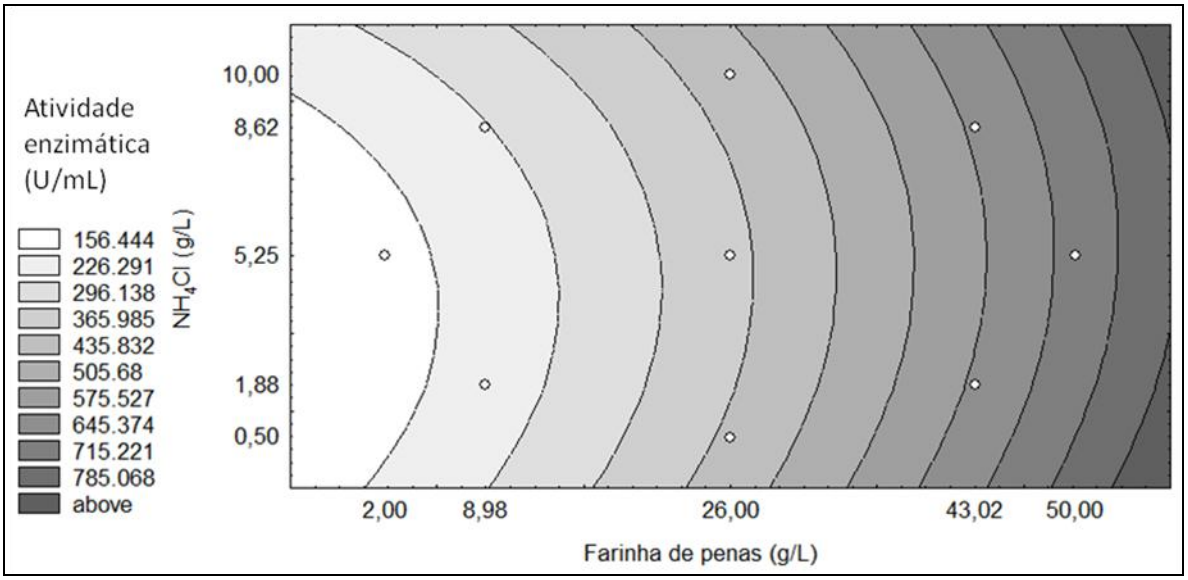

Figura 3. Superfície de contorno para os efeitos da concentração de farinha de penas (FP) e NH_4Cl sobre a produção de queratinases por *Bacillus* sp. P45 em cultivos submersos (30 °C, 48 h, 125 rpm).

Considerando o alto custo de produção de enzimas e, além disso, a necessidade da reciclagem de resíduos e energia (RAO et al., 2008; GUPTA et al., 2002a; IYER & ANANTHANARAYAN, 2008), a utilização de FP representa um meio de cultivo de baixo custo para a obtenção de queratinases de *Bacillus* sp. P45, bem como uma forma de agregação de valor ao resíduo queratinoso empregado como substrato, uma vez que, além da obtenção da enzima, os hidrolisados protéicos resultantes e a biomassa microbiana produzida podem ser utilizados em rações animais e/ou fertilizantes (GUPTA & RAMNANI, 2006).

A caracterização parcial da queratinase bruta produzida por *Bacillus* sp. P45 nos cultivos em meio otimizado foi então realizada. A atividade ótima foi observada a 50 °C e pH 7,0 (Seção 3.2). A atividade nesta temperatura é esperada para enzimas produzidas por microrganismos mesófilos. Embora

enzimas termofílicas normalmente demonstrem maior velocidade na hidrólise de substratos e diminuam a possibilidade de contaminação do processo, enzimas mesofílicas apresentam a vantagem de necessitar menores aportes energéticos para sua atuação (BRANDELLI et al., 2010), bem como maior facilidade no controle do processo de hidrólise (RAO et al., 1998). Ainda, destaca-se a atividade ótima em pH neutro, contrastando com a vasta literatura que reporta a produção de proteases alcalinas por representantes do gênero *Bacillus* (RAO et al., 1998; KUMAR & TAKAGI, 1999; GUPTA et al., 2002a,b).

Diversas proteases foram detectadas no sobrenadante do cultivo de *Bacillus* sp. P45 (Seções 3.1 e 3.2), que podem estar relacionadas com o processo de queratinólise e, consequentemente, à nutrição microbiana (BRANDELLI et al., 2010; RIFFEL et al., 2011). A produção de queratinases e a bioconversão de penas e FP por *Bacillus* sp. P45 em cultivos realizados em condições moderadas de temperatura (30 °C) podem sugerir o estabelecimento de um bioprocesso sustentável com baixas necessidades energéticas.

Uma das proteases extracelulares produzidas durante o cultivo foi purificada e caracterizada (Seção 3.3). Esta enzima, de aproximadamente 26 kDa, apresentou atividade ótima a 55 °C e pH 8,0, características comumente observadas em queratinases microbianas (Tabelas 2 e 3). Observa-se a predominância de estudos envolvendo queratinases bacterianas produzidas por representantes dos gêneros bacterianos *Bacillus* e *Streptomyces* (Tabela 2). Queratinases fúngicas são menos exploradas do ponto de vista biotecnológico (Tabela 3), o que pode ser devido, pelo menos em parte, ao potencial patogênico dos fungos dermatófitos.

Tabela 2. Propriedades de algumas queratinases bacterianas

Microrganismo	Tipo catalítico	Massa molecular (kDa)	pH ótimo	Temperatura ótima (°C)	Referência
Bacillus sp. P45	Serino	26	8,0	55	Este estudo
Bacillus sp. SCB-3	Metalo	134	7,0	40	LEE et al., 2002
Bacillus cereus DCUW	Serino	80	8,5	50	GHOSH et al., 2008
Bacillus halodurans PPKS-2	Serino	I: 30	I: 11,0	I: 60-70	PRAKASH et al., 2010b
	Serino	II: 66	II: 11,0	II: 60-70	
Bacillus halodurans JB 99	Serino	29	11,0	70	SHRINIVAS & NAIK, 2011
Bacillus licheniformis ER-15	Serino	58	11,0	70	TIWARY & GUPTA, 2010
Bacillus licheniformis FK14	Serino	35	8,5	60	SUNTORNSUK et al., 2005
Bacillus licheniformis K-508	Tiol	42	8,5	52	ROZS et al., 2001
Bacillus licheniformis MSK103	Serino	26	9,0-10,0	60-70	YOSHIOKA et al., 2007
Bacillus licheniformis PWD-1	Serino	33	7,5	50	LIN et al., 1992
Bacillus licheniformis RPk	Serino	32	9,0	60	FAKHFAKH et al., 2009
Bacillus pumilus	Serino	65	8,0	65	KUMAR et al., 2008
Bacillus pumilus A1	Serino-metalo	34	9,0	60	FAKHFAKH-ZOUARI et al., 2010a
Bacillus pumilus KS12	Serino	45	10,0	60	RAJPUT et al., 2010
Bacillus pseudofirmus FA30-01	Serino	27,5	8,8-10,3	60	KOJIMA et al., 2006
Bacillus subtilis KD-N2	Serino	30,5	8,5	55	CAI et al., 2008
Bacillus subtilis KS-1	Serino	25,4	7,5	–	SUH & LEE, 2001
Bacillus subtilis MTCC (9102)	Metalo	64-69	6,0	40	BALAJI et al., 2008
Bacillus subtilis RM-01	Serino	20,1	9,0	45	RAI et al., 2009

(continua)

Continuação - **Tabela 2.** Propriedades de algumas queratinases bacterianas

Microrganismo	Tipo catalítico	Massa molecular (kDa)	pH ótimo	Temperatura ótima (°C)	Referência
Bacillus subtilis S14	Serino	27	8,0-9,0	–	MACEDO et al., 2008
Brevibacillus sp. AS-S10-II	–	83,2	12,5	45	RAI & MUKHERJEE, 2011
Clostridium sporogenes	–	28,7	8,0	55	IONATA et al., 2008
Chryseobacterium sp. kr6	Metalo	64	8,5	50	RIFFEL et al., 2007
	Metalo	20	7,4-9,2	35-50	SILVEIRA et al., 2009, 2010
Chryseobacterium L99 sp. nov	Serine	33	8,0	40	LV et al., 2010
Chryseobacterium indologenes	Metalo	P1: 56	P1: 10,0	P1: 30-50	WANG et al., 2008a
TKU014	Metalo	P2: 40	P2: 7,0-8,0	P2: 40	
	Metalo	P3: 40	P3: 8,0-9,0	P3: 40-50	
Fervidobacterium islandicum AW-1	Serino	>200	9,0	100	NAM et al., 2002
Fervidobacterium pennavorans	Serino	130	10,0	80	FRIEDRICH & ANTRANIKIAN, 1996
Kocuria rosea	Serino	240	10,0	40	BERNAL et al., 2006
Kytococcus sedentarius	Serino	30-50	7,0-7,5	40-50	LONGSHAW et al., 2002
Lysobacter sp. NCIMB 9497	Metalo	148	–	50	ALLPRESS et al., 2002
Microbacterium sp. kr10	Metalo	42	7,5	50	THYS & BRANDELLI, 2006
Nesterenkonia sp. AL-20	Serino	23	10,0	70	GESSESSE et al., 2003
Nocardiopsis sp. TOA-1	Serino	20	>12,5	60	MITSUIKI et al., 2004
Pseudomonas aeruginosa KS-1	Serino	45	9,0	60	SHARMA & GUPTA, 2010b
	Serino	33	7,0	50	SHARMA & GUPTA, 2010c
Stenotrophomonas maltophilia DHHJ	Serino	35,2	7,8	40	CAO et al., 2009

(continua)

Continuação - **Tabela 2.** Propriedades de algumas queratinases bacterianas

Microrganismo	Tipo catalítico	Massa molecular (kDa)	pH ótimo	Temperatura ótima (°C)	Referência
Stenotrophomonas (*Xanthomonas*) *maltophilia*	Serino	36	8,0	60	DE TONI et al., 2002
Streptomyces sp. S7	Serino-metalo	44	11,0	45	TATINENI et al., 2008
Streptomyces sp. AB1	Serino	29,8	11,5	75	JAOUADI et al., 2010
Streptomyces sp. strain 16	Serino	KI: 203,2	KI: 9,0	KI: 50	XIE et al., 2010
	Serino	KII: 100,8	KII: 9,0	KII: 50	
	Serino	KIII: 31,8	KIII: 9,0	KIII: 50	
	Serino	KIV: 19,2	KIV: 9,0	KIV: 60	
Streptomyces albidoflavus	Serino	18	6,0-9,5	40-70	BRESSOLLIER et al., 1999
Streptomyces gulbargensis DAS 131	–	46	9,0	45	SYED et al., 2009
Streptomyces pactum	Serino	30	7,0-10,0	40-75	BÖCKLE et al., 1995
Streptomyces thermoviolaceus SD8	–	40	8,0	55	CHITTE et al., 1999
Thermoanaerobacter sp. 1004-09	Serino	150	9,3	60	KUBLANOV et al., 2009b
Thermoanaerobacter keratinophilus	Serino	135	8,0	85	RIESSEN & ANTRANIKIAN, 2001

Tabela 3. Propriedades de algumas queratinases fúngicas

Microrganismo	Tipo catalítico	Massa molecular (kDa)	pH ótimo	Temperatura ótima (°C)	Referência
Aspergillus fumigatus	Serino	–	6,5-9,0	45	SANTOS et al., 1996
Aspergillus oryzae	Metalo	60	8,0	50	FARAG & HASSAN, 2004
Doratomyces microsporus	Serino	30-33	8,0-9,0	50	GRADISAR et al., 2000
Myrothecium verrucaria	Serino	22	8,3	37	MOREIRA-GASPARIN et al., 2009
Paecilomyces marquandii	Serino	33	8,0	60-65	GRADISAR et al., 2005
Penicillium sp. Morsy1	Metalo	Ahm1: 19	Ahm1: 7,0-8,0	Ahm1: 50	EL-GENDY, 2010
	Metalo	Ahm2: 40	Ahm2: 10,0-11,0	Ahm2: 60-65	
Scopulariopsis brevicaulis	Serino	36-39	8,0	40	ANBU et al., 2005
Trichoderma atroviride F6	Serino	21	8-9	50-60	CAO et al., 2008
Trichophyton sp. HA-2	Serino	34	7,8	40	ANBU et al., 2008
Trichophyton mentagrophytes	Serino	38-41	4,5	–	TSUBOI et al., 1989
Trichophyton mentagrophytes var. erinacei	Serino	38	5,5	50	MUHSIN & AUBAID, 2000
Trichophyton schoenleinii	–	38	5,5	50	QIN et al., 1992
Trichophyton vanbreuseghemii	Serino	37	8,0	–	MOALLAEI et al., 2006

Resultados obtidos em ensaios com inibidores de proteases sugerem que a enzima purificada pertence ao grupo das serino proteases, dependento de íons metálicos para sua atividade ótima e/ou estabilidade. De fato, a maioria das queratinases produzidas por espécies de *Bacillus* é classificada no grupo das serino proteases (Tabela 2). As queratinases produzidas por fungos também são majoritariamente classificadas como serino proteases, apresentando atividade ótima em faixas neutras ou ácidas de pH (Tabela 3), enquanto que queratinases bacterianas apresentam, comumente, pH ótimo em faixas neutras ou alcalinas (Tabela 2).

A enzima purificada foi submetida à hidrólise com tripsina e os fragmentos resultantes foram seqüenciados. As sequências de aminoácidos dos oito peptídeos trípticos recuperados (Seção 3.3; Tabela 4) demonstraram homologia elevada (>90%) quando comparadas às sequências de diversas proteases semelhantes à subtilisina produzidas por representantes do gênero *Bacillus* (CHOI et al., 2004; GUPTA & RAMNANI, 2006; KIM et al., 2006, 2007, 2009; CHEN et al., 2007), incluindo subtilisina BPN' de *Bacillus amyloliquefaciens* (WELLS et al., 1983). Estas análises também indicaram que os peptídeos 2 a 8 são adjacentes na estrutura da enzima (Tabela 4). Contudo, homologias de aproximadamente 65% foram observadas quando da comparação dos fragmentos sequenciados com queratinases de *Bacillus licheniformis* PWD-1 (LIN et al., 1995) e subtilisina Carlsberg (JACOBS et al., 1985). A partir dos resultados de inibição, sequenciamento de peptídeos trípticos e o sequenciamento parcial do gene codificante para a enzima

(resultado não mostrado), a protease queratinolítica purificada foi identificada como uma serino protease semelhante à subtilisina.

Tabela 4. Sequência dos peptídeos trípticos da protease queratinolítica de *Bacillus* sp. P45

Peptídeo	Sequência*
1	VAVIDSGIDSSHPDLK
2-8	AVASGVVVVVAAAGNEGTSGGS**ST**VGYPGKYPSVIAVGAVNS
	SNQRASFSSVGSELDVMAPGVSIQSTLPGNKYGAYNGTSMAS
	PHVAGAAALILSKHPNWTNTQVRSSLENTTTKLGDAFYYGK

***** Com base nas sequências de proteases semelhantes à subtilisinas, □ indica os resíduos que fazem parte da tríade catalítica (SIEZEN & LEUNISSEN, 1997); ___ indica um dos resíduos que compõe o sítio de ligação forte de cálcio, e ___ indica resíduos que fazem parte do sítio de ligação fraca de cálcio (PANTOLIANO et al., 1988); os resíduos em negrito representam um dos sítios de autólise descritos para proteases semelhantes à subtilisina (BRAXTON & WELLS, 1992); _____ indica região conservada próxima ao resídio de asparagina (N) da cavidade do oxiânion (*oxyanion hole*) (GUPTA & RAMNANI, 2006).

As serino proteases, presentes em todos os domínios da vida celular e mesmo em genomas virais, são assim nomeadas por ser um resíduo de serina o principal responsável pela catálise. Estas enzimas contituem o maior grupo de proteases, representando aproximadamente um terço das enzimas proteolíticas conhecidas e identificadas (~66 mil; PAGE & DI CERA, 2008b). Entre as serino proteases mais estudadas estão a quimotripsina, tripsina,

elastase e subtilisina, cujas atividades dependem de uma tríade catalítica composta por resíduos de serina (S), histidina (H) e ácido aspártico (D). A serina atua como nucleófilo, a histidina participa como ácido/base geral e o ácido aspártico auxilia na orientação correta do resíduo de histidina em relação à serina (POLGÁR, 2005; EKICI et al., 2008). Embora o mecanismo catalítico destas enzimas seja semelhante, a estrutura tridimensional e a ordem dos resíduos na tríade catalítica das subtilisinas (D/H/S) são distintas em relação à quimotripsina, tripsina e elastase (H/D/S), indicando convergência evolutiva (BARRETT & RAWLINGS, 1995; PERONA & CRAIK, 1995). Nas proteases semelhantes à subtilisina, a tríade catalítica apresenta-se, usualmente, como D32/H64/S221 (SIEZEN & LEUNISSEN, 1997).

De forma breve, a catálise realizada por estas proteases inicia com o posicionamento do substrato no sítio ativo, de forma que a ligação peptídica a ser hidrolisada seja inserida próxima ao resíduo de serina. O grupamento nucleofílico (-OH) do resíduo de serina ataca a carbonila da ligação peptídica, resultando em um intermediário tetraédrico oxianiônico ligado covalentemente à serina. Este ataque nucleofílico é auxiliado pela presença do resíduo de histidina, que atua como uma base geral, aceitando o próton do grupo -OH da serina. A histidina agora tua como um ácido geral, protonando o grupo amina do intermediário tetraédrico, o que resulta na liberação do primeiro produto da reação, ou seja, o equivalente à porção C-terminal do substrato. Concomitantemente, há a formação de um complexo acil-enzima, que por sua vez sofre um ataque nucleofílico realizado por uma molécula de água, dando origem a um novo intermediário tetraédrico. Neste processo, a histidina aceita

um próton da molécula de água. A ligação formada entre serina e o grupo carbonila do substrato ataca o hidrogênio adquirido pela histidina, liberando a porção N-terminal do substrato e regenerando a enzima (KRAUT, 1977; PERONA & CRAIK, 1995; POLGÁR, 2005; EKICI et al., 2008).

Os intermediários tetraédricos observados durante a catálise são estabilizados por pontes de hidrogênio formadas entre o oxigênio do grupo carbonila (oxiânion) do substrato e um resíduo de asparagina (usualmente N155) presente em uma estrutura da enzima denominada cavidade do oxiânion (*oxyanion hole*) (Tabela 4; PERONA & CRAIK, 1995; SIEZEN & LEUNISSEN, 1997). Resíduos envolvidos na ligação de íons cálcio foram também identificados (Tabela 4), processo este que está relacionado à estabilidade das proteases semelhantes à subtilisina (PANTOLIANO et al., 1988; BRYAN, 2000). Além disso, foi possível identificar um dos sítios de autoproteólise postulados para estas enzimas (BRAXTON & WELLS, 1992).

O efeito de diversos reagentes (sais, detergentes, solventes, agente redutor) sobre a atividade da queratinase purificada foi avaliado. Os íons Ca^{2+} e Mg^{2+} apresentaram efeito positivo leve sobre a atividade enzimática, bem como o detergente aniônico Triton X-100, etanol, e dimetil-sulfóxido (DMSO). Usualmente, a atividade de queratinases microbianas é estimulada por íons Ca^{2+} e Mg^{2+}, através da manutenção da conformação ativa da enzima e proteção contra desnaturação térmica (GUPTA & RAMNANI, 2006).

A enzima purificada foi capaz de hidrolisar diversos substratos protéicos naturais, com destaque para caseína, farinha de penas e farinha de peixe. Com base na atuação sobre estes substratos, e também resultados de

outras investigações, podem ser sugeridas aplicações potenciais para esta enzima. A partir da hidrólise de caseinato ovino utilizando queratinase de *Bacillus* sp. P7 foram obtidos hidrolisados com promissoras atividades antioxidantes, antihipertensivas e antimicrobianas (CORRÊA et al., *no prelo*). A aplicação de queratinases na degradação de queratina para produção de fertilizantes foliares foi previamente relatada (VESELÁ & FRIEDRICH, 2009), bem como a possibilidade de utilizar proteases microbianas na produção de insumos e rações para a aquacultura (ESAKKIRAJ et al., 2009) e a adição de queratinases como suplemento em rações para animais monogástricos (WANG et al., 2006, 2008b).

A hidrólise de materiais queratinosos não ocorreu de forma extensa nos ensaios realizados. Diversos estudos corroboram os resultados obtidos, que podem ser devidos à presença de pontes dissulfeto nestes substratos, o que torna a proteína compacta e dificulta o acesso da enzima às ligações peptídicas (BRANDELLI, 2008). Neste sentido, apesar de estudos relatarem a ativação de queratinases na presença de agentes redutores (GUPTA & RAMNANI, 2006), este incremento na capacidade catalítica seja provavelmente provocado por efeitos do reagente na estrutura da queratina (substrato), e não sobre a queratinase (BRANDELLI et al., 2010).

A avaliação da hidrólise de peptídeos sintéticos indica a preferência por peptídeos longos (pelo menos quatro resíduos de aminoácidos) contendo aminoácidos aromáticos e/ou hidrofóbicos na posição P1, bem como a interferência de aminoácidos nas posições P2-P4 sobre a capacidade catalítica. Queratinases microbianas comumente demonstram o mesmo

comportamento (BRANDELLI et al., 2010). De acordo, a preferência das subtilisinas por diferentes substratos é amplamente determinada pela interação das cadeias laterais dos aminoácidos P4-P1 do substrato com os sítios de ligação S4-S1 na enzima, respectivamente, com destaque para os sítios S4 e S1, que aparentam dominar a relação de especificidade enzima-substrato (SIEZEN & LEUNISSEN, 1997; PAGE & DI CERA, 2008b).

A biocatálise utiliza a habilidade de enzimas na realização da conversão de substratos em produtos. A grande variedade de biocatalisadores, juntamente com a utilização de diferentes condições de reação para a bioconversão e a aplicação de técnicas de engenharia para a caracterização dos processos, permitem a implementação de sistemas biocatalíticos. Entre as vantagens da biocatálise, podem ser citadas a alta seletividade e eficiência catalítica das enzimas, a operação em condições moderadas e a possibilidade de modificações visando incrementar atividade, seletividade e estabilidade dos biocatalisadores (ILLANES, 2008; FERNANDES, 2010).

Neste sentido, o efeito positivo da adição de íons metálicos sobre a atividade enzimática pode ser uma variável importante na exploração do potencial tecnológico desta enzima. Os efeitos de Ca^{2+} e Mg^{2+} sobre a atividade e estabilidade térmica da queratinase purificada foram investigados e comparados às atividades na ausência destes íons. A presença de Ca^{2+} (3mM) ou Mg^{2+} (4 mM) resultou em aumento da atividade enzimática (26%) a 55 °C. No caso da adição de cálcio, a temperatura ótima, nas condições utilizadas, foi de 60 °C. Nesta temperatura, a atividade enzimática foi 42% maior do que nos controles (55 °C, sem adição de íons). Estas concentrações (3 mM para Ca^{2+}

ou 4 mM para Mg^{2+}) foram então utilizadas em ensaios de estabilidade térmica. Na ausência de íons metálicos adicionados e na presença de Mg^{2+}, a inativação da enzima foi avaliada durante aquecimento em temperaturas de 40 a 50 °C, enquanto que na presença de cálcio os ensaios foram realizados no intervalo de 55 a 62 °C.

Enzimas são catalisadores instáveis, e sua inativação usualmente envolve processos de desnaturação, ou seja, a desordenação da estrutura terciária da enzima (ILLANES, 2008). Altas temperaturas, por exemplo, atuam na quebra de pontes de hidrogênio na estrutura terciária da enzima, dando origem a uma molécula altamente desordenada que perde sua funcionalidade (IYER & ANANTHANARAYAN, 2008). Os resultados indicaram que a presença do íon cálcio é essencial para a estabilização da enzima em altas temperaturas. Sítios específicos para ligação de Ca^{2+}, relacionados à manutenção e estabilização da estrutura da enzima em temperaturas elevadas, estão presentes em serino proteases semelhantes à subtilisina (BRYAN, 2000; ALEXANDER et al., 2001; FISHER et al., 2007; Tabela 4). Portanto, o efeito estabilizante promovido pelo íon cálcio pode ser explorado para aumentar a vida-útil deste biocatalisador em processos tecnológicos de hidrólise protéica.

A atividade proteolítica diminuiu com o aumento dos tempos de incubação e também com o aumento das temperaturas, nos ensaios realizados. Os dados obtidos nestes experimentos foram avaliados através de seis diferentes modelos de inativação cinética, e a equação de primeira ordem foi aceita como a mais adaptada para descrever a inativação térmica da queratinase de *Bacillus* sp. P45. De forma análoga, a inativação térmica de

proteases microbianas é normalmente descrita como um processo exponencial de primeira ordem (ALEXANDER et al., 2001; SUBBA RAO et al., 2009; SILVEIRA et al., 2010). A modelagem de inativação térmica da queratinase pode ser utilizada na predição do comportamento cinético da enzima em condições de aplicação.

Parâmetros cinéticos podem auxiliar na determinação do potencial de aplicação da enzima. A partir da energia de ativação para desnaturação (E_a), obtida através da Equação de Arrhenius, foi possível estimar as constantes cinéticas $\Delta G^{\#}$ (energia livre), $\Delta H^{\#}$ (entalpia) e $\Delta S^{\#}$ (entropia) para o processo de inativação. A análise destes parâmetros corrobora o aumento da instabilidade cinética observada com o incremento da temperatura, indicando que este processo apresenta natureza endotérmica ($\Delta H^{\#}$ positivo), bem como o efeito estabilizante dos íons magnésio e cálcio. Nos sistemas avaliados na ausência/presença de íons metálicos adicionados, $\Delta S^{\#}$ parece ser o principal fator na determinação da inativação da enzima purificada. Aparentemente, a presença de íons metálicos reduziu os valores de $\Delta S^{\#}$, compensando os maiores valores de $\Delta H^{\#}$ na ausência de íons (o que indicaria maior estabilidade). Isto resultou em valores maiores de $\Delta G^{\#}$ em comparação com os dados da enzima na ausência de íons adicionados, ou seja, foram necessários maiores aportes energéticos (temperatura) para o aumento da desordem ($\Delta S^{\#}$) e consequente inativação catalítica da enzima (BROMBERG et al., 2008).

Em um contexto amplo, tecnologia enzimática e biotecnologia industrial podem ser consideradas como sinônimos. A aplicação desta tecnologia é particularmente interessante em indústrias de alimentos, rações,

papel, couros, entre outras, onde biomoléculas podem ser produzidas, degradadas ou modificadas através de processos catalisados por enzimas (VAN BEILEN & LI, 2002). A importância comercial destes biocatalisadores é demonstrada pelo mercado de enzimas industriais, que alcançou aproximadamente US$ 5 bilhões em 2009 (SANCHEZ & DEMAIN, 2011).

Contudo, enzimas podem ser desnaturadas durante sua produção, armazenagem e aplicação, o que contribui para o aumento dos custos dos processos nos quais estes biocatalisadores podem ser empregados. Neste contexto, a investigação da estabilidade enzimática é fundamental para a exploração destes biocatalisadores em nível industrial, podendo resultar em enzimas mais eficientes, aumentando o potencial biotecnológico e econômico de processos enzimáticos existentes e mesmo permitindo o desenvolvimento de novos processos, ora impedidos pela instabilidade enzimática (Ó'FÁGÁIN, 2003; IYER & ANANTHANARAYAN, 2008).

5 CONCLUSÕES

A bactéria mesofílica *Bacillus* sp. P45, previamente isolada do intestino do peixe Amazônico *Piaractus mesopotamicus*, foi capaz de utilizar penas inteiras e farinha de penas como única fonte de carbono, nitrogênio, enxofre e energia em cultivos submersos, sendo observada a extensa degradação destes substratos durante o cultivo. A atividade queratinolítica desta linhagem em temperaturas moderadas pode ser potencialmente utilizada no bioprocessamento destes resíduos queratinosos, com a concomitante obtenção de hidrolisados protéicos, biomassa microbiana e enzimas proteolíticas e queratinolíticas. Portanto, a bioconversão pode ser empregada como uma alternativa ao manejo dos resíduos queratinosos, atuando também na agregação de valor a estes resíduos.

A utilização de farinha de penas (FP) e NH_4Cl demonstrou ser a melhor combinação de substratos para a produção de queratinases extracelulares por *Bacillus* sp. P45 em cultivos submersos. Esta formulação representa um meio de cultivo simples e de baixo custo para a obtenção de queratinases visando potenciais aplicações.

A queratinase purificada, caracterizada como uma serino protease similar à subtilisina, apresentou atividade em amplas faixas de temperatura e

pH, com valores ótimos a 55 °C e pH 8,0. Estas características podem ser vantajosas para aplicações industriais, necessitando baixos aportes energéticos e evitando a geração de efluentes alcalinos. A baixa estabilidade térmica da enzima pode ser importante para o controle da atividade enzimática em bioprocessos.

A modelagem cinética dos dados de estabilidade térmica demonstrou a melhor adequação do modelo de primeira ordem para a representação da inativação enzimática. A presença de íons cálcio foi crucial para o aumento da estabilidade térmica da enzima, corroborando o fato de esta enzima ser relacionada às subtilisinas produzidas por *Bacillus* spp. Esta propriedade pode ser útil visando a aplicação deste biocatalisador na hidrólise de proteínas. Embora não tenha sido possível identificar o processo (ou processos) responsável pela inativação da enzima, o fenômeno da perda de atividade é fundamental do ponto de vista tecnológico.

De acordo com suas propriedades, a enzima pode ser potencialmente empregada na hidrólise controlada de proteínas alimentares, como caseínas e gelatina, bem como de proteínas como colágeno e β-queratina (após rompimento de ligações dissulfeto), entre outras, visando a modificação das propriedades funcionais destas e/ou a obtenção de peptídeos bioativos.

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