UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
MESTRADO EM BIOQUÍMICA E FISIOLOGIA

Produção de protease pelo *Penicillium aurantiogriseum* URM4622

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Recife, 2008

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Dissertação apresentada para o cumprimento parcial das exigências para obtenção do título de Mestre em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco

Aprovado por: ____

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Data: ____/____/______

Ata da defesa de dissertação da Mestranda **Priscila Maria de Barros Rodrigues**, realizada em 22 de fevereiro de 2008, como requisito final para obtenção do título de Mestre em Bioquímica e Fisiologia da UFPE.

Às 14:00 horas, do dia vinte e dois de fevereiro de 2008, foi aberto, no Auditório Prof. Marcionilo Lins – Depto. de Bioquímica do Centro de Ciências Biológicas da Univesidade Federal de Pernambuco, o ato de defesa de dissertação da mestranda **Priscila Maria de Barros Rodrigues**, aluna do Curso de Mestrado em Bioquímica e Fisiologia/CCB/UFPE. Iniciando os trabalhos a Profa. Dra. **Vera Lúcia de Menezes Lima** fez a apresentação da aluna, de sua orientadora Profa. Dra. Maria das Graças Carneiro da Cunha, da co-orientadora Profa. Dra. Ana Lúcia Figueiredo Porto, bem como da Banca Examinadora composta pelos professores doutores: Maria das Graças Carneiro da Cunha, na qualidade de Presidente, Ranilson de Souza Bezerra, ambos do Depto. de Bioquímica da UFPE, Nelson Manuel Viana da Silva Lima, da Universidade do Minho, e Keila Aparecida Moreira da Unidade Acadêmica de Garanhuns/Universidade Federal Rural de Pernambuco. Após as apresentações, a Profa. Dra. Maria das Graças Carneiro da Cunha convidou a aluna para a apresentação de sua dissertação intitulada: **"Produção de Protease pelo *Penicillium aurantiogriseum* (URM 4622)"**, e informou que de acordo com o Regimento Interno do Curso, a candidata dispõe de até 50 (cinqüenta) minutos para apresentação do trabalho e o tempo de argüição para cada examinador, juntamente com o tempo gasto pela aluna para responder às perguntas será de 30 (trinta) minutos. A aluna procedeu à explanação e comentários acerca do tema em **25 (vinte e cinco) minutos**. Após a apresentação da mestranda, a Sra. Presidente convidou a Banca Examinadora para ocupar seus lugares e passou a palavra ao primeiro examinador, Profa. Dra. Keila Aparecida Moreira, que agradeceu ao convite, fez alguns comentários e sugestões, iniciando sua argüição. Ao final, a referida professora deu-se por satisfeita. Em seguida, a Sra. Presidente passou a palavra para o Prof. Dr. Nelson Manuel Viana da Silva Lima, que agradeceu o convite, fez alguns comentários e sugestões, e iniciou sua argüição. Ao final, o referido professor deu-se por satisfeito. Logo após, a Sra. Presidente passou a palavra para o Prof. Dr. Ranilson de Souza Bezerra que agradeceu o convite, fez alguns comentários e sugestões, e iniciou sua argüição. Ao final, o referido professor deu-se por satisfeito. Em seguida, a Sra. Presidente, na qualidade de orientadora, usou da palavra para tecer alguns comentários a respeito do trabalho da aluna, agradeceu à Banca Examinadora e parabenizou a candidata. Em seguida, a Sra. Presidente passou a palavra para a co-orientadora Profa. Dra. Ana Lúcia Figueiredo Porto, que fez alguns comentários sobre o trabalho da aluna. Finalmente, a sessão foi suspensa para proceder ao julgamento pela Banca Examinadora, a qual se reuniu na Secretaria do Curso. Após alguns comentários, a Banca decidiu, por unanimidade, conceder a menção "**Aprovada com Distinção**". Nada mais havendo a tratar, lavrei a presente ata que vai assinada por mim, Secretário, e demais membros da Banca Examinadora. Recife, 22 de fevereiro de 2008.

Rodrigues, Priscila Maria de Barros

Produção de protease pelo *Penicillium aurantiogriseum* URM4622 / Priscila Maria de Barros. – Recife: O Autor, 2008.

56 folhas : il., graf., tab.

Orientadores: Maria das Graças Carneiro da Cunha, Ana Lúcia Figueiredo Porto e José António Teixeira.

Dissertação (Mestrado) – Universidade Federal de Pernambuco. Centro de Ciências Biológicas. Pós-Graduação em Bioquímica e Fisiologia, 2008.

Inclui bibliografia e anexos.

1. Enzimas proteolíticas - Biotecnologia 2. Enzimas proteolíticas – Planejamento experimental I. Título.

572.76 CDD (22.ed.) **UFPE**

CCB – 2010- 054

ÍNDICE

AGRADECIMENTOS...	I
LISTA DE FIGURAS..	II
LISTA DE TABELAS..	III
RESUMO..	IV
ABSTRACT..	V
1. INTRODUÇÃO..	01
1.1. Proteases..	01
1.2. Proteases microbianas..	03
1.3. Aplicações das proteases...	06
2. OBJETIVOS..	09
2.1. Geral..	09
2.2. Específicos...	09
3. REFERÊNCIAS BIBLIOGRÁFICAS...	10
4. ARTIGO..	14
Abstract..	16
Introduction..	17
Materials and Methods...	19
Materials..	19
Methods..	20
Culture medium..	20
Bioreactor studies...	20
Biomass Determination...	21
Protein assay..	21
Protease activity..	21
Effect of pH on the activity and stability of the protease	22
Effect of temperature on the activity and stability of the protease..	22
Effect of metal ions on activity..	22

Effect of oxidizing agent and surfactant................................	23
Effect of inhibitors..	23
Protease activity on different substrates................................	23
Compatibility with various commercial detergents……..........	24
Results and discussion..	24
Bioreactor Studies...	24
Effect of pH on the activity and stability of the protease................	25
Effect of temperature on the activity and stability of the protease.	26
Effect of metal ions on protease activity..	26
Effect of oxidizing agent and surfactant...	27
Effect of inhibitors..	28
Protease activity on different substrates..	29
Compatibility with various commercial detergents.........................	29
Conclusion..	30
Acknowledgments...	31
References..	32
Table 1..	36
Table 2..	37
Table 3..	38
Table 4..	39
Table 5..	40
Table 6..	41
Figure Captions...	42
Figure 1...	43
Figure 2...	44
Figure 3...	45
Figure 4...	46
Figure 5...	47
5. CONCLUSÕES...	48
6. ANEXOS	49
6.1. Normas para a revista: “*World Journal of Microbiology and Biotechnology*”	49
6.2. Indicadores de produção 2006-2008 (Resumos em congressos)	56

AGRADECIMENTOS

Agradeço primeiramente a Deus, por me dar tudo aquilo de que sou merecedora.

À minha mãe, pelo apoio em todos os momentos da minha vida, o que foi fundamental para que eu atingisse este objetivo.

À Profa. Dra. Maria das Graças Carneiro da Cunha e a Profa. Dra. Ana Lúcia Figueiredo Porto pela dedicação, orientação e amizade.

Ao Prof. Dr. José António Teixeira pelo ótimo acolhimento e oportunidade de adquirir novos conhecimentos na área de biotecnologia.

Ao Prof. José Luiz de Lima Filho, responsável pelo Laboratório de Biotecnologia/Lika, por disponibilizar os recursos e infra-estrutura para a realização deste trabalho.

À Profa. Dra. Cristina Maria de Souza Motta, do Departamento de Micologia da UFPE, por ter gentilmente cedido o microrganismo utilizado neste trabalho.

Aos meus amigos que fazem parte do Laboratório de Fermentações, de Enzimologia e Instalações Piloto (LIP) do Departamento de Engenharia Biológica da Universidade do Minho (Portugal) pela ajuda e acolhimento.

Aos meus amigos que fazem parte do laboratório de Biotecnologia/Lika, em especial a Carolina Lima, Daniela Viana e Tatiana Porto, pela grande ajuda no desenvolvimento deste trabalho.

A todos os amigos e funcionários do LIKA, em especial aos meus amigos da Patologia: Jorge Luiz, Mário Ribeiro, Vasco Malta, Luciano Melo, Rodrigo Bacelar e Renata Veiga.

Ao meu fiel companheiro Marcos Machado pela compreensão, amizade e amor.

E a todos que direta ou indiretamente contribuíram para a realização deste trabalho.

MUITO OBRIGADA!

LISTA DE FIGURAS

Figura 1. Macromorfologia do *Penicillium*.
(Fonte: http://www.microbiologia.vet.br/imagensmicologia.htm) 5
(acessado em 12/11/2007)
Figura 2. Conídios de *Penicillium* agrupados em forma de pincel.
(Fonte: http://www.microbiologia.vet.br/imagensmicologia.htm) 6
(acessado em 12/11/2007)

Artigo

Fig. 1 Pareto bar chart of effects of variables temperature (1), pH (2) and O_2 concentration (3) on (a) Biomass (g/L) and (b) on Specific activity (U/mg). The extension of bars across the vertical dotted line (p=0.05) represents the dimensions of significance. 43
Fig. 2 Effect of pH on the activity (a) and on the stability after incubation for 120 min (b) of the protease produced by *P. aurantiogriseum*. Buffers: (■) 0.1 M Sodium-phosphate; (▲) 0.1 M Tris-HCl; (●) 0.1 M Gliycine-NaOH. Each data point is an average of three experiments and the error bars show the standard deviation. 44
Fig. 3 Effect of temperature on the activity (a) and on the stability (b) of the protease produced by *P. aurantiogriseum*. Temperatures: 25 °C (◆); 30 °C (■); 40 °C (▲); 50 °C (○); 60 °C (□) and 70 °C (△).Each data point is an average of three experiments and the error bars show the standard deviation. 45
Fig. 4 Effect of oxidizing agent H_2O_2 on the activity of protease at different concentrations: (○) 5 %; (■) 10 % and (▲) 15 %. Each data point is an average of three experiments and the error bars show the standard deviation. 46
Fig. 5 Stability of protease in various commercial detergents. (◆); Bem-te-vi® (■); Ala® (○); Surf® (▲); Omo Multi Ação® (□) and Ace® (△). Activity of control sample devoid of any detergent, incubated under similar conditions, was taken as100 %. Each data point is an average of three experiments and the error bars show the standard deviation. 47

LISTA DE TABELAS

Artigo

Table 1. Factor levels used in the 2^3 full factorial design. 36

Table 2. Results of the 2^3 full factorial design. 37

Table 3. Effect of metal ions on protease activity. 38

Table 4. Effect of various surfactants on protease activity of *P. aurantiogriseum*. 39

Table 5. Effect of various inhibitors on protease activity of *P. aurantiogriseum* 40

Table 6. Substrate specificity of the protease from *P. aurantiogriseum.* 41

RESUMO

As proteases microbianas representam cerca de 60 % do total mundial de vendas de enzimas. Estas proteases têm sido extensivamente estudadas, devido à necessidade de novas proteases com diferentes características, para atender o rápido crescimento das indústrias baseadas na tecnologia de produção de enzimas. O presente trabalho objetivou a produção e caracterização parcial da protease produzida pelo *Penicillium aurantiogriseum* URM4622 em biorreator utilizando um planejamento experimental (2^3), visando sua aplicação em detergentes. A produção da protease ocorreu em bioreator (Fermenter RALF 2,0 L) de tanque agitado com 1,5 L de volume de trabalho, equipado com controles de temperatura, pH e oxigênio dissolvido. Amostras foram coletadas a cada 12 horas, para determinações da biomassa, curva de pH e atividade proteásica. As melhores condições para a produção da enzima, correspondente a maior atividade específica (43,67 ±1,98 U/mg) foram 26 ^{o}C; pH 7,0 e 25 % O_2. A protease do caldo fermentado mostrou-se estável em uma ampla faixa de pH 5,8 - 9,5 e a temperaturas de 25 – 40 °C. A atividade proteolítica decresceu cerca de 26 % na presença do íon Zn^{2+} e aumentou 29 % com o íon Mn^{2+}. Cerca de 96,2 % e 70,8 % da atividade proteolítica foram mantidas após 90 min de incubação com H_2O_2 a 5 % e 10 % (v/v), respectivamente. A inibição pelo PMSF revelou a presença de proteases do tipo serina; nenhuma inibição ocorreu em presença de Tween 80 e Triton X-100 e mais de 50% de sua atividade foi retida em presença de vários detergentes comerciais. Os resultados obtidos sugerem que o *Penicillium aurantiogriseum* URM4622 é uma fonte viável de produção de protease alcalina com potencial interesse na indústria de detergentes.

Palavras chave: *Penicillium aurantiogriseum*; produção, protease; biorreator; detergente; planejamento experimental.

ABSTRACT

Microbial proteases hold a world-wide position of about 60 % of the total enzyme sales. These proteases have been widely studied due to the need of new proteases with different characteristics to attend to the fast growth of the industry based on the technology of enzyme production. This work addressed to the partial characterization of proteases produced by *Penicillium aurantiogriseum* (URM4622) in bioreactor, using a 2^3 experimental statistical full design, with a view to a biotechnological application. The production of proteases occurred in bioreactor (Fermenter RALF 2.0 L) of stirred tank with 1.5 L of work capacity, provided with controls of temperature, pH and dissolved oxygen. Samples were collected every 12 hours for determinations of biomass, pH curve and protease activity. The best conditions for the production of the enzyme, corresponding to the highest specific activity values (43.67 ± 1.98 U/mg), were 26º C, pH 7.0 and 25 % dissolved O_2 concentration. The protease from fermented broth showed to be stable over a large pH range of 5.8 – 9.5 and at temperatures of 25 – 40 ºC. The proteolytic activity decreased about 26 % in the presence of Zn^{2+} ion and increased 29 % with the Mn^{2+} ion. About 96.2 % and 70.8 % of proteolytic activity were maintained after 90 min of incubation with 5 % H_2O_2 and 10 % (v/v), respectively. The inhibition with PMSF revealed the presence of peptidases of serine type; no protease inhibition occurred in the presence of Tween 80 and Triton X-100 and more than 50 % of its activity was retained in the presence of several commercial detergents. These results suggest that *Penicillium aurantiogriseum* is a viable production source of alkaline protease with potential interest of detergent industry.

Keywords: *Penicillium aurantiogriseum*; production, protease; bioreactor; detergent, statistical design

1. INTRODUÇÃO

1.1. Proteases

As enzimas são proteínas, portanto são constituídas por cadeias de aminoácidos unidas por ligações peptídicas. São biocatalizadores de alta especificidade e estão envolvidas nos principais processos bioquímicos que ocorrem nos organismos vivos, tanto nas alterações estruturais de biomoléculas como na expressão gênica e no processamento de nutrientes de natureza macromolecular. Enzimas proteolíticas, proteases, proteinases ou peptidases, são sinônimos utilizados para definir as enzimas pertencentes ao grupo das hidrolases, que têm em comum, o consumo da água na formação do produto. Catalisam as reações de hidrólise nas ligações peptídicas dos substratos protéicos (Lehninger, 2002).

As proteases executam uma grande variedade de funções nos organismos vivos, compreendendo a ativação e a participação em cascatas biológicas, como na digestão, homeostasia e inflamação em diversos sistemas, sendo imprescindíveis para o perfeito funcionamento celular (Gavrilescu e Chisti, 2005).

De acordo com o Comitê de Nomenclatura da União Internacional de Bioquímica e Biologia Molecular, as proteases estão classificadas no subgrupo quatro do grupo três das hidrolases. No entanto, as proteases não são facilmente agrupadas no sistema geral da nomenclatura enzimática, devido à sua enorme diversidade de ação e estrutura. As proteases são classificadas de acordo com os seguintes três critérios: (i) quanto ao tipo de reação catalisada, em exopeptidases e endopeptidases, que clivam, respectivamente, as ligações peptídicas próximas ou distantes das regiões terminais das proteínas; (ii) quanto à natureza química do sítio catalítico, as quais de acordo com a composição do sítio catalítico, dividem-se em serina, cisteína, aspartato e metalo proteases e (iii) quanto às relações evolutivas, onde são agrupadas em famílias e subdivididas em "clãs", de acordo com a convergência ou divergência de um ancestral comum. Baseando-se nestes critérios, as famílias das proteases são denominadas com uma letra, S, C, A, M e U para os tipos serina, cisteína, aspartato, metalo e tipo desconhecido, respectivamente (Barret et al., 2001).

As serino proteases são caracterizadas pela presença de um resíduo de serina no seu sítio ativo. São bastante numerosas, amplamente distribuídas entre vírus, procariotos

e eucatiotos, sugerindo uma importância vital para todos os organismos. De acordo com as suas similaridades estruturais, estão divididas em 20 famílias e seis "clãs". Outro aspecto interessante é a presença de resíduos conservados de glicina (Gly) próximos do resíduo de serina (Ser) no sítio ativo, formando a sequência característica Gly-Xaa-Ser-Yaa-Gly, onde Xaa e Yaa podem ser qualquer aminoácido. As serino proteases podem ser reconhecidas pela sua inibição irreversível pelos compostos 3,4-dicloroisocumarina (DCI), diisopropilfluorfosfato (DFP), 3-carboxitrans-2,3-epoxipropil-leucilamido e fluoreto de fenilmetilsulfonil (PMSF) (Rao et al., 1998).

As aspartil proteases, também são conhecidas como proteases ácidas e dependem da presença de resíduos ácidos do ácido aspártico (Asp) para a atividade catalítica. A maioria delas apresenta atividade máxima a valores baixos de pH e estão agrupadas em três famílias. O resíduo de ácido aspártico no sítio ativo das aspartil proteases, está situado geralmente dentro da sequência Asp-X-Gly onde X pode ser o aminoácido serina ou treonina. Estas proteases são inibidas pela pepstatina (Rao et al., 1998).

As cisteína proteases apresentam cerca de 20 famílias conhecidas e a atividade de todas depende da presença de uma díade de cisteína (Cys) e histidina (His) no sítio ativo, sendo que a ordem, Cys-His ou His-Cys, difere entre as famílias. Geralmente, estas enzimas são ativas somente em condições redutoras. São na sua maioria ativas em pH neutro, mas algumas, encontradas em lisossomos, têm o pH ótimo na faixa ácida. Outra característica destas proteases é a inibição por agentes sufidrila como o *p*-cloromercurilbenzoato (PCMB) e a iodoacetamida (Rao et al., 1998).

As metalo proteases são as enzimas que apresentam a maior diversidade de sítios catalíticos. São caracterizadas pela necessidade da presença de um íon metálico divalente para a sua atividade e estão distribuídas em cerca de 30 famílias, sendo 17 com atividade endopeptidase, 12 com atividade exopeptidase e uma contendo ambas. Estão agrupadas em "clãs" de acordo com a natureza dos aminoácidos que compõem o sítio de ligação ao metal. As metalo proteases são inibidas por agentes quelantes como o ácido etileno diamino tetracético (EDTA) e o ácido etileno glicol tetracético (EGTA) (Rao et al., 1998).

As proteases podem ser extraídas e/ou obtidas de várias fontes biológicas. A impossibilidade das proteases de plantas e animais atenderem à demanda mundial de enzimas tem levado a um interesse cada vez maior pelas proteases de origem microbiana. Além disto, as proteases de origem microbiana são preferidas, em relação

às enzimas de plantas e de animais, uma vez que possuem a maioria das características desejadas para a aplicação em biotecnologia, e o seu custo de produção, de uma maneira geral, é menor do que o das enzimas de origem vegetal ou animal. Aproximadamente 60 % da produção mundial de proteases é obtida de microrganismos (Sana et al*.,* 2006).

1.2. Proteases microbianas

Um grande número de microrganismos como as bactérias, leveduras e os fungos, são produtores de proteases (Potumarthi et al., 2007), quer sejam extracelulares ou intracelulares, ligadas ou não à membrana (Said e Pietro, 2002). Os microrganismos representam uma fonte atrativa de proteases devido à sua ampla diversidade bioquímica, por poderem ser cultivados em grandes quantidades num tempo relativamente curto por métodos estabelecidos de fermentação e por serem susceptíveis à manipulação genética. Além disto, as proteases microbianas têm uma vida mais longa e podem ser armazenadas, sob condições ideais por semanas, sem perda significativa da sua atividade (Gupta et al., 2002a).

A maioria das enzimas produzidas pelos microrganismos estão envolvidas em processos celulares. As enzimas extracelulares são capazes de digerir substratos solúveis e insolúveis, tais como a celulose, e os produtos digeridos são transportados para a célula onde são utilizados como nutrientes para o crescimento. Em geral, as proteases microbianas extracelulares são excretadas diretamente no meio de cultura pelo produtor, simplificando assim o processo de "dowstream" da enzima quando comparado com o das proteases obtidas de plantas e de animais. Apesar da diversidade de microrganismos produtores de proteases, somente alguns são considerados como produtores apropriados para a exploração industrial (Gupta et al., 2002b).

Na literatura, são relatados vários estudos sobre a produção de proteases por diversos microrganismos tais como, a bactéria *Serratia marcescens* (Ustáriz et al., 2004), o *Bacillus* sp. isolado do seu habitat natural (Genckal e Tari, 2006), o fungo *Beauveria bassiana* (Rao et al., 2006) e o *Aspergillus tamarii* (Anandan et al*.,* 2007).

Em relação às bactérias, os fungos produzem uma ampla variedade de enzimas e apresentam muitas vantagens como produtores de enzimas, considerando que as suas enzimas são normalmente extracelulares, tornando fácil a sua extração do caldo

fermentado e podem, ainda, ser cultivados utilizando meios de cultura de baixo custo como por exemplo, meios à base de resíduos agrícolas como a soja, o bagaço de cana, o farelo de trigo e o farelo de arroz (Soccol e Vandenberghe, 2003; Bergquist et al., 2002).

A tecnologia para a obtenção da produção de enzimas por microrganismos, consiste essencialmente em processos que incluem a seleção dos microrganismos da natureza, desenvolvimento da espécie por modificação genética, para a obtenção de altos rendimentos, cultivo do organismo selecionado no meio de cultura sob condições que induzam a produção de enzima e, finalmente, clarificação, concentração e estabilização do caldo fermentado, a fim de se obter a preparação enzimática (Dasu et al., 2003).

A produção de enzimas por fermentação é geralmente realizada em batelada. Para o cultivo de microrganismos em grandes quantidades e sob condições controladas são utilizados os biorreatores. Os biorreatores são reatores de tanques agitados, equipados com várias unidades de controle. Uma configuração básica é composta de unidades de controle automático para o pH, oxigênio, temperatura e a velocidade de agitação. Todos estes sistemas de controle funcionam sem qualquer contato com o ambiente externo, evitando assim a contaminação da cultura e a liberação de, por exemplo, organismos patogênicos, além de se ter um melhor controle de todo o processo de produção de enzimas. A importância destes sistemas de controle torna-se evidente considerando que num bioprocesso aeróbio, o consumo total de energia é compartilhado, em partes iguais, pela aeração, resfriamento e mistura (Dasu et al., 2003).

Todo o processo de fermentação é influenciado significativamente por parâmetros físicos (agitação, aeração e temperatura), químicos (pH, constituintes do meio) e biológicos (natureza do microorganismo). A produção de enzimas depende da temperatura, mas nem sempre a temperatura ótima da síntese de uma determinada enzima coincide com a temperatura ótima de crescimento do microrganismo. É preciso considerar ainda que, a temperatura ótima da atividade de uma enzima para um determinado substrato, pode diferir da temperatura ótima de crescimento e da síntese enzimática. As enzimas contêm grupos ionizáveis e, portanto, o pH do meio de cultura afeta a sua estrutura e a sua função. Assim sendo, o crescimento das células microbianas também é influenciado pelo pH, uma vez que a síntese dos constituintes básicos do

desenvolvimento celular depende dos íons. Assim como para a temperatura, o pH ótimo de crescimento celular e de produção podem diferir (Said e Pietro, 2002).

Diversos trabalhos descrevem a produção eficiente de proteases através de processos fermentativos por vários fungos pertencentes aos gêneros: *Aspergillus* (Anandan et al., 2007; Tunga et al., 2003), *Rhizopus* (Haq e Mukhtar, 2004), *Humicola* (Aleksieva e Peeva, 2000) e *Penicillium* (Agrawal et al., 2004; Germano et al., 2003; Silva et al., 2003).

O *Penicillium* é considerado um gênero universal de fungos, encontrado em todo o planeta. A maioria das espécies é saprófita e comumente ou ocasionalmente encontradas no solo, vegetais em putrefação, sementes, grãos e até no ar (Larsen et al., 2000). Estes fungos raramente são relatados como agentes etiológicos de micoses em humanos, sendo considerados patógenos oportunistas (Guglielminetti et al., 2000).

Os fungos do gênero *Penicillium* (Figura 1) pertencem à família Trichocomaceae, à ordem Eurotiales, à classe Eurotiomycetes e ao filo Ascomicota.

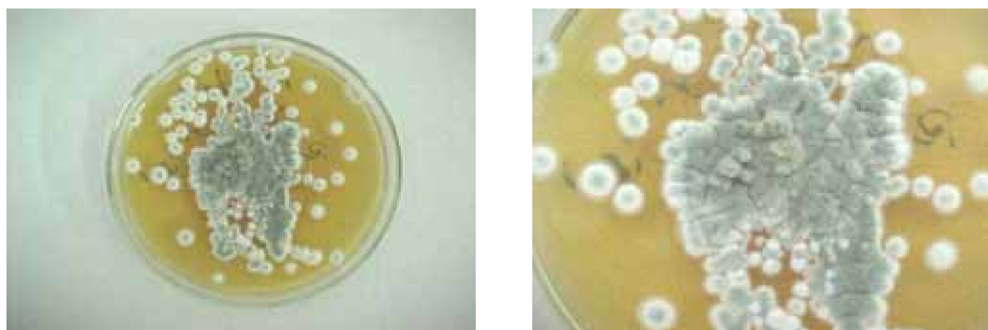

FIGURA 1. Macromorfologia do *Penicillium*
Fonte: www.microbiologia.vet.br/imagensmicologia.htm
(acessado em 12/11/2007)

A divisão citada acima é caracterizada morfologicamente por hifas septadas e reprodução assexuada através de propágulos denominados conídios. Por esta razão, são conhecidos como fungos imperfeitos. Apresentam corpo de frutificação, dentro do qual se desenvolvem os conidióforos com os seus conídios. Os conidióforos apresentam a forma semelhante à de um pincel (Figura 2), o que deu origem ao nome do gênero.

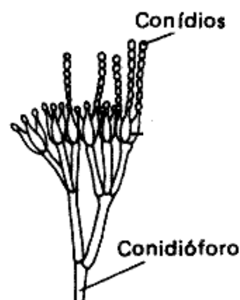

FIGURA 2. Conídios de *Penicillium* agrupados em forma de pincel.
Fonte: www.microbiologia.vet.br/imagensmicologia.htm
(acessado em 12/11/2007)

1.3. Aplicações das proteases

Sob o aspecto biotecnológico, as proteases compreendem as enzimas de maior relevância industrial (Gavrilescu e Chisti, 2005), representando cerca de 60 % do total de enzimas comercializadas mundialmente (Sana et al., 2006).

As proteases possuem uma grande variedade de aplicações, sendo utilizadas em diversas áreas da indústria como: alimentícias (Casaburi et al., 2008), farmacêuticas (Daviet e Colland, 2008), de curtumes (Kumar et al*.,* 2008) e de detergentes (Mukherjee et al., 2008).

Na indústria de detergentes as proteases são um dos ingredientes padrão adicionados a todos os detergentes, principalmente por causa da especificidade para alguns substratos constituintes do material a ser removido. Cerca de 30 % do total das vendas mundiais de enzimas são direcionadas às indústrias de detergentes, que adquirem proteases alcalinas para serem incorporadas como aditivos (Sana et al., 2006).

A primeira preparação de detergentes contendo proteases data de 1913 e consistia em carbonato de sódio e extrato de pâncreas (Rao et al., 1998). Somente a partir da década de 60, proteases extraídas de microrganismos começaram a ser utilizadas (Maurer et al., 2004). Nos últimos 30 anos, os estudos acerca de proteases industriais cresceram consideravelmente, embora ainda não haja sucessores para as proteases alcalinas versáteis e universalmente aplicadas como aditivos de detergentes como as substilisinas que são produzidas por bactérias do gênero *Bacillus* (Vasconcelos et al., 2006).

Atualmente, a maioria das enzimas proteolíticas utilizadas como aditivos de detergentes são serino-proteases produzidas por bactérias, embora as metodologias aplicadas para a sua obtenção impliquem um custo elevado (Vasconcelos et al., 2006). Paralelamente, a utilização de fungos não apresenta este problema, entretanto, existe a necessidade de ajustar o meio para a produção de proteases (Gavrilescu e Christi, 2005).

A utilização de proteases como aditivos tem crescido substancialmente devido ao fato de serem biodegradáveis e haver um aumento na razão entre o desempenho do detergente e o custo do processo quando estas biomoléculas são utilizadas nas formulações de detergentes (Saisubramanian et al., 2006). Atuam sobre manchas específicas ao degradarem os resíduos orgânicos de origem protéica, permitindo assim a substituição ou a redução da utilização de produtos cáusticos, ácidos e solventes tóxicos, que agridem o meio ambiente e causam o desgaste dos tecidos e de equipamentos (Vasconcelos et al., 2006).

As propriedades envolvidas na escolha de uma protease para a sua utilização como aditivo de detergente incluem: (1) atividade em pH alcalino e a elevadas temperaturas (Mukherjee et al., 2008), (2) estabilidade em presença de agentes oxidantes (Saeki et al., 2007), (3) eficiência na remoção de manchas em baixas concentrações em relação à concentração da solução de detergente (Vasconcelos et al., 2006), (4) especificidade a vários substratos (Moreira et al., 2003), e (5) aumento do potencial de limpeza de um detergente em 30 a 40 % (Sana et al., 2006).

Os detergentes modernos apresentam um espectro de ação e de utilização bastante amplo, havendo, conseqüentemente, necessidade de especialização das formulações (Mittidieri et al., 2006). Por este motivo, a indústria de detergentes tem emergido como a maior compradora de várias enzimas hidrolíticas alcalinas em todo o mundo, com a finalidade de aplicá-las como aditivos (Gavrilescu e Christ, 2005). Deste modo, no ambiente altamente competitivo do mercado de detergentes para lavanderia, os fabricantes são constantemente pressionados para a busca de proteases viáveis, não só sob o aspecto industrial, mas também financeiro, para a sua aplicação como aditivos a fim de satisfazerem, cada vez mais, os consumidores e se consolidarem industrialmente (Saisubramanian et al., 2006).

Uma outra aplicação das proteases é na indústria do couro em substituição do processamento tradicional das peles que envolve uma série de operações: a depilação, a qual trata da limpeza das peles, o curtimento, onde as peles são estabilizadas tornando-se resistentes a putrefação e, o acabamento, onde são asseguradas as características

estéticas do produto, gerando grande quantidade de resíduos químicos (Thanikaivelan et al., 2004).

Neste contexto, a depilação ocorre em condições extremamente alcalinas com utilização de grandes quantidades de sulfeto de sódio, altamente tóxico, gerando cerca de 80 % de toda poluição dos curtumes. A substituição por proteases, no processo, leva à redução da quantidade de sulfeto empregado, ao aumento na qualidade do couro, à recuperação dos pêlos e à eliminação da necessidade de banhos extras para a remoção dos agentes responsáveis pela depilação (Huang et al., 2003).

Um processo enzimático efetivo para a remoção dos pêlos deve liberar os pêlos do sistema epidérmico e abrir a estrutura fibrosa das peles. Experimentos utilizando enzimas microbianas têm atingido este objetivo (Huang et al., 2003). Estudos utilizando proteases produzidas por *Aspergillus tamarii* (Anandan et al., 2007), *Bacillus pumilus* (Huang et al., 2003), *Bacillus subtilis* (Macedo et al., 2005) e algumas bactérias queratinolíticas (Riffel et al., 2003) têm proporcionado resultados satisfatórios para um processo de depilação efetivo sem danos para a qualidade das peles.

Contudo, o aumento na demanda de proteases com propriedades específicas para os diversos setores industriais tem estimulado os biotecnologistas a explorar novas fontes destas enzimas (Mei e Jiang, 2005). Justifica-se, assim, o presente trabalho, no contexto biotecnológico, devido à procura de novas fontes de proteases com características específicas requeridas para a utilização nos diversos setores industriais.

2. OBJETIVOS

2.1. Objetivo geral

Produzir e caracterizar parcialmente a protease produzida por *Penicillium aurantiogriseum* URM4622, visando a sua aplicação em detergentes.

2.2. Objetivos específicos

- Estudar a influência das variáveis (pH, temperatura e aeração) na produção da protease em biorreator utilizando um planejamento experimental completo 2^3.
- Estudar o efeito da temperatura e do pH na atividade e na estabilidade da protease;
- Avaliar o efeito de inibidores na atividade proteásica;
- Determinar a atividade proteolítica frente a diferentes substratos protéicos;
- Avaliar a estabilidade da protease na presença de agentes oxidantes e de íons metálicos;
- Avaliar a compatibilidade da protease frente a diferentes detergentes.

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4. ARTIGO

PRODUCTION AND PARTIAL CHARACTERIZATION OF PROTEASE FROM *Penicillium aurantiogriseum* URM 4622 AS A LAUNDRY DETERGENT ADDITIVE

Artigo a ser submetido para publicação no periódico: *World Journal of Microbiology and Biotechnology*

PRODUCTION AND PARTIAL CHARACTERIZATION OF PROTEASE FROM *Penicillium aurantiogriseum* URM 4622 AS A LAUNDRY DETERGENT ADDITIVE

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Abstract

The increasing need for efficient and low cost proteases requires the development of high productivity fermentation processes with minimum downstream requirements for protease production. In this work, the optimal fermentation conditions for the production of a protease from *Penicillium aurantiogriseum* dierchx (URM4622) are presented together with the characterization of the catalytic properties. The best conditions for the production of the enzyme, corresponding to the highest specific activity values (43.67 ± 1.98 U/mg), were 26º C, pH 7.0 and 25 % dissolved O_2 concentration. The obtained enzyme, a serine protease, is stable over a wide range of pH (5.8 to 9.5) and temperature (25 to 40ºC) values; the presence of Zn^{2+} reduces its proteolytic activity being the opposite observed for Mn^{2+}; the protease maintains 96.2 and 70.8% of its activity after 90 min incubation in 5 and 10% (v/v) H_2O_2 aqueous solutions, respectively. Moreover, it is able to hydrolyze different proteins, is not inhibited by surfactants like Tween 80 and Triton X-100 and retains more than 50% of its activity in the presence of several commercial detergents. The possibility of using the entire fermentation broth (without further processing) as a detergent additive was also evaluated, being clearly demonstrated that only biomass removal is needed for the successful application of the produced proteases.

Keywords: *Penicillium aurantiogriseum*; production; characterization; protease; bioreactor; detergent.

Introduction

Proteases, also known as peptidyl-peptide hydrolases, are responsible for approximately 60 % of world enzyme sales being extensively used in a variety of industries, including food, pharmaceutical, leather and detergents (Chellappan et al. 2006; Rao et al. 2006).

The detergent industry has emerged as a major consumer of several hydrolytic enzymes acting at alkaline pH. They are primarily used as detergent additives since they are biodegradable and increase performance/cost ratios (Kumar and Takagi 1999; Gupta et al. 2002). The use of these enzymes as detergent additives stimulated their commercial development and resulted in a considerable increase in fundamental research (Chellappan et al. 2006; Rao et al. 2006).

Alkaline proteases added to laundry detergents play a specific catalytic role in the hydrolysis of proteins present in blood, milk, human sweat, etc. The increased usage of proteases as a detergent additive is mainly due to its cleaning capabilities in environmentally acceptable, nonphosphate detergents. The performance of alkaline proteases is influenced by several factors such as the pH of washing solution, the washing temperature and the detergent composition. Ideally, proteases used in detergent formulations should have a high activity and stability over a broad range of pH and temperature values and should also be compatible with various detergent components along with oxidizing and sequestering agents (Mei and Jiang 2005).

Microbes represent an excellent source of enzymes because of their broad biochemical diversity (Bhaskar et al. 2007; Najafi et al. 2005). Protease production is an inherent capacity of all microorganisms, however only those microbes that produce substantial amounts of extracellular enzyme are of industrial importance (Beg et al. 2003). Microbial proteases are gaining importance compared to conventional chemicals

as lower productions costs are possible and renewable resources can be used. Microbial proteases can be produced from bacteria, fungi and yeast using solid-state fermentation as well as submerged fermentation (Portumarthi et al. 2007).

Bacterial proteases have long been used in detergents, but the cost-intensive technologies required to obtain a microorganism free enzyme preparation remain an important drawback. On the other hand, fungal proteases have proved to be advantageous because mycelium can easily be removed by common filtration. In any case, it is essential to optimize the fermentation medium for the growth and production of proteases (Germano et al. 2003).

Any fermentation process is significantly influenced by the operating parameters and different results can be obtained as we move from shake flask to large bioreactor fermentation as different mixing and mass transfer patterns may occur as scale is increased (Dasu et al. 2003). It is well known that microbial extracellular protease production is greatly influenced by medium components, physical factors such as aeration, agitation, temperature, inoculum density, dissolved oxygen and incubation time. Also, industrial fermentation is moving away from traditional and largely empirical operation towards knowledge based and better-controlled process (Portumarthi et al. 2007).

Process optimization is a topic of central importance in industrial production processes. With particular regard to biotechnological production processes, in which even small improvements can be decisive for commercial success, process optimization is presently an undisputed component of the agenda of any commercial plant. In fermentation technology, improvements in the productivity of the microbial metabolite are achieved, in general, via the manipulation of nutritional and physical parameters and by strain improvement as result of selection by mutation or by genetic engineering.

Nowadays, taking in account the advantages associated with its application, statistical methodologies are widely applied for process optimization (Reddy et al. 2008).

Although there has been a number of studies on protease production by *Penicillium* species (Germano et al. 2003; Agrawal et al. 2004), little information on its characterization and optimization of submerged fermentation parameters is available in literature. This work addresses the optimization of the fermentation conditions for protease production by *Penicillium aurantiogriseum* dierchx (URM4622) and the characterization of the enzyme catalytic properties, as well as their compatibility with commercial laundry detergents, oxidants and surfactant agents.

Materials and methods

Materials

The *Penicillium aurantiogriseum* dierchx (URM4622) was supplied by the Micoteca of the Mycology Department of the Universidade Federal de Pernambuco (UFPE). Bovine serum albumin (BSA), Haemoglobin, Ovalbumin, Azocasein, Azocoll, Phenylmethylsulphonyl fluoride (PMSF), Ethylenediamine tetraacetic acid (EDTA) and Iodoacetic acid were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade. The enzyme free commercial laundry detergents Ace® (Protec & Gamble), Bem-te-vi® (Asa Ltd), Ala®, Omo Multi Ação® and Surf® (both from UniLever) were obtained from local supermarkets.

Methods

Culture medium

The *Penicillium aurantiogriseum* dierchx (URM4622) was maintained at 28 ºC in malt extract agar, consisting of: 2% (w/v) malt extract, 0.1% (w/v) peptone, 2% (w/v) glucose and 1.5 % (w/v) agar.

Soy flour medium (SM), as described by Porto et al. (1996), was used for protease production. SM was composed by: 1 % (w/v) filtered soy flour, 0.1% (w/v) NH_4Cl, 0.06% (w/v) $MgSO_4.7H_2O$, 0.435% (w/v) K_2HPO_4, 0.01% (w/v) glucose and 0.8 mL mineral solution. The composition of the mineral solution, per 100 mL of distilled water, was: 100 mg of $FeSO_4.7H_2O$; 100 mg of $MnCl_2.4H_2O$; 100 mg of $ZnSO_4.H_2O$; 100 mg of $CaCl_2.H_2O$. The fermentation medium was sterilized in autoclave at 121 ºC, for 20 min.

Bioreactor studies

The effects and interactions of the variables pH, temperature and O_2 concentration on the protease production in the bioreactor were evaluated using a 2^3 full factorial design. In this design, a set of 11 experiments, with three replicates at the central points, was performed. The range and levels of the components under study is given in Table 1.

The experiments were performed in a stirred tank reactor (Fermenter RALF 2.0 L) with 1.5 L working volume, equipped with temperature, pH and dissolved oxygen measurement and control. The pre-inoculum was prepared in 250 mL Erlenmeyer flasks containing 50 mL of the Soy flour medium with 10^6 spores/mL and incubated at 28 ºC, 150 rpm for 24 h. After this, the culture was added in bioreactor containing 1.45 L of the Soy flour medium previously sterilized in an autoclave at 121 ºC, for 30 min. The

pH of the medium was adjusted with NH_3 (6% v/v) and/or H_3PO_4 (21% v/v). Samples were collected at regular time intervals of 12 h during 84 h of growth for determination of the biomass, total protein concentration and protease activity. All statistical and graphical analyses were carried out with the "Statistica 8.0" software (StatSoft, Inc. 2008, USA).

Biomass determination

The growth of the *Penicillium aurantiogriseum* was accompanied by measuring biomass dry weight. The fermented broth was filtered through previously weighted 0.45 µm porosity membranes (after drying at 80 ºC, for 1 h). After the filtration of the fermented broth, the membranes were dried again at 80 ºC for 2 h. The dry weight of the samples was obtained by the difference of the weight of the membranes, after and before filtration.

Protein assay

The protein concentration was determined according to Bradford (1976), using bovine serum albumin (BSA) as standard.

Protease activity

The protease activity was determined according to Leighton et al. (1976) using 1% (w/v) azocasein as substrate in 0.1 M Tris-HCl buffer (pH 7.2). One unit of protease activity was defined as the amount of enzyme that produces an increase in the optical density of 1.0 in 1 h at 440 nm and was expressed as U/mL. The specific activity was calculated as the ratio between the protease activity (U/mL) and the total protein in the sample (mg/mL) and expressed as U/mg.

All the experiments concerning protease characterization were made using the whole fermentation broth after biomass removal by vacuum filtration through 0.45 µm nitrocellulose membranes.

Effect of pH on the activity and stability of the protease

The optimum pH of protease activity was determined with 1% (w/v) azocasein as substrate in different buffers. The buffers used were: 0.1 M sodium-phosphate (pH 5.8, 6.2 and 7.2); 0.1 M Tris-HCl (pH 7.2, 8.6 and 9.0) and 0.1 M glycin-NaOH (pH 8.6, 9.0 and 9.5).

The pH stability of the protease was determined by incubating the enzyme solution with the different mentioned buffers in a proportion of 1:1 (v/v) during 120 min at 25 ºC. Samples were collected every 30 min, for protease activity determination.

Effect of temperature on the activity and stability of the protease

The protease activity was assayed at various temperatures (25 - 70 ºC) to determine the optimum temperature, using 1% (w/v) azocasein as substrate in 0.1 M Tris-HCl buffer (pH 9.0).

For thermal stability determination, the enzyme was pre-incubated at different temperatures for 120 min, being samples taken every 30 min for determination of the protease activity. The shelf life at low temperatures (-20 ºC and 4 ºC) was determined after 30 days of incubation.

Effect of metal ions on activity

Different metal ions - $CaCl_2$, $AlCl_3$, LiCl, $ZnCl_2$, $MnCl_2$, KCl, and NaCl - dissolved in 0.1 M Tris-HCl buffer (pH 9.0) were added to the 1% (w/v) azocasein

solution, at a final concentration of 1 mM. Enzyme activities in the presence of metal ions were compared with the control (without metal ions).

Effect of oxidizing agent and surfactant

The enzyme was incubated with different concentrations of hydrogen peroxide from 5 to 15% (v/v) at 40 ºC for 120 min. Samples were collected every 30 min for protease activity determination.

Different solutions of surfactants like Tween 80, Triton X-100 and Sodium dodecyl sulphate (SDS) were tested at concentrations of 0.5 and 1% (v/v) for 60 min at 40 ºC. Samples were collected every 30 min for protease activity determination.

Effect of inhibitors

To determine the type of the protease, the enzyme was pre-incubated for 15 min at 37 ºC in with different specific protease inhibitors at a 0.1 M concentration and protease activity was determined. Tested inhibitors were: phenylmethylsulphonyl fluoride (PMSF), ethylenediamine tetraacetic acid (EDTA) and iodoacetic acid. The relative activity was determined as a percentage of the activity in control sample (without inhibitors).

Protease activity on different substrates

The protease activity, corresponding to the protease activity of the fermentation broth after biomass removal, was measured in different substrates. For bovine serum albumin (BSA), haemoglobin and ovalbumin, a modification of the method described by Pokorny et al. (1979) was used: for BSA and haemoglobin, 0.5 mL of protease sample were added to 1.0 mL of 1 % (w/v) BSA or 1 % (w/v) haemoglobin in 0.1 M

Tris-HCl buffer pH 9.0, followed by incubation at 37 °C for 10 min; in the case of ovalbumin, a 5 mL 1 % (w/v) protein solution in the same buffer used and incubation was done at 40 ºC for 10 min. For azocasein, the procedure described by Leighton et al. (1973) was applied while for azocoll the used protocol was the one by Chavira et al. (1984). The different reactions were stopped by the addition of 2 mL TCA (0.003 M) followed by centrifugation at 5000 g for 5 min.

Compatibility with various commercial detergents

The commercial detergents: Ala®, Bem-te-vi®, Omo Multi Ação®, Surf® and Ace®, were diluted in distilled water to give a final concentration of 7 mg/ml to simulate washing conditions. Protease from *Penicillium aurantiogriseum* URM4622 in a concentration of 0.05 mg/mL was incubated at 40 ºC for 90 min in various detergents (7mg/mL) and every 15 min samples were collected for protease activity determination. The relative activity in each sample was determined at 25 ºC, assayed and compared with the control sample incubated at 40 ºC without detergent.

Results and discussion

Bioreactor studies

The results obtained with the 2^3 full factorial design are shown in Table 2, being observed that the experiments with numbers 2, 5 and 10 showed the highest values for the protease specific activity 44.6, 41.3 and 45.0 (U/mg), respectively. These results showed that the central point of the experimental design (26 ºC, pH 7 and 25% O_2 concentration) was the best condition for the protease production.

The analysis of the Pareto bar chart (Figure 1), demonstrated that only the pH value was significant for biomass (Figure 1a) and enzyme production (Figure 1b). A pH

significant negative effect was observed, suggesting that the decrease of the pH value improves both the protease production and cell growth. It has been noted that the important characteristic of most microorganisms is their strong dependence on extracellular pH for cell growth and enzyme production (Kumar and Takagi 1999).

Similar results were previously obtained about the influence of controlled-pH and uncontrolled-pH conditions together with the initial pH in serine alkaline protease production by recombinant *Bacillus licheniformis* (Çalik et al. 2003).

Effect of pH on the activity and stability of the protease

The effect of pH on the enzymatic activity was determined at 25 ºC in the range 5.8 to 9.5. The relative activities at various pH values are shown in Figure 2a. Regarding the effect of the buffers themselves, differences of activity with different buffers were observed. The protease exhibited a maximum activity at pH 9.0 in 0.1 M Tris-HCl buffer. This is comparable with a detergent protease from *Conidiobolus coronatus*, with an optimum pH at 8.5, and the *Aspergillus parasiticus* protease which had an optimum pH of 8.0 and the activity decreased with increasing pH (Phadatare et al. 1993; Tunga et al. 2003). More than 90% of the maximum activity was detected for the protease between pH 7.2 – 9.5. The results are in accordance with several earlier reports showing an optimum pH of 8.5 – 9.0 for protease from *Penicillium* sp., *Aspergillus tamari* and *Aspergillus clavatus* (Agrawal et al. 2004; Phadatare et al. 1993; Tunga et al. 2003). It must pointed out that protease and other enzymes currently used in detergent formulations should be alkaline in nature with a high optimum pH because the pH of laundry detergents is commonly in the range of 9.0 – 11.0 (Mei and Jiang 2005; Banerjee et al. 1999; Anwar and Saleemuddin 1998). The important detergent enzymes, subtilisin Carlberg and subtilisin Novo or BpN also showed maximum

activity at pH 10.5 (Banerjee et al. 1999). Protease was very stable in a broad pH range from acid to basic, maintaining over 90% of its initial activity between pH values of 5.8 and 9.5, after incubation for 2 h (Figure 2b). Similar results were described for several *Penicillium* sp. and *Aspergillus parasiticus* strains in literature with the optimum pH range being between 6.0 and 9.0 (Germano et al. 2003; Tunga et al. 2003).

Effect of temperature on the activity and stability of the protease

The optimum temperature for the protease was found to be 50 ºC (Figure 3a). The protease was stable between 25 and 40 ºC after 2 h incubation retaining above 100% of the activity, which allows lower washing temperatures when added to detergents. More than 25% of maximal activity was maintained after 30 min pre-incubation at 50 ºC but total inactivation occurred above 60 ºC (Figure 3b). This result is similar to that described for proteases from *Penicillium* sp., *Aspergillus tamari, Aspergillus parasiticus, Streptomyces* sp. and *Norcadiopsis* sp. (Agrawal et al. 2004; Tunga et al. 2003; Anandan et al. 2007; Azeredo et al. 2004; Moreira et al. 2003). The *P. aurantiogriseum* protease possesses a maximum activity at high pH and a temperature range that indicates its usefulness in wide range of washing temperature programmes.

At low temperatures (-20 and 4 ºC), the protease retained 54.5% of its activity after 1 month of storage. The proteases from *Penicillium* sp. and *Streptomyces* sp. retained 98% and 75%, respectively, of its activity after 1 month at 4ºC (Germano et al. 2003; Azeredo et al. 2003).

Effect of metal ions on protease activity

The effect of metal ions on the activity of the protease was investigated and the results are shown in Table 3. The proteolytic activity decreased about 26% in the presence of Zn^{2+} ion and increased 29 % in the presence of Mn^{2+}. The presence of Li^{+}, Ca^{2+} and K^{+}, resulted in a discrete increase in the proteolytic activity. Similar effects of Mn^{2+} on the activity of protease were previously found (Agrawal et al. 2004) when studying the production of alkaline protease by *Penicillium* sp.

In the process of detergent formulation, where alkaline proteases are commonly added, chelating agents are included to overcome the problem of water hardness. However, in the presence of such chelating agents, the Ca^{2+} from the weak-binding site of the alkaline protease can easily be stripped-off, thus greatly affecting the thermal stability of the detergent enzyme under application conditions (Genckal and Tari 2006). Therefore, enzymes such as the one described in this study, where the effect of Ca^{2+} at 40 ºC was not significant, could offer tremendous benefit for detergent application.

Effect of oxidizing agent and surfactant

In order to be effective during washing, a good detergent protease must be compatible and stable with all commonly used detergent compounds such as surfactants, bleaches, oxidizing agents and other additives that could be present in the detergent formulation (Gupta et al. 2002). The results obtained with the stability of the protease in the presence of hydrogen peroxide (Figure 4) showed that at concentrations of 5, 10 and 15% (v/v) the protease retained 96.70, 81.20 and 67.35% of its activity, respectively, after 60 min of incubation. For the same hydrogen peroxide concentrations, after 120 min of incubation, the retained protease activity was 77.30, 46.50 and 27.90%, respectively. In the detergent industry, several oxidizing agents are used, like sodium perborate, which may release hydrogen peroxide and hence bleach

stable enzymes (Johnvesly and Naik 2001). The protease from *Aspergillus parasiticus* (Tunga et al. 2003) was not influenced by hydrogen peroxide, while the protease from *Penicillium* sp. (Germano et al. 2003) showed a good stability for a concentration of 5% (v/v) for 1 h of incubation. These results reinforce the interest on the application of the reported protease on the detergent industry.

As shown in Table 4, the enzyme was not inhibited in the presence of the non-ionic surfactants like Tween 80 and Triton X-100. The protease maintained 92.2 and 71.6% of its activity in the presence of 0.5% Tween 80 and Triton X-100 solutions after 60 min of incubation. Alkaline protease from *Aspergillus clavatus* was also reported as being highly stable in the presence of 5% Tween 80 and Triton X-100 solutions and SDS was the only detergent that totally inhibited its activity (Hajji et al. 2007). The *P. aurantiogriseum* protease was inhibited by SDS and retained only 12 and 6% of its activity in the presence of SDS (0.5 and 1.0%) after 60 min of incubation. A similar result was reported for *Penicillium* sp. (Agrawal et al. 2004). Also, proteases from *Aspergillus clavatus* had a low activity (33%) in the presence of SDS (0.5%) after 1 h of incubation (Hajji et al. 2007).

Effect of inhibitors

In order to determine the nature of the protease, enzyme activity was measured in the presence of different protease inhibitors (Table 5). No inhibition was detected when the cystein type inhibitor, i.e. iodoacetamide was added. Almost no inhibition was observed with EDTA. In contrast, protease was strongly inhibited by the serine protease inhibitor PMSF (0.1 M). This result shows that *P. aurantiogriseum* URM4622 secreted a serine type protease during submerged fermentation. This finding was similar to those

previously found (Germano et al. 2003; Anandan et al. 2007) for proteases produced by *Penicillium* sp. and *Aspergillus tamarii*, respectively.

Protease activity on different substrates

The ability to hydrolyze diverse protein substrates is one criterion of protease power. In this study, the enzyme was examined for the ability to hydrolyze several proteins (Table 6). Protease activity on bovine serum albumin (BSA) and hemoglobin was of 26 and 29%, respectively, as compared to its activity on azocasein. The protease showed the best activity against azocoll (144%). Results previously obtained studying proteases from *Aspergillus tamarii* showed a maximum activity with casein (Anandan et al. 2007). Bovine serum albumin was digested to a small extent as also shown using protease from *Norcadiopsis* sp. (Moreira et al. 2003). The enzyme reported in this work is capable of degrading hemoglobin, which indicates that it can be used for the removal of recalcitrant blood stains. The broader specificity of the protease from *P. aurantiogriseum* URM4622 may be advantageous for its use in detergents against a wide variety of stains.

Compatibility with various commercial detergents

The suitability of an enzyme preparation to be used in detergents depends on its compatibility with the detergents under a wide alkaline pH and temperature range. An ideal detergent enzyme should be stable and active in the detergent solution for a longer period of time and should have adequate temperature stability to be effective in a wide range of washing temperatures (Banik and Prakash 2004). The studies on compatibility with commercial detergents are shown in Figure 5. The protease produced by *P. aurantiogriseum* URM4622 retained more than 50% of its activity when incubated for

90 min at 40 ºC with all tested detergents and the maximum stability (80%) was achieved with Omo Multi Ação®. Serine alkaline protease from *Bacillus cereus* retained more than 80% of its activity in all of the commercial detergents tested even after 1h of incubation at 40 ºC and 50 ºC (Banik and Prakash 2004). Results previously obtained studying serine alkaline protease from *Bacillus* sp. SSR1 showing nearly 70 – 80% of activity in most of the detergents at 40 ºC (Singh et al. 2001). They reported that above 40 ºC, the addition of an additive like $CaCl_2$ is required to maintain enzyme stability. Which was also reported that protease retained a high activity in commercial detergents after supplementation of $CaCl_2$ and glycin (Banerjee et al. 1999). A comparison of our results with those reported in the literature shows that *P. aurantiogriseum* URM4622 protease presents an important advantage as it retains the enzyme activity for a long period in the presence of laundry detergents and no addition of additives is required. As hand washing or machine-washing of cloths normally takes 60 – 90 min, it is important that the enzyme present in detergent remains active during the washing period – this is the case of the reported protease that retained 83.6% of its activity in the presence of Omo Multi Ação® after incubation for 90 min at 40 ºC. *Penicillium* sp. protease has been reported to show compatibility with Omo Máquina® and Omo Multi-ação® (Germano et al. 2003).

Conclusion

The alkaline protease produced by *P. aurantiogriseum* dierchx (URM 4622) is a serine type peptidase, stable under a wide range of pH (5.8 to 9.5) and temperature (25 to 40 ºC) values. The protease activity was enhanced in the presence of Mn^{2+}, it was not inhibited by surfactants like Tween 80 and Triton X-100, SDS being an exception, and it was able to hydrolyze different proteins. Moreover, it remained stable at high H_2O_2

concentrations and in the presence of several commercial detergents (Bem-te-vi®, Ala®, Surf®, Omo Multi Ação® and Ace®). These results demonstrate the potential usefulness of the application of this protease in laundry detergents and are even more relevant if we consider that the whole fermentation broth (after biomass removal by filtration) can be used and, in this way, the production cost significantly improved.

Acknowledgements

This work was supported by CNPq/MCT (RENEBRA/nº 552410/2005-5), CAPES and ALFA- Valnatura project (AML/B7-311/97/0666/II-0440-FA).

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Table 1. Factor levels used in the 2^3 full factorial design.

Variables	Levels		
	Lower (-1)	Central (0)	Higher (+1)
Temperature (ºC)	22	26	30
pH	5.0	7.0	9.0
O_2 concentration (%)	10	25	40

Table 2. Results of the 2^3 full factorial design.

Run	A	B	C	BM	TP	PA	SA
1	30	9.0	10	0.69	0.714	0.053	0.07
2(*)	26	7.0	25	1.82	0.038	1.696	44.63
3	22	5.0	40	1.31	0.048	0.079	1.65
4	30	5.0	10	1.71	0.074	0.093	1.26
5(*)	26	7.0	25	2.22	0.054	2.235	41.39
6	30	5.0	40	1.80	0.034	0.142	4.18
7	22	9.0	10	0.72	0.526	0.040	0.08
8	22	5.0	10	3.08	0.036	0.682	18.94
9	30	9.0	40	0.86	0.412	0.062	0.15
10(*)	26	7.0	25	1.68	0.036	1.620	45.00
11	22	9.0	40	0.70	0.437	0.111	0.25

A- temperature (ºC); B- pH; C- O_2 concentration (%); BM- biomass (g/L); TP- total protein (mg/mL); PA- protease activity (U/mL); SA- specific activity (U/mg); (*) - central points.

Table 3. Effect of metal ions on protease activity.

Metal ions (1mM)	Relative activity (%)
Control	100
$AlCl_3$	99
$MnCl_2$	129
LiCl	105
$CaCl_2$	105
KCl	103
NaCl	100
$ZnCl_2$	74

Table 4. Effect of various surfactants on protease activity of *P. aurantiogriseum*.

Surfactants	Concentration (% v/v)	Residual activity (%)	
		after 30 min	after 60 min
Tween 80	0.5	93.5	92.2
	1.0	88.5	72.0
Triton X-100	0.5	95.9	71.6
	1.0	82.3	68.4
SDS	0.5	36.0	12.3
	1.0	15.2	6.1

Table 5. Effect of various inhibitors on protease activity of *P. aurantiogriseum*

Inhibitors	Relative activity (%)
Control	100.0
EDTA	94.8
PMSF	7.0
Iodoacetic acid	94.9

Table 6. Substrate specificity of the protease from *P. aurantiogriseum.*

Substrates	Concentration (%, w/v)	Specific activity (U/mg)	Relative activity (%)
BSA	1.0	21.3	26
Hemoglobin	1.0	24.2	29
Ovalbumin	1.0	9.9	12
Azocoll	0.2	118.2	144
Azocasein	1.0	82.3	100

Figure captions

Fig. 1 Pareto bar chart of effects of variables temperature (1), pH (2) and O_2 concentration (3) on (a) Biomass (g/L) and (b) on Specific activity (U/mg). The extension of bars across the vertical dotted line (p=0.05) represents the dimensions of significance.

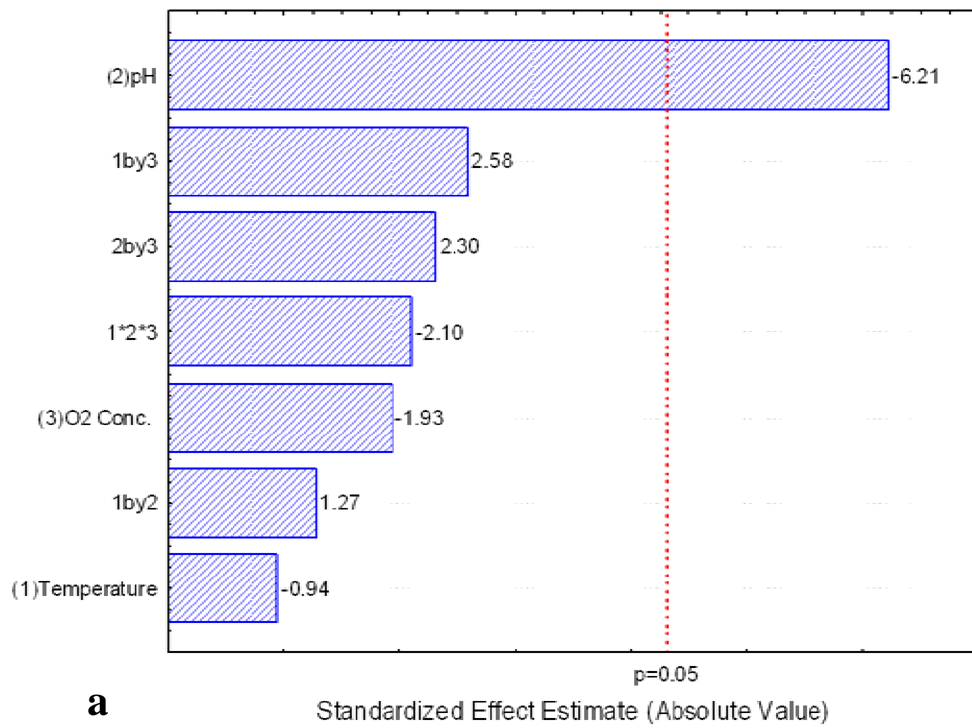
Fig. 2 Effect of pH on the activity (a) and on the stability after incubation for 120 min (b) of the protease produced by *P. aurantiogriseum*. Buffers: (■) 0.1 M Sodium-phosphate; (▲) 0.1 M Tris-HCl; (●) 0.1 M Gliycine-NaOH. Each data point is an average of three experiments and the error bars show the standard deviation.

Fig. 3 Effect of temperature on the activity (a) and on the stability (b) of the protease produced by *P. aurantiogriseum*. Temperatures: 25 °C (◆); 30 °C (■); 40 °C (▲); 50 °C (○); 60 °C (□) and 70 °C (△).Each data point is an average of three experiments and the error bars show the standard deviation.

Fig. 4 Effect of oxidizing agent H_2O_2 on the activity of protease at different concentrations: (○) 5 %; (■) 10 % and (▲) 15 %. Each data point is an average of three experiments and the error bars show the standard deviation.

Fig. 5 Stability of protease in various commercial detergents. (◆); Bem-te-vi® (■); Ala® (○); Surf® (▲); Omo Multi Ação® (□) and Ace® (△). Activity of control sample devoid of any detergent, incubated under similar conditions, was taken as100 %. Each data point is an average of three experiments and the error bars show the standard deviation.

Figure 1



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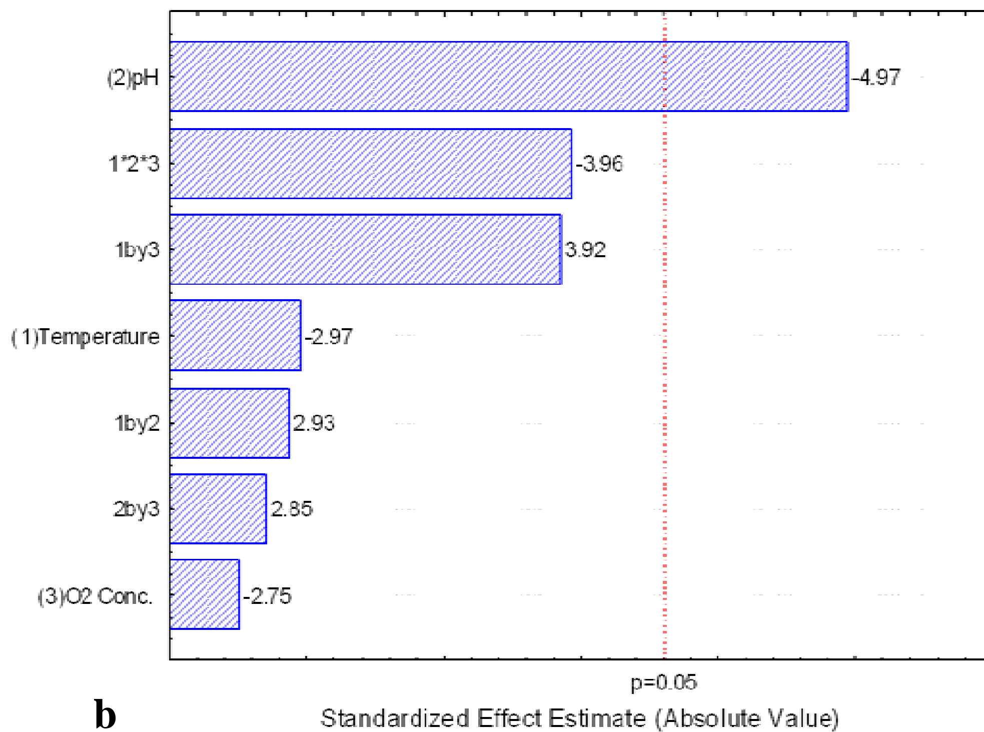
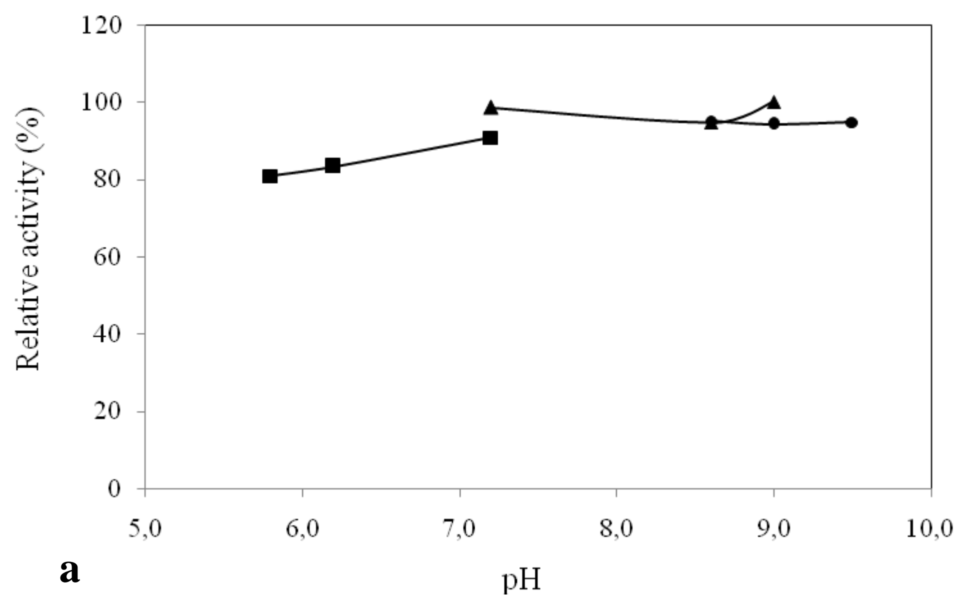


b

Figure 2



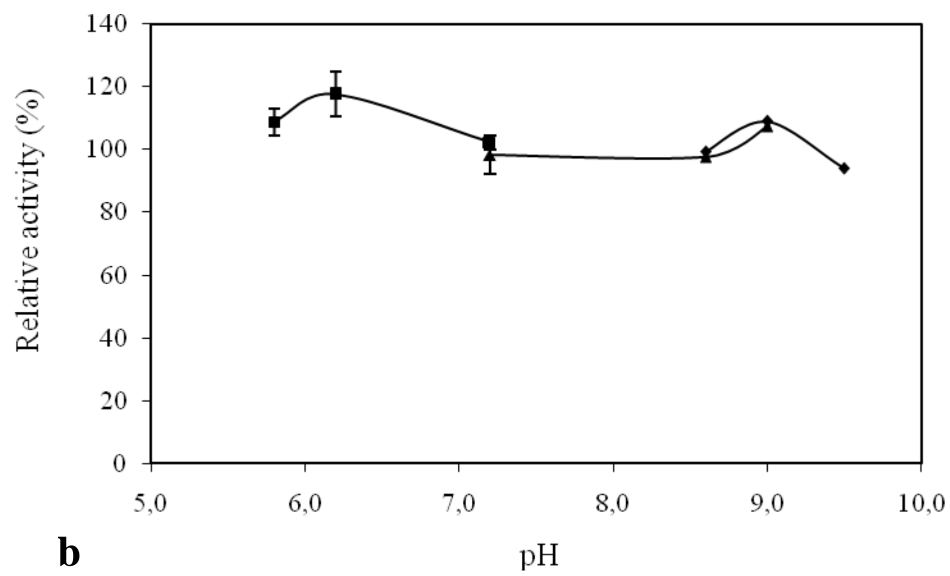
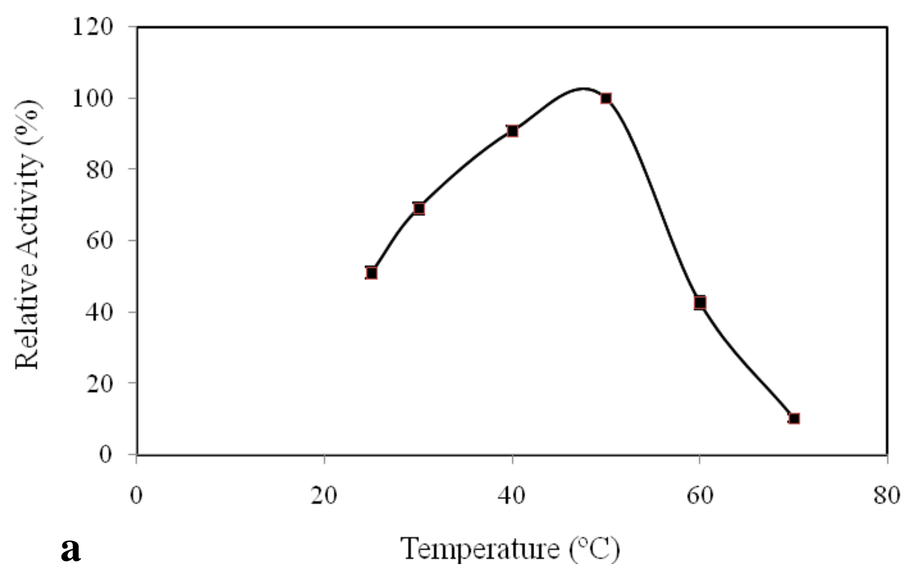


Figure 3



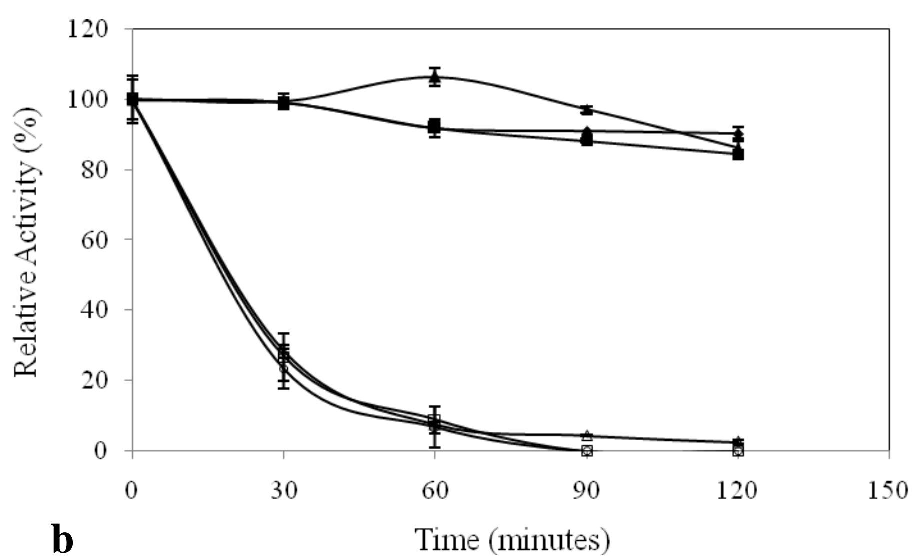


Figure 4

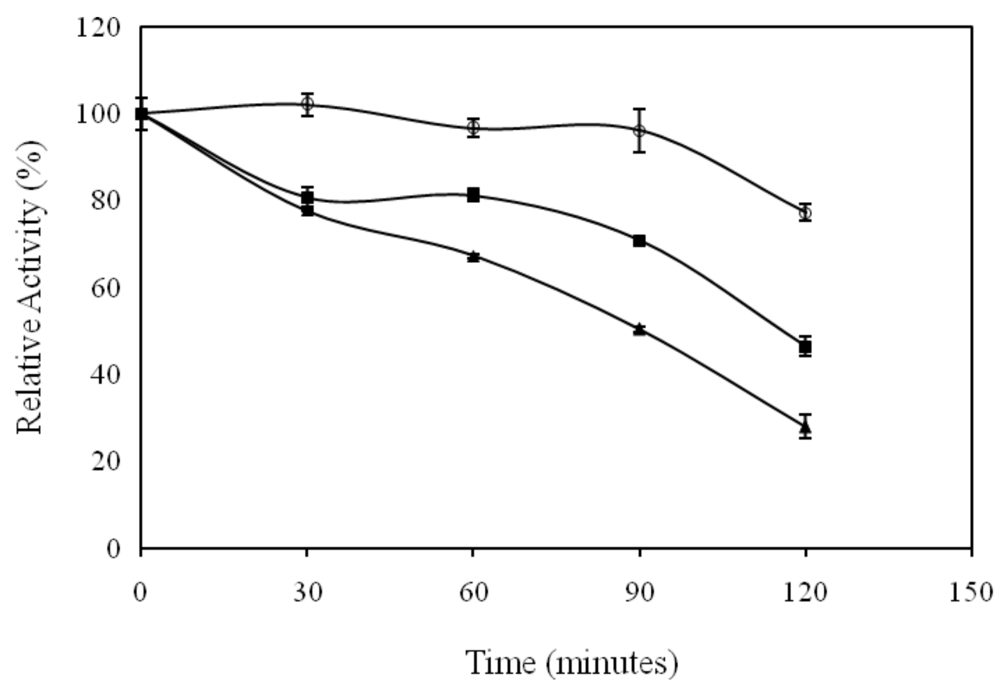
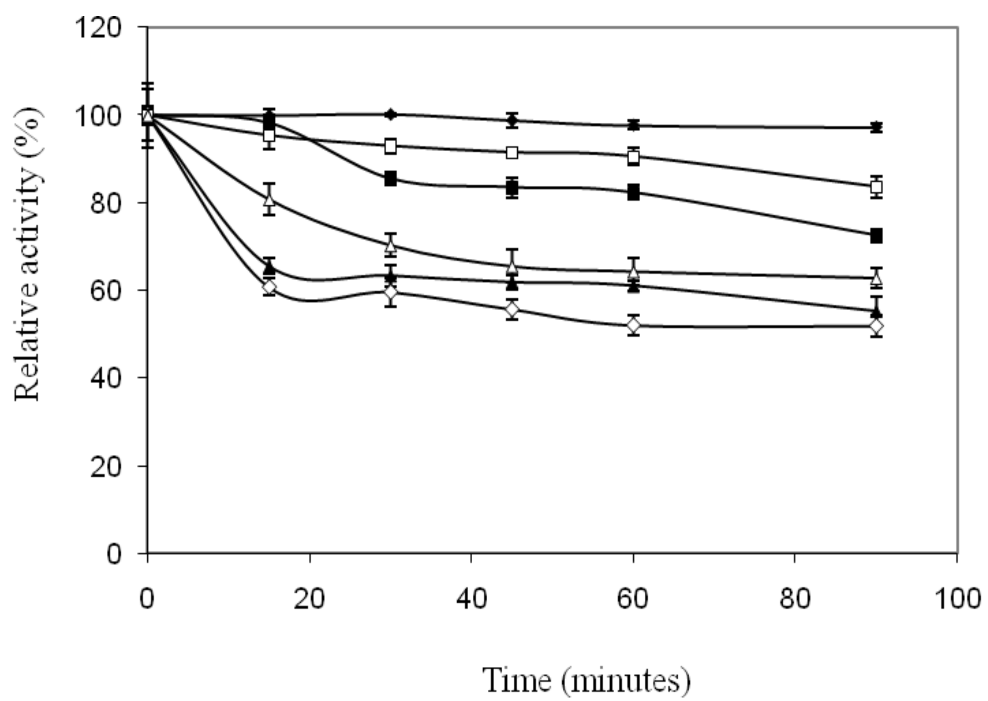


Figure 5



5. CONCLUSÕES

A partir dos resultados apresentados foi possível concluir que:

- As melhores condições para a produção da protease, correspondente a maior atividade específica (43,67 ±1,98 U/mg) foram 26 °C; pH 7,0 e 25 % O_2.
- A protease produzida apresentou : atividade máxima ao pH 9,0 e temperatura de 50 °C; estável nas faixas de pH 5,8 a 9,5 e de temperaturas de 25 ºC a 40 ºC, após 120 minutos; reteve 54,5 % de sua atividade a 0 °C e a 4 °C, após 30 dias de armazenamento.
- A atividade proteásica foi estimulada na presença do íon Mn^{2+};
- A protease demonstrou ser do tipo serina, sendo compatível ao H_2O_2 e a vários detergentes comerciais; foi ativa frente a diferentes proteínas e apresentou uma alta atividade colagenolítica;

Tais resultados sugerem que o *Penicillium aurantiogriseum* URM4622 é uma fonte viável de produção de proteases alcalinas de interesse industrial, apresentando características específicas para fins biotecnológico nas indústrias de detergentes.

6. ANEXOS

6.1. Normas para a revista: “*World Journal of Microbiology and Biotechnology*”

Instructions for Authors

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

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- Negotiation research spans many disciplines (Thompson 1990).
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  Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. doi: 10.1007/s00421-008-0955-8

  Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

  Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

- Article by DOI

  Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086

- Book

  South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

  Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

- Online document

  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

- Dissertation

  Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see

- www.issn.org/2-22661-LTWA-online.php

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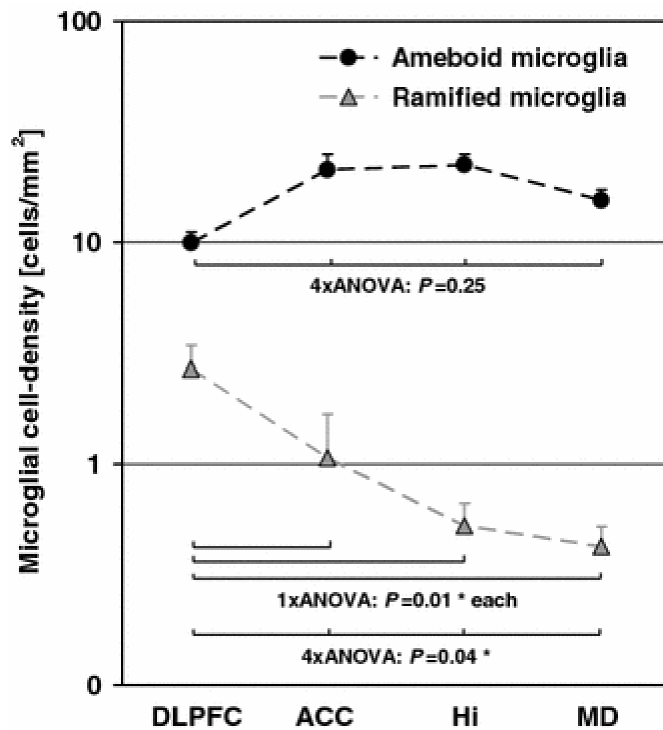

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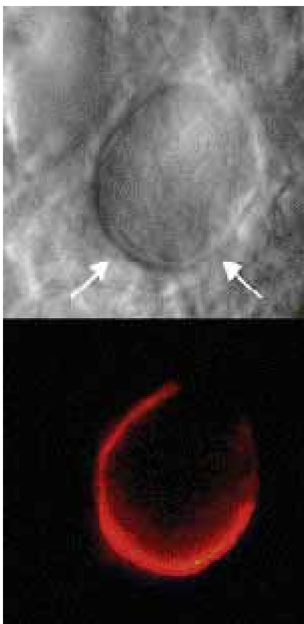

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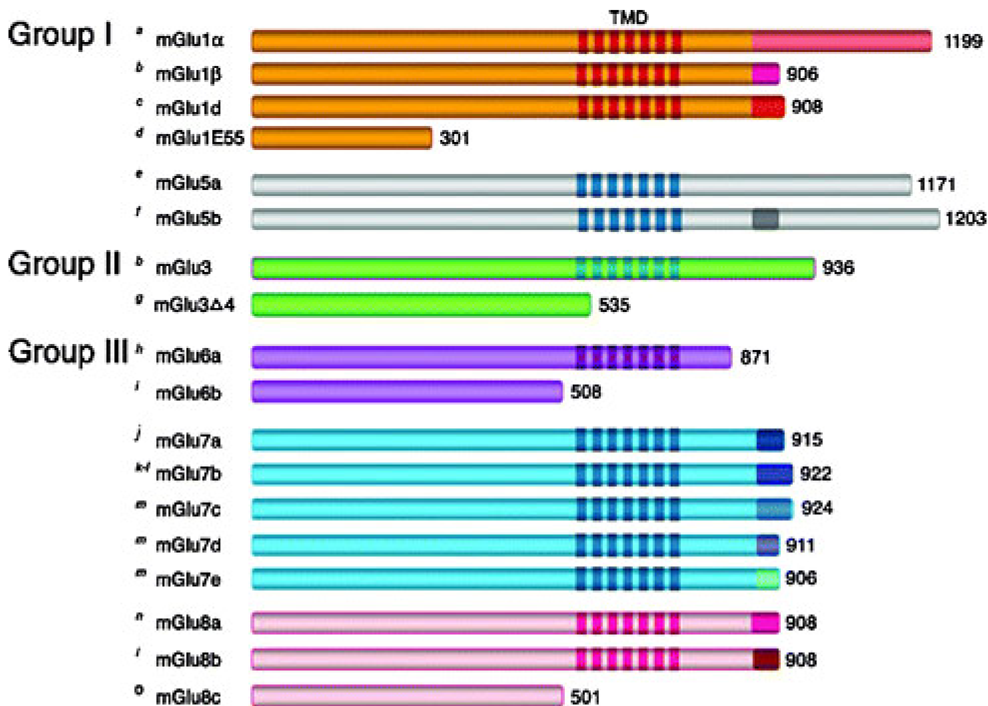

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6.2. Indicadores de produção 2006-2008 (Resumos em congressos)

RODRIGUES, P.M.B.; LIMA, C. A.; MOREIRA, K..A.; MOTTA, C.M.S. ; LIMA FILHO, J.L.; PORTO, A.L.F.; CARNEIRO-DA-CUNHA, M.G. Production and partial characterization of protease from *Peniccillium aurantiogriseum*. VII Reunião Regional Nordeste da Sociedade Brasileira de Bioquímica e Biologia Molecular-SBBq, realizada em Natal, Rio Grande do Norte, no período de 6 a 8 de Dezembro de 2006.

LIMA, C. A; **RODRIGUES, P.M.B**; BEZERRA, R. S.; CARNEIRO-DA-CUNHA, M.G. Pré-purification of an alkaline protease from intestine of Nile Tilapia (*Oreochromis niloticus)* using reversed micelles. VIII Reunião Regional Nordeste da Sociedade de Brasileira de Bioquímica e Biologia Molecular-SBBq, realizada em Natal, Rio Grande do Norte, no período de 6 a 8 de Dezembro de 2006.

RODRIGUES, P.M.B.; MOTTA, C.M.S.; PORTO, A.L.F.; PORTO, T. S; TEIXEIRA, J.A.; CARNEIRO-DA-CUNHA, M.G. Study of the production of protease in bioreactor by *Penicillium aurantiogriseum.* V Congresso Brasileiro de Micologia, realizado em Recife, Pernambuco, no período de 2 a 6 de Novembro de 2007.