Instituto Superior de Agronomia
Universidade Técnica de Lisboa

NOVEL WILD BACTERIAL ENZYMES FOR APPLICATIONS IN THE WOOL INDUSTRY

TESE APRESENTADA PARA OBTENÇÃO DO GRAU DE DOUTOR EM BIOLOGIA

ORIENTADOR:
Doutor Francisco Xavier Delgado Domingos Antunes Malcata
CO-ORIENTADOR:
Doutora Maria da Glória Calado Inglês Esquível

JÚRI

Presidente:
Reitor da Universidade Técnica de Lisboa

Vogais:
Doutor Francisco Xavier Delgado Domingos Antunes Malcata, professor catedrático do Instituto Superior da Maia, Porto

Doutor Luís Manuel dos Anjos Ferreira, professor catedrático da Faculdade de Medicina Veterinária da Universidade Técnica de Lisboa

Doutor Artur Cavaco-Paulo, professor associado da Escola de Engenharia da Universidade do Minho

Doutora Maria da Glória Calado Inglês Esquível, professora auxiliar do Instituto Superior de Agronomia da Universidade Técnica de Lisboa

Doutora Maria Luísa Lopes de Castro e Brito, professora auxiliar do Instituto Superior de Agronomia da Universidade Técnica de Lisboa

Doutora Lígia Maria de Oliveira Martins, professora auxiliar convidada do Instituto de Tecnologia

Ana Catarina Gonçalves Carvalho **Queiroga** Santos

LISBOA
2011

Universidade Técnica de Lisboa

Instituto Superior de Agronomia

NOVEL WILD BACTERIAL ENZYMES FOR APPLICATIONS IN THE WOOL INDUSTRY

TESE APRESENTADA PARA OBTENÇÃO DO GRAU DE DOUTOR EM BIOLOGIA

ORIENTADOR: Doutor Francisco Xavier Delgado Domingos Antunes Malcata

CO-ORIENTADOR: Doutora Maria da Glória Calado Inglês Esquível

JÚRI

Presidente:

Reitor da Universidade Técnica de Lisboa

Vogais:

Doutor Francisco Xavier Delgado Domingos Antunes Malcata, professor catedrático do Instituto Superior da Maia, Porto;

Doutor Luís Manuel dos Anjos Ferreira, professor catedrático da Faculdade de Medicina Veterinária da Universidade Técnica de Lisboa;

Doutor Artur Cavaco-Paulo, professor associado da Escola de Engenharia da Universidade do Minho;

Doutora Maria da Glória Calado Inglês Esquível, professora auxiliar do Instituto Superior de Agronomia da Universidade Técnica de Lisboa;

Doutora Maria Luísa Lopes de Castro e Brito, professora auxiliar do Instituto Superior de Agronomia da Universidade Técnica de Lisboa;

Doutora Lígia Maria de Oliveira Martins, professora auxiliar convidada do Instituto de Tecnologia Química e Biológica da Universidade Nova de Lisboa.

Ana Catarina Gonçalves Carvalho **Queiroga** Santos

LISBOA

2011

To Ginja, Rui and ZéZé, who taught me how to live curious.

Acknowledgements

Developing a PhD is a true marathon event – and I would not have been able to successfully complete this quest and conquer this academic achievement without the effort, support and collaboration of a countless number of people – who followed my work, and motivated and helped me in so many different ways over the latest years. It is, therefore, a great pleasure (and even honor) to hereby acknowledge them.

I must first express my gratitude to Escola Superior de Biotecnologia (ESB), for receiving me as a graduate student and providing me with good lab premises to perform a major part of my research, as well as promoting my participation in scientific meetings. I also acknowledge Instituto Superior de Agronomia, for taking me as a PhD student at a later stage of my program, and providing me with the necessary conditions to finalize my thesis and defend it.

My sincere gratitude goes obviously to Prof. F. Xavier Malcata, my supervisor from the very beginning, for his consistent and strong enthusiasm, for his everlasting motivation and for his extensive and strict scientific guidance. His recognized leadership, unique support, attention to detail, hard work and vast culture have all set an example for me and all those that have worked under his guidance. I keep reminding what he once told me: "Catarina, why do you think not everyone gets a PhD? – If getting advanced training were as simple as "following the basics", everyone would surely achieve it" surprisingly, this has helped me put things in perspective over the last years – after all, *perspective* is the essence, and only someone with his intellectual status would give me such a seminal advice. I acknowledge him for understanding and helping me strive in my multidisciplinary approach, and for trusting me – so that, despite a number of troubling features along the way, I would be able to focus on the essentials, and hence finish up my project successfully. I would also like to thank him for proposing me such great challenges as helping in the organization of National Scientific Meetings, and in the edition of a national peer-reviewed journal – those experiences really made a difference in my early career, in stimulating my learning and structuring my growth. I also acknowledge Dr. Glória Esquível, my co-supervisor, for having accepted me and reviewed my thesis. To Prof. F. Xavier Malcata and Dr. Glória Esquível, I am indeed grateful for the promptness in providing suggestions to improve my thesis, despite their many professional commitments.

I would like to express my gratitude to Dr. Manuela Pintado, for her continuous operational support during my research path at ESB, and to Dr. Ana Gomes, for her prompt

availability and guidance to solve unexpected problems, irrespective of the topic. For their kindness and friendship, and their special care in setting a good working environment during the years I spent there (I still remember all the moments of joy, especially in Badajoz, and the very many "birthday-cake party" in our offices) – for all that, I thank them.

A particularly special recognition to Dr. Paula Tamagnini, from Instituto de Biologia Molecular e Celular (IBMC), for the availability to receive me in her lab where I was able to perform an important part of my experimental work, as well as for all the specific and technical support made available in terms of molecular biology issues – it was indeed a pleasure to work in said lab. I also acknowledge her sustained friendship, continuous motivation and insightful advices. Likewise, I am grateful to Prof. Pedro Moradas Ferreira, from IBMC, for always treating me as if I were part of his research group, and for his wise advice that made a difference early after my graduation.

I acknowledge financial support from Fundação para a Ciência e Tecnologia (SFRH/BD/19212/2004), granted for development of this PhD project, and for complementary participation in international scientific meetings – where I presented my results, and was able to learn from international experts (who so often offered interesting feedback and valuable suggestions). In particular, I acknowledge Fundação Calouste Gulbenkian for granting me a fellowship to present an invited oral communication at an international scientific meeting held in South Korea.

Orlanda Martins, my favorite "doctor" at ESB, deserves a special mention: she inspired me because of her professionalism and commitment to work. I would like to thank her for being so supportive and positive – about work, and about life in general, and for all the effort she put forth to ensure I had all required consumables and reagents on time, so as not to delay my experiments.

I am also indebted to Escola Profissional de Economia Social (epESaJMS), where I have been working for the last 4 years, for the opportunity to grow both as a professional and as a person; this experience has been fantastic, and made a difference in my education by helping me face many of the PhD project difficulties in a more positive manner. Finishing writing of my thesis would ultimately have not been possible if its Directors, namely Fernando Martinho, Justino Santos and Lucília Alves, had not believed in my capacity to efficiently couple pedagogic management and finalization of a research project – I thank them wholeheartedly, for their everlasting support and friendship, and for providing me the chance to prove they were right.

I could not pass without showing my sincere appreciation for the effort of many school collaborators, to help relief my professional burden via their dynamism and competence – particularly Ana Sá, David Alves and Paula Marques. To the other collaborators and teachers at epESaJMS, I thank them for allowing me – via increasing their dedication and commitment to epESaJMS vision and mission, to reduce the need for permanent monitoring.

My recognition also goes:

To Ana Ferreira, Camino Fontan, Catarina Barbosa, Joana Teodósio and Sérgio Maia, with whom I had the pleasure to share the lab benches since the very beginning of my research program, and who helped me develop a critical judgment throughout such a "simple" task of thinking about the world as I see it and confronting it with the perception by others.

To Susana Miranda, for helping to run the lab smoothly, and for assisting me in so many different ways – her contribution and dedication surely allowed me to focus better on my research project, and certainly less on lab management tasks.

To my dear “senior” researcher fellows, namely Ana Paula Carvalho, Freni Tavaria and Susana Caldas, for providing a stimulating and fun environment that aided to learn and progress.

To Alison Machado, Simone Abrunhosa and Manuel Teibão, who have contributed to my work in the scope of their undergraduate courses.

To my many colleagues and friends, within the research groups which I had the pleasure to work with – Ana Amaro, Ana Catarina Guedes, Ana Ferreira, Ana Figueiredo, Ana Pintado, André Borges, Camino Fontan, Catarina Barbosa, Catarina Guedes, César Rodrigues, Cláudia Pereira, Ivone Moreira, Isabel Franco, Joana Carvalho, Joana Inácio, Joana Odila, Joana Teodósio, João Fernandes, Lígia Pimentel, Manuela Amorim, Maria Gião, Óscar Ramos, Patrícia Moreira, Patrícia Reis, Raquel Branquinho, Raquel Madureira, Ricardo Pereira, Sérgio Maia, Sérgio Sousa, Tânia Tavares, Teresa Braga, Vanessa Ralha, Vânia Figueira and Vicenza Ferraro from ESB; and Ângela, Catarina Pacheco, Daniela, Filipe, Patrícia Cardoso, Pedro, Sara and Zille from IBMC. Each of them helped make the time invested in this PhD project more fun and interesting. I also acknowledge the enjoyable lunches together at University cantina and “D. Júlia”.

To Manu, João, Sérgio, Tânia and Vanessa, for their companionship during many nights and weekends spent on the lab.

Throughout my life, I have been fortunate to come across with so many funny and good friends, without whom life would have been void and dull. I would like to thank them all for distracting me joyously, and for the constant reminder that there are more important things in life than only a PhD thesis.

To Ana Ferreira, Sérgio Maia and Vanessa Ralha, I thank all the “after-work” parties – it was sometimes exactly what I needed after a day spent fighting with data (or absence thereof).

To Joana Inácio, Isabel Franco and César Rodrigues, for their friendship and ever-present support, and with whom I could always have a great chat (regardless of subject).

To Tânia Tavares, for inspiring me with her strong personality and continuous search for happiness. I thank her for the long telephone calls about science (and others), especially during the thesis-writing period – when we were physically far away from each other.

To Catarina Pacheco, Daniela, Filipe, Marta, Paula Tamagnini, Pedro and Sara, for sharing so many happy moments, namely great summer gatherings at Prof. Pedro Moradas Ferreira’s home, during our great and challenging canyoning adventure and several dinner parties.

To Júlio Cabral, Miguel Polónia and Susana Pedro – my friends for life, for the huge amounts of laughing and unconditional sharing.

Finally, I would like to express my gratitude to my closest family for providing me with a loving environment to.

To Fernando and Lucília, I thank the love, support, entertainment and comradeship made available during so many weekday dinners spent together.

To my mom, for instilling me with a drive for the PhD pursuit and to my dad, for showing me that art is everywhere and is available to anyone who is open to learn from it – this really assisted me in so many different ways. Ginja and Zézé, you raised me, supported me, taught me and loved me – I thank you from the bottom of my heart, for your continuous and unconditional support. From the thesis-writing period, you can be sure I will retain for life the memories of our weekends – “invariably” spent together at home

with our laptops: Ginja hitting hard on her keyboard, Rui working hard on his PhD with his headphones always on and high, Zézé checking his colorful and funny e-mails, and Tarzan Babayaga (our "Prince of the White Russia") always sleeping in the couch.

To Rui, my life companion, my friend, my lover: I am grateful for the sharing of so many happy moments, and for helping me through so many difficult times – for his sustained emotional support, love and caring. Throughout the writing of this thesis, you provided me encouragement, sound advices, crisis support during a few "brain" meltdowns, good teaching, excellent company and lots of good ideas. I would have been lost without you.

Título da tese: Novas enzimas bacterianas para aplicação na indústria dos lanifícios

Resumo

A investigação descrita nesta dissertação foi desenvolvida com o objectivo de desenvolver processos enzimáticos para aplicação na indústria dos lanifícios, através da pesquisa de novas atividades enzimáticas produzidas por estirpes bacterianas isoladas de amostras de lã crua – recolhida de 3 zonas distintas do corpo de ovelhas Merino Portuguesa.

De entre os 156 isolados, 115 mostraram possuir atividade proteolítica, dos quais foram selecionados os dois apresentando atividades proteolíticas superiores – 24.6 and 15.9 U/mL, e identificados como *Bacillus* sp. HTS 102 e *Bacillus* sp. HTS 119. Os sobrenadantes livres de células desses dois isolados foram testados sobre malha de lã, no intuito de avaliar o seu potencial efeito benéfico sobre as características da fibra – tendo sido usadas força de rebentamento, perda de peso e área de encolhimento como parâmetros quantitativos, enquanto que a análise da microestrutura por microscopia electrónica de varrimento foi utilizada como parâmetro qualitativo.

Qualquer bioprocesso industrial requer uma cuidada optimização das condições de processamento para ser economicamente viável. Como os dois isolados anteriormente referidos causaram menores perda de peso e área de encolhimento do que as registadas usando uma enzima comercial (como referência), sob idênticas condições de processamento – e sem perdas significativas na força de rebentamento, o isolado apresentando maior

produtividade proteolítica (i.e. *Bacillus* sp. HTS 102) foi selecionado para os ensaios de otimização de síntese de proteases. Após uma pesquisa inicial dos parâmetros mais relevantes com efeito sobre a referida síntese, foi aplicado um planeamento factorial fraccionado do tipo 2_{VI}^{6-1} para determinar os efeitos da concentração de extracto de levedura, do nível de peptona, do tamanho do inóculo, da taxa de agitação, da temperatura e do pH. Este ensaio preliminar revelou que 4 factores (i.e. extracto de levedura, peptona, temperatura e pH) desempenham papéis importantes, e que existe uma interação relevante entre a concentração de extracto de levedura e o pH. A influência desses 4 factores sobre a produtividade de protease(s) foi então investigada através de um planeamento factorial completo (2^4), juntamente com metodologias de resposta de superfície. O modelo polinomial de segunda ordem sugerido mostrou-se adequado para prever a síntese de protease(s) (R^2=0.944, P<0.01), sendo que a atividade máxima atingida foi de 56.8 U/mL – o que traduz uma mais que duplicação da atividade registada em meio basal.

A protease extracelular sintetizada pelo *Bacillus* sp. HTS 102 foi caracterizada bioquimicamente, a par da avaliação da sua capacidade para ser usada na formulação de detergentes – bem como a de funcionar como ferramenta no tratamento anti-encolhimento de artigos de lã. Considerando os bons resultados obtidos em termos de estabilidade e atividade na gama de pH 7-9, bem como de estabilidade adequada em relação a agentes tensioativos e oxidantes, conclui-se que a protease produzida por aquela estirpe é uma boa candidata para os referidos detergentes – que são concebidos para boa eficiência a baixas temperaturas de lavagem. Peças de malha de lã tratadas com extracto enzimático de *Bacillus* sp. HTS 102 mostraram melhorias significativas nas suas

características físico-químicas – designadamente molhabilidade e grau de suavidade aumentados, a par de baixa área de encolhimento e perda de força aceitável.

Por fim, dos 115 isolados proteolíticos, 5 estirpes bacterianas capazes de degradar a lã foram selecionadas pelas suas notáveis atividades queratinolíticas. As estirpes selecionadas foram identificadas como pertencentes ao género *Bacillus*; cresceram em meio com penas e lã (inteiras e trituradas); e foram capazes de os hidrolisar, bem como em meio contendo cabelo e unhas – embora nestes últimos o grau de hidrólise tenha sido inferior. Estes resultados revelam a possibilidade da sua utilização no aproveitamento e valorização de desperdícios sólidos ricos em queratina, incluindo a preparação de rações animais.

Palavras-chave: novas estirpes, lã, enzima proteolítica, queratinase, caseínase, anti-feltragem, encolhimento, planeamento experimental factorial fraccionado, metodologia de resposta de superfície, biodegradação.

Abstract

The research reported in this thesis was aimed at developing enzyme-based processes for application in the wool industry, via screening novel enzyme activities from wild bacterial strains isolated directly from raw wool samples – collected at distinct parts of healthy Portuguese Merino sheep.

Among the 156 isolates obtained, 115 exhibited proteolytic activity, and 2 were eventually chosen owing to their particularly high proteolytic activities – 24.6 and 15.9 U/mL, and identified as *Bacillus* sp. HTS 102 and *Bacillus* sp. HTS 119. The cell-free supernatants of the isolates exhibiting the best performance were tested on knitted wool, to assess their potential beneficial role upon fiber features – viz. bursting strength, weight loss and area shrinkage were used as quantitative parameters, whereas microstructure via scanning electron microscopy was used as qualitative parameter.

Economically feasible industrial bioprocesses require in general careful preliminary optimization of processing conditions. Since the aforementioned isolates produced lower weight loss and area shrinkage than those brought about by a commercial enzyme (used as reference), under similar operating conditions – and without significantly compromising bursting strength, the isolate exhibiting the best proteolytic performance in basal medium (i.e. *Bacillus*

sp. HTS 102) was selected for exploratory optimization of protease synthesis. After preliminary screening for the most relevant factors affecting protease synthesis, a 2_{VI}^{6-1} fractional factorial design was applied to ascertain the effects of yeast extract concentration, peptone level, inoculum size, stirring rate, temperature and pH. This preliminary optimization attempt revealed that 4 factors (i.e. yeast extract, peptone, temperature and pH) played significant roles, as well as a 2-factor interaction between yeast extract level and pH. The influence of said factors upon protease productivity was further investigated via a 2^4 full factorial, central composite design, coupled with a response surface methodology. The empiric second-order model postulated was adequate to predict protease productivity (R^2=0.944, P<0.01); the maximum protease activity attained was 56.8 U/mL – i.e. more than twice that exhibited in the basal medium.

The (crude) extracellular protease synthesized by *Bacillus* sp. HTS 102 was biochemically characterized – with special care toward assessment in detergent formulation, and as a tool in attempts to increase the shrink-resistance of woolen fabrics. Considering its good activity and stability in the pH range 7-9, and its relative good stability to surfactants and oxidizing agents, the protease(s) produced by said strain is an interesting candidate for formulation of detergents intended for efficiency at low washing temperatures. Woven wool fabrics treated with crude enzyme from *Bacillus* sp. HTS 102 exhibited noticeable improvements in their fabric features – viz. increased wettability and softness degree, together with low area shrinkage and acceptable strength loss.

Finally, among the 115 proteolytic isolates, five wool-degrading bacterial strains were selected because of their noteworthy keratinolytic activity. All said

five strains, belonging once again to the genus *Bacillus*, were able to grow and hydrolyze feathers and wool (both native and milled), and hair and nails to a lesser extent. A potential for upgrading keratin-rich solid wastes was thus unfolded, including animal feed formulation.

Keywords: novel strains, wool, proteolytic enzyme, keratinase, caseinase, anti-felting, shrinkage, fractional factorial design, response surface methodology, biodegradation.

Scope and Outline

This PhD dissertation is organized in chapters – each divided in one or two sections, which parallel the progress of my research. The sections are related to each other, and the methodology chosen in each section was (unsurprisingly) dependent on the conclusions conveyed in previous one(s).

Most work described hereby encompasses screening and optimization of a novel bacterial proteolytic enzyme, suitable for industrial applications in the wool industry. Additionally, the need to assess the applicability of such an enzymatic system to wool processing led to tuning of several technical tests.

In **Chapter 1** – "General Introduction", an overview of current and novel approaches is conveyed, encompassing the search for novel proteolytic enzymes aimed at textile and agro-industrial applications.

Attempts to find novel bacterial sources, originating in raw Portuguese Merino wool, and able to produce proteolytic enzyme(s) suitable for application in (selected) textile processes, are described in **Chapter 2**. The methodology followed and the results obtained for isolation, selection and identification of bacterial isolate(s) with desired proteolytic activities are described in **Section 2.1** – whereas **Section 2.2** pertains to preliminary assessment of enzyme applicability to wool processing.

To be of industrial/commercial interest, an enzyme should to advantage be excreted to high levels or its rate of production optimized. After isolation, selection and identification of the best bacterial source of protease, synthesis of said enzyme was optimized using several approaches (**Chapter 3**): **Section 3.1** describes the preliminary optimization of several nutritional and processing factors affecting protease production based on one-at-a-time and fractional factorial design approaches, whereas use of a central composite design and response surface methodology to optimize protease synthesis by a novel strain of *Bacillus* sp. is described in **Section 3.2**.

Several additional tests for assessment of (crude) enzyme applicability for wool processing, as well as its biophysical characterization were conducted as detailed in **Chapter 4**: while **Section 4.1** relates to the potential applications of the protease in wool processing and laundry, **Section 4.2** focuses on the potential use of said protease(s) for biodegradation of keratinous materials.

Final remarks are presented in **Chapter 5**, which is divided in "General Conclusions" and "Future Perspectives" – as **Section 5.1** and **Section 5.2**, respectively.

Most information provided in the 9 sections of this thesis has been already submitted to international peer reviewing, via publication in international scientific journals – as detailed bellow:

Chapter 1

Queiroga, A.C., Pintado, M.E. & Malcata, F.X. (2012) "Search for novel proteolytic enzymes aimed at textile and agro-industrial applications: an overview of current and novel approaches", Biocatalysis and Biotransformation 30: 154-169

Soares, J.C., Moreira, P.R., **Queiroga, A.C.**, Malcata, F.X. & Pintado, M.E. (2011) "Application of immobilized enzyme technologies for the textile industry: a review", Biocatalysis and Biotransformation 29: 223-237

Chapter 2

Queiroga, A.C., Pintado, M.E. & Malcata, F.X. (2011) "Wool-associated proteolytic bacteria, isolated from the Portuguese Merino breed", Enzyme and Microbial Technology [***submitted***]

Queiroga, A.C., Pintado, M.M. & Malcata, F.X. (2007) "Novel microbial-mediated modifications in wool", Enzyme and Microbial Technology 40:1491-1495

Chapter 3

Queiroga, A.C., Pintado, M.E. & Malcata, F.X. (2011) "Processing for maximum extracellular protease productivity by *Bacillus subtilis* HTS 102 – a novel wild strain isolated from Portuguese Merino Wool", Applied Microbiology and Biotechnology [***submitted***]

Queiroga, A.C., Pintado, M.E. & Malcata, F.X. (2011) "Use of response surface methodology to optimize protease synthesis by a novel strain of *Bacillus* sp., isolated from Portuguese sheep wool", Biochemical Engineering Journal [***submitted***]

Chapter 4

Queiroga, A.C., Pintado, M.E. & Malcata, F.X. (2011) "Protease from a novel *Bacillus* strain: potential industrial application in wool processing and laundry", Journal of Industrial Microbiology and Biotechnology [***submitted***]

Queiroga, A.C., Pintado, M.E. & Malcata, F.X. (2012) "Potential use of wool-associated *Bacillus* species for biodegradation of keratinous substrates", International Biodeterioration and Biodegradation 70: 60-65

TABLE OF CONTENTS

Chapter 1

General Introduction

Section 1.1

Search for novel proteolytic enzymes aimed at textile and agro-industrial applications: an overview of current and novel approaches

1.1.1. Abstract

Types and sources of proteolytic enzymes, assays commonly used for proteases, strategies for protease yield-improvements, and novel proteases and applications in industrial sectors are covered in this review, in a balanced and critical manner – with an emphasis on alkaline proteases employed in the textile and detergent industries, and on degradation of keratin-rich wastes thereby. Current constraints pertaining to industrial applicability of these enzymes, and tentative solutions to be pursued are highlighted as well.

1.1.2. Introduction

Proteolytic enzymes are ubiquitous in terms of occurrence; they are found in all living organisms, and are essential for cell growth and differentiation. Although protease production is an inherent feature of the Earth biota, only those producing substantial amounts of extracellular protease(s) have been exploited commercially (Gupta et al. 2002b); and microorganisms – especially strains of *Bacillus* sp., dominate the industrial sector at present (Fujinami & Fujisawa 2010; Rao et al. 1998).

Various isolation methods have been described and discussed that enable screening and selection of promising organisms for industrial production; in addition, there are many possibilities for modifying biocatalysts through molecular approaches. For instance, strain improvement using mutagenesis and/or recombinant DNA technology has been applied to magnify the efficiency of producer strains to commercially feasible forms. Search for microbial sources of novel alkaline proteases within the natural biodiversity – using metagenomic approaches, has also unfolded a wide molecular diversity (Gupta et al. 2002b). These fascinating developments will eventually allow the biotechnological exploitation of uncultured microorganisms, which outnumber by far the species accessible by cultivation (regardless of their source habitat).

To develop efficient enzyme-based processes suitable for industry, prior knowledge of various fermentation parameters, purification strategies and properties of the biocatalyst itself are of the utmost importance. Furthermore, the method of assay for proteolytic performance – including selection of

substrate and analytical protocol, depends on the ultimate industrial application. A large array of assays are indeed available in the literature; however, with the advent of molecular approaches aimed at producing better biocatalysts, less conventional substrates and assay protocols have become increasingly important – chiefly those that can be conducted at micro/nano-scale (Gupta et al. 2002a).

Fermentation of proteases is usually mastered by varying the C/N ratio, and the presence and level of metabolizable sugars, e.g. glucose; successful scale-up has been attained using fed-batch, continuous and chemostat approaches, via extending the stationary phase of the culture (Joshi et al. 2008). Conventional purification strategies encompass concentration by bulk chromatographies, or aqueous two-phase systems (solid/liquid via precipitation, or liquid/liquid via solvent extraction).

Keratinases form a unique group of proteolytic enzymes; they display the ability to degrade (the insoluble protein) keratin, and have accordingly become important since they bring about hydrolysis of the highly rigid, strongly cross-linked structural polypeptides in keratin – which is a well-known recalcitrant to most proteases (Gupta & Ramnani 2006). Such enzymes are often produced in the presence of keratinous substrates, e.g. hair, feather, wool, nail and horn, either milled or intact; they form a class of particularly robust enzymes, with wide temperature and pH activity ranges – and most are serine- or metallo-proteases. Sequence homologies exhibited by keratinases have indeed indicated their relatedness to the subtilisin family (Adıgüzel et al. 2009).

The aforementioned enzymes stand out among proteases because they can attack keratin wastes – hence finding applications in cost-effective feather

by-product upgrading, toward feed and fertilizer formulations. Their application can also be extended to the detergent and leather industries, where they serve as specialty enzymes (Gupta & Ramnani 2006); and they also find uses in wool and silk cleaning, whereas their enhanced dehairing potential has led to development of greener hair-saving and dehairing technology in the leather industry, as well as of personal care products in the cosmetic industry.

1.1.3. Types and sources of proteolytic enzymes

As detailed by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB), all existing enzymes are divided into six distinct classes: oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. Proteolytic enzymes (or peptidases, a synonymous term recommended by NC-IUBMB) constitute a class of hydrolases – and are defined as enzymes that can hydrolyze peptide bonds, thus forming a distinct subclass, EC 3.4.

Recall that a further two sets of sub-subclasses of peptidases are recognized: those of exopeptidases (EC 3.4.11-19), which act only near either terminus of a polypeptide chain; and endopeptidases (EC 3.4.21-24 and EC 3.4.99), which act preferentially away from those termini; a comprehensive account of this classification is provided in Table 1.1.1. Hence, peptidases are not classified purely on the basis of the reactions they catalyze, but also based on their catalytic type. As a consequence, different proteins can be regarded as distinct peptidases, even when they express similar or identical peptidase activities (Beynon & Bond 2001).

Table 1.1.1 – The EC system of classification of peptidases. Adapted from Beynon & Bond (2001), updated with data available at www.chem.qmul.ac.uk/iubmb/enzyme, on March 2011)

Sub-subclass	Type of peptidase	Mode of action	Number of entries
3.4.11-19	**exopeptidases**	acting only near the ends of polypeptide chains	**80**
		at a free N-terminus	
3.4.11	aminopeptidases	liberating a single amino acid residue	21
3.4.14	dipeptidyl-peptidases	liberating a dipeptide or a tripeptide	9
		at a free C-terminus	
3.4.15	peptidyl-dipeptidases	liberating a dipeptide	4
3.4.16-18	carboxypeptidases	liberating a single amino acid residue	
3.4.16	serine-type carboxypeptidases	—	4
3.4.17	metallocarboxypeptidases	—	20
3.4.18	cysteine-type carboxypeptidases	—	1
		specific for dipeptides	
3.4.13	dipeptidases	—	12
		removing terminal residues that are substituted, cyclized or linked by isopeptide bonds	
3.4.19	omega peptidases	—	9
3.4.21-25 + 3.4.99	**endopeptidases**	cleavage of internal bonds in polypeptide chain	**280**
3.4.21	serine endopeptidases	—	99
3.4.22	cysteine endopeptidases	—	58
3.4.23	aspartic endopeptidases	—	40
3.4.24	metalloendopeptidases	—	81
3.4.25	threonine endopeptidases	—	2
3.4.99	endopeptidases of unknown type	—	0

Besides the type of reaction catalyzed and the chemical nature of the catalytic site, a third classification criterion has been proposed – MEROPS, in attempts to group peptidases according to a criterion that reflects essential structural features, and evolutionary relationships referred to those structural features (Rawlings et al. 2010). In the MEROPS system, each peptidase is assigned to a family on the basis of statistically significant similarities in its amino acid sequence; and families thought to be homologous are in turn grouped together in a clan (Rawlings et al. 2010). A more detailed description of the

intrinsic features of each peptidase sub-subclass was addressed elsewhere to some length (Rao et al. 1998; Beynon & Bond 2001).

Since peptidases are of a major physiological importance in all living organisms, they are found in nature at large (Rao et al. 1998). Owing to their metabolic, and consequently commercial importance, proteolytic enzymes have drawn the attention of researchers for quite some time – so there is a vast array of literature on the biochemical and biotechnological aspects of peptidases, including their animal, plant or microbial sources (Anwar & Saleemuddin 1998; Rao et al. 1998; Kumar & Takagi 1999; Niehaus et al. 1999; Demirjian et al. 2001; Gupta et al. 2002b; Antão & Malcata 2005; Gupta & Ramnani 2006; Dubey et al. 2007; Brandelli 2008; Klomklao 2008; Esposito et al. 2009; Brandelli et al. 2010; Fujinami & Fujisawa 2010).

Plant sources have conveyed many useful peptidases, viz. papain (Dubey et al. 2007), bromelain (Benucci et al. 2011) and ficin (Devaraj et al. 2008) – which have proven successful in medicine and food industry applications. Nevertheless, use of plants as sources of commercially relevant peptidases is typically constrained by availability of land for cultivation and suitability of weather conditions for growth (Rao et al. 1998). Moreover, production of proteases by plants is a time-consuming process (Rao et al. 1998), so it will hardly become economically feasible for applications requiring substantial amounts of said enzymes.

The most commonly studied peptidases of animal origin are gastrointestinal varieties, e.g. pepsin, trypsin, chymotrypsin and rennin (Klomklao 2008; Esposito et al. 2009; Mendes et al. 2009). These peptidases are prepared in pure form to large numbers, yet their production rate is critically

dependent on the availability of livestock for slaughter – which, in turn, is a direct function of political and agricultural policies, besides tightening ethical considerations (Rao et al. 1998).

Although proteolytic enzymes can be (and were originally) obtained from plants and animals, microorganisms are a much more favorable source if industrial applications are targeted – due to their much broader biochemical diversity, coupled with their susceptibility for genetic manipulation; additionally, they can be cultured to large amounts, require limited space for cultivation and regular supply can be assumed on a worldwide basis (Gupta et al. 2002b). The most convenient peptidase-producing microorganisms belong to three domains: Eukarya, Bacteria and Archae (Brandelli et al. 2010). These microorganisms have been isolated from the most distinct habitats on Earth, ranging from Antarctic soils (Marshall 1998) to hot springs (Pillai & Archana 2008), and from acidic (Yallop et al. 1997) to extremely alkaline environments (Singh et al. 1999; Genckal & Tari 2006) – besides both aerobic and anaerobic ecosystems; therefore, they are capable of producing proteolytic enzymes with biochemical features that allow similar environmental conditions to be used during processing, including ability to withstand harsh industrial setups. A number of reviews have detailed information on a comparative basis, and emphasized the microbial diversity among peptidase producers (Anwar & Saleemuddin 1998; Kumar & Takagi 1999; Niehaus et al. 1999; Gupta et al. 2002b; Haki & Rakshit 2003; Gupta & Ramnani 2006; Brandelli 2008; Brandelli et al. 2010); information on the most relevant genera is depicted in Table 1.1.2, especially pertaining to those proteolytic enzymes eventually more useful in textile and agro-industrial processing.

A significant fraction of commercially available bacterial peptidases are produced by the *Bacillus* genus (Rao et al. 1998; Gupta et al. 2002b); they are predominantly neutral and alkaline. This should come as no surprise, since those species are known for their wide metabolic versatility – including production of highly resistant dormant endospores, which has allowed them to strive in extreme environments (Alcaraz et al. 2010). In particular, several *Bacillus* spp. have recently been described to synthesize highly thermostable and alkaline peptidases (Haddar et al. 2009; Cheng et al. 2010; Fujinami & Fujisawa 2010; Rachadech et al. 2010; Shrinivas & Naik 2011) – which make them appropriate and advantageous for textile and agro-industrial applications, e.g. detergent additives, leather processing, silk degumming and wool finishing, besides general purpose formulations of food and feed. In fact, among alkalophiles, *Bacillus* species and their extracellular peptidases have been the focus of several studies because of their wide appearance, non-pathogenicity (except for *Bacillus cereus*) and remarkable production capacity in terms of peptidases – which are secreted across their single membrane system directly into the culture medium (Gupta et al. 2002b; Burg 2003; Fujinami & Fujisawa 2010). Despite the advantages derived from *Bacillus* secretory systems (Westers et al. 2004; Araújo et al. 2008; Vavrová et al. 2010), their use to express heterologous proteins has faced a number of shortcomings; their intrinsic quality control systems on the cellular level can efficiently remove misfolded or incompletely synthesized proteins, but they also represent bottlenecks for production of heterologous proteins to significant levels (Li et al. 2004; Zweers et al. 2008).

Table 1.1.2 – Microbial genera containing species able to synthesize proteolytic enzymes with potential industrial applications (adapted from Anwar & Saleemuddin 1998; Kumar & Takagi 1999; Niehaus et al. 1999; Breithaupt 2001; Demirjian et al. 2001; Gupta et al. 2002; Haki & Rakshit 2003; Gupta & Ramnani 2006; Brandelli 2008; Brandelli et al. 2010).

Eukarya	Bacteria		Archae
	Gram positive	Gram negative	
Thermonospora	*Bacillus*	*Vibrio*	*Pyrococcus*
Engyodontium (formerly *Tritirachium*)	*Lysobacter*	*Xanthomonas*	*Staphylothermus*
Conidiobolus	*Nesternokia*	*Stenotrophomonas*	*Thermococcus*
Aspergillus	*Kocuria*	*Chryseobacterium*	*Sulfolobus*
Doratomyces	*Microbacterium*	*Thermotoga*	*Desulfurococccus*
Myrothecium	*Kurthia*	*Pseudomonas*	*Halobacterium*
Paecilomyces	*Thermoanaerobacter*	*Fervidobacterium*	*Pyrobaculum*
Seopulariopsis	*Clostridium*	*Alcaligenes*	
Thrichoderma	*Kytococcus*	*Janthinobacterium*	
Thricophyton	*Nocardiopsis*	*Halomonas*	
Cephalosporium	*Streptomyces*	*Thermus*	
Chrysosporium	*Thermoactinomyces*		
Entomophthora	*Coprothermobacter*		
Fusarium	*Microbispora*		
Penicillium	*Terrabacter*		
Rhizopus	*Oerskovia*		
Scedosporium	*Arthrobacter*		
Dendryphiella			
Scolebasidium			
Candida			
Yarrowia			
Aureobasidium			
Malbranchea			
Torula			

Fungi synthesize a wider variety of proteolytic enzymes than do bacteria – including acid, neutral and alkaline peptidases (Rao et al. 1998). Even though fungal peptidases (mainly keratinases) with interesting biochemical properties have been described as produced by non-dermathophytic fungi (Brandelli et al. 2010), this group has attracted little commercial interest so far – probably because fungal peptidases exhibit low reaction rates and poor heat tolerance, when compared with their bacterial counterparts (Rao et al. 1998).

As emphasized before, keratinases (EC 3.4.21-24) constitute a special group among peptidases that have the ability to degrade insoluble keratin substrates (Gupta & Ramnani 2006; Brandelli 2008); several potential applications have been claimed encompassing keratinous substrates, which

allow them to find uses in such traditional industrial sectors as detergent, leather and feed, but also in newer fields, viz. prion degradation, biodegradable plastic manufacture and feather meal production (Onifade et al. 1998; Gupta & Ramnani 2006). Thermophilic and alkalophilic microorganisms (mainly Bacteria and Archae, see Table 1.1.2) are thus of great interest for industrial textile and agro-processing entailing keratin degradation – especially because the process is facilitated by high temperature and pH (Brandelli et al. 2010).

Despite the growing body of uses of proteolytic enzymes as biocatalysts in various fields, commercially available peptidases for industrial applications have reduced essentially to *Bacillus* members; consequently, a demand exists from the industrial side for novel proteases, which is yet not matched by the capacity of supply of most enzyme-producing companies. The vast majority of enzymes available to date have limited biotechnological applications owing to their poor stability at high temperatures and extreme pH, and when exposed to organic solvents – coupled with the need for co-factors and a narrow substrate range (Breithaupt 2001; Mansfeld & Ulbrich-Hofmann 2007). Current attempts to address these limitations, as part of application-oriented research programs, will be dealt with below.

On the other hand, industry has engaged in the production of new biocatalysts synthesized by novel organisms only if they are secreted to significantly superior extents than currently available ones – with the issue of alternative specificities and biochemical features coming only second. Furthermore, isolation of novel microbial enzymes is not an easy undertaking – it actually requires much more than bringing a field sample into the laboratory; microbiologists estimate that a mere 10% of all microorganisms in a given

environment are actually cultivable (Breithaupt 2001). Several approaches have been followed in efforts to find novel enzymatic systems – some of which will be discussed in the next section.

1.1.4. Search for novel systems – classical and improved approaches

New technologies of manipulation, coupled with an increased understanding of fundamental biology and bioinformatics, have been shaping the discovery, purification and application of enzymes (Beilen & Li 2002) – with obvious expansion in number and performance of enzyme applications in industry. The first step is the identification of a target reaction within an existing industrial process that can benefit from use of enzymes (Beilen & Li 2002); based on existing reaction constraints, an ideal biocatalyst is hypothesized – and then actively sought, characterized and even modified, as depicted in Figure 1.1.1, encompassing either biocatalyst screening or biocatalyst engineering (or a combination of both).

1.1.4.1 Biocatalyst screening

The genetic diversity of nature is still the major asset in terms of proteolytic enzymes to be used in textile and agro-industries (Burton et al. 2002); the great many habitats deserve comprehensive efforts of bioprospection – especially the microbial communities established in harsh environments in terms of temperature, salinity, pressure and pH (Niehaus et al. 1999; Breithaupt 2001; Demirjian et al. 2001; Burg 2003). Although thermophilic extremophiles have attracted most attention, psychrophiles have become also interesting

sources of industrial enzymes because of the ongoing efforts to decrease energy consumption worldwide (Burg 2003). Thermophilic peptidases have found application in detergent and controlled hydrolyses of food and feed, psychrophilic peptidases as detergent additives, halophilic peptidases in peptide synthesis, alkalophilic peptidases also in detergent formulation, and acidophilic peptidases in feed formulation (Niehaus et al. 1999; Breithaupt 2001; Burg 2003; Fujinami & Fujisawa 2010).

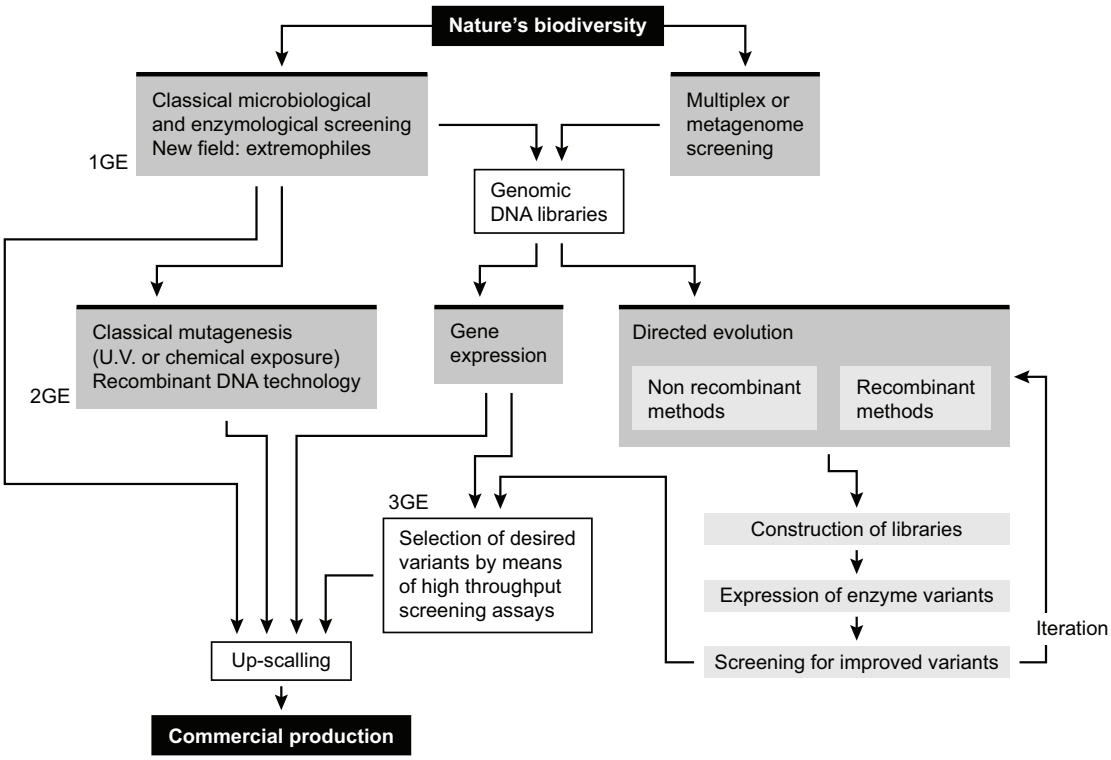


Figure 1.1.1 - Schematic representation of classical and novel "research-flows" leading to commercial protease production. 1GE, 2GE and 3 GE correspond to first, second and third generation enzymes, respectively (adapted from Kirk et al. 2002).

Despite the intensive research efforts to find and characterize new microbial sources of enzymes, one realizes that a rather high proportion of microbial species are currently uncultivable – which restricts access to microbial genomes and gene products (Burton et al. 2002). Modern bioprospecting

methods, e.g. multiplex or metagenome cloning, have been more and more often employed to directly access environmental genomes, whether culturable or not (Burton et al. 2002) – and constitute an alternative way to screen for biodiversity in nature. Therefore, high-throughput screening methods for biocatalysts are in order (Wahler & Reymond 2001) – which are needed to rapidly and inexpensively screen genomic DNA libraries, previously obtained either from biosphere sampling (through classical or genomic approaches) or from diversity generation methods (e.g. error-prone PCR, or gene shuffling of an existing enzyme gene or gene family). Recent progresses in high-throughput enzyme assays have included analytical instruments for parallel screening, thermistor arrays, and new fluorogenic and chromogenic substrates – as well as novel applications of pH indicator methods (Wahler & Reymond 2001). Resorting to high-throughput screening approaches depends directly on the cost of the technology involved, but will likely become less expensive as robotics and the like become more and more widely available.

Directed enzyme evolution techniques (see Figure 1.1.1) have emerged recently as a tool to create biological diversity, by generating enzymes with unconventional and improved features – tailored to production processes; such techniques are increasingly used, both in academic and industrial settings (Arnold & Volkov 1999).

1.1.4.2 Biocatalyst engineering

Although search for novel proteolytic enzymes is a successful strategy, several methodologies are available to improve existing ones (Breithaupt 2001). Enzymes found in nature are quite often not readily available in quantities

sufficient for industrial use, so use of gene expression methods to express recombinant proteins in suitable homo- or heterologous expression systems is required (Araújo et al. 2008). In addition, protein engineering – based on random or site-directed mutagenesis, may also aid in improving the turnover number of a particular enzyme (Gupta et al. 2002b). These account for relatively fast and inexpensive approaches to the incremental modification of selected enzyme features, with a reduced risk of losing desirable enzyme characteristics in the process (Burton et al. 2002). Furthermore, rapid developments in DNA sequencing will permit the genome of many more industrially relevant microorganisms to be completely resolved in the near future, thus providing ready access to useful genes – provided they are carefully annotated, in terms of relationships between sequence and function (Beilen & Li 2002).

Despite the recent development of sophisticated molecular engineering and screening technologies, the ability to move around the sequence space in search of the ideal biocatalyst for a given process is still limited (Burton et al. 2002); new technologies of biocatalyst screening and engineering will out-compete classical ones, yet the combined use of rational protein engineering, directed evolution and nature own biodiversity will be far superior to any standing-alone technology (Kirk et al. 2002).

1.1.5. Peptidase assays

The pre-requirements of any novel protease to be labeled as useful vary obviously with its envisaged application: textile and agro-industrial applications

impose specific restrictions upon the features of proteases – including robust, selective and highly active enzymes (Beynon & Bond 2001; Gupta et al. 2002a).

The ability to ascertain the products of protein hydrolysis, or of residual protein itself constitutes the basis of any attempt to assay for peptidases (Gupta et al. 2002a). Proteolytic activity may indeed be assessed using a wide range of available techniques, either qualitative or quantitative, which can be applied to a large variety of natural and synthetic proteinaceous substrates. Quantification of proteolytic activity then depends on the nature of the substrate, the type and the amount of activity being detected, and the sensitivity and precision needed (Beynon & Bond 2001). Both natural and synthetic substrates can be used for peptidase assays, but a few constraints may apply – depending on the nature of the enzyme at stake.

Natural substrates, viz. gelatin, casein and hemoglobin, are commonly used in endopeptidase assays, but not in routine assaying for exopeptidases (Beynon & Bond 2001). Since most peptidases of interest for textile and agro-industrial applications are of a serine- or metallo-endopeptidase type, assays on natural substrates are usually employed. The great many natural substrates available do not, however, promote an easy choice; actually, the greatest problem in assaying endopeptidases is the choice of substrate, because the enzyme is often not well characterized, or its existence is only inferred from general experimental data (Beynon & Bond 2001). Under these conditions, use of synthetic substrates is not recommended, so development of a suitable assay is more complex. If the nature of the enzyme is already known, then the substrate best suited for the assay is readily found based on said nature – e.g. casein for caseinase, keratin for keratinase or gelatin for gelatinase (Beynon & Bond 2001).

Several methods have been proposed in the literature to assay for peptidase activity; an overview of the most relevant qualitative, semi-quantitative and quantitative methods in routine use is conveyed in Table 1.1.3 and 1.1.4.

1.1.5.1 Qualitative and semi-quantitative methods

In academic research pertaining to peptidases, qualitative methods are commonly employed in initial screening of proteolytic activity. Most such assays resort to solid-phase matrices, on which either the enzyme or the substrate are immobilized; this is the case of electrophoresis and plate assays (Beynon & Bond 2001).

Screening procedures based on agar plate assays – in which enzymes diffuse into a gel matrix containing a hydrolysable substrate, have been in use for long; they are aimed mainly at detection of extracellular proteolytic activity in microorganisms (Frazier & Rupp 1928; Šafařík & Šafaříková 1994). However, such detection is sometimes difficult due to the low contrast between un-hydrolyzed and hydrolyzed areas on the agar plate. Therefore, it has been recommended to overlay a suitable precipitating agent to improve resolution – even though some damage may result upon the colonies (Šafařík & Šafaříková 1994; Saran et al. 2007b). Moreover, when the aim of a given study is not only to find protease producers but also to isolate them for *a posteriori* studies, use of a precipitating agent is by no means adequate.

Many protein substrates have been employed in agar plate assays, viz. skim milk, casein, calcium caseinate and feathers (Kanekar et al. 2002; Tatineni et al. 2008; Zhang et al. 2009) – but the underlying principle is the same, i.e.

creation of a clearance zone as a result of the enzyme-mediated hydrolysis of substrate (Gupta et al. 2002a).

Table 1.1.3 - Overview of qualitative and semi-quantitative assays for proteases applicable in textile and agro-industrial processes.

Type of assay: Qualitative or Semi quantitative

Method	Substrate	References
Protein agar plate Radial diffusion assays	casein	Tatineni et al. 2008
	feather	Mabrouk 2008; Zhang et al. 2009
	skim milk	Mehrotra et al. 1999; Kanekar et al. 2002; Nadeem et al. 2007; Saran et al. 2007; Devi et al. 2008; Tang et al. 2008; Abusham et al. 2009; Fang et al. 2009; Mala & Srividya 2010
Liquid medium	intact feathers	Tatineni et al. 2008; Xu et al. 2009; Jaouadi et al. 2010
Zymography	azocasein	Reddy et al. 2008
	casein	Prakash et al. 2005; Tang et al. 2008; Moradian et al. 2009
	feather meal	Riessen & Antranikian 2001
	gelatin	Prakash et al. 2005; Kim et al. 2007; Tatineni et al. 2008; Chen et al. 2011
	keratin	Kainoor & Naik 2010
	skim milk	Kim et al. 2007

As can be observed from inspection of Table 1.1.3 several authors have used skim milk agar to screen for protease producers; one disadvantage is that acid-forming bacteria can produce clearance zones on such medium that are not a result of proteolysis proper (Martley et al. 1970). Consequently, use of distinct protein agar formulations, e.g. calcium caseinate agar, can be suggested – because it allows good visualization of the digestion halos, while their opaque carbohydrate-free agar does not permit clearing due in particular to acid production. A similar solid-phase qualitative (and even semi-quantitative) method is the radial diffusion assay – whereby protease is detected via observation of the hydrolysis zone building around small wells cut in agar plates containing immobilized substrate (Gupta et al. 2002a).

Table 1.1.4 – Overview of spectrophotometric assay conditions for proteases applicable in textile and agro-industrial processes.

Type of assay: Quantitative (spectrophotometric)

	Assay conditions (ranges)			
Substrate	Temp (ºC)	Wavelength (nm)	Time	References
azocasein	25 - 55	340 - 450	6 min - 3 h	Manczinger et al. 2003; Moreira et al. 2003; Najafi et al. 2006; Olivera et al. 2006; Thys et al. 2006; Dienes et al. 2007; Meza et al. 2007; Chen & Wang 2008; Abusham et al. 2009; Infante et al. 2010; Chen et al. 2011
azokeratin	37 - 60	440 - 450	15 min - 30 min	Riffel et al. 2003; Xu et al. 2009; Zhang et al. 2009; Chen et al. 2011
casein	30 - 60	280 - 660	10 min - 1 h	Kamal et al. 1995; Garcia-Kirchner et al. 1998; Matta & Punj 1998; Mehrotra et al. 1999; Riessen & Antranikian 2001; Geok et al. 2003; Banik & Prakash 2004; Gupta et al. 2005; Li et al. 2005; Prakash et al. 2005; Rahman et al. 2005; Genckal & Tari 2006; Tari et al. 2006; Zhang et al. 2006; Anandan et al. 2007; Kim et al. 2007; Nadeem et al. 2007; Saran et al. 2007; Wang et al. 2007; Huang et al. 2008; Reddy et al. 2008; Tang et al. 2008; Vonothini et al. 2008; Wang et al. 2008a; Wang et al. 2008b; Jaouadi et al. 2009; Moradian et al. 2009; Syed et al. 2009; Zhou et al. 2009; Jaouadi et al. 2010; Mala & Srividya 2010; Manni et al. 2010; Romsomsa et al. 2010
keratin	37 - 75	280 - 660	10 min - 1 h	Garcia-Kirchner et al. 1998; Cai & Zheng 2009; Kainoor & Naik 2010
keratin azure	28 - 50	595	30 min - 24 h	Al-Sane et al. 2002; Cai et al. 2008; Mabrouk 2008; Tatineni et al. 2008; Jaouadi et al. 2009; Syed et al. 2009; Elíades et al. 2010
other substrates	37 - 75	245 - 595	15 min - 1 h	Al-Sane et al. 2002; Manczinger et al. 2003; Riffel et al. 2003; Anbu et al. 2005; Najafi et al. 2006; Anandan et al. 2007; Jaouadi et al. 2009; Jaouadi et al. 2010; Vishwanatha et al. 2010

Besides radial diffusion and protein agar plate assays, zymography is a semi-quantitative solid-phase technique that has been extensively used in attempts to detect proteolytic activity – owing to its simplicity and sensitivity (Quesada et al. 1996); zymograms further allow identification of a protease of interest within a complex mixture of enzymes. Zymography is essentially a gel electrophoresis method, where the substrate is incorporated in a gel matrix;

protease-containing zones will show up as areas where the proteinaceous substrate has been depleted from the gel. Several proteinaceous substrates have proven adequate in zymography, yet gelatin and casein are the ones used more frequently (see Table 1.1.3). Proteases that possess the ability to renature and exert proteolytic activity on a copolymerized substrate, upon removal of SDS, can be analyzed by this method (Leber & Balkwill 1997). While other qualitative and semi-quantitative methods are used almost exclusively for screening purposes, zymography has also been often employed when a certain degree of purification was previously attained – to rapidly assess presence and level of activity of proteases in the purified extract.

1.1.5.2 Quantitative methods

Methods aimed at quantifying the proteolytic activity actually measure the extent of proteolysis – and, in general, consist of liquid-phase assays: spectrophotometry, fluorimetry, radiometry, chromatography (HPLC), capillary electrophoresis and enzyme-linked immunosorbent assays (ELISA) are commonly used, with natural or synthetic substrates. Most attention has been paid to medical applications, where methods with very low detection limits, but very robust and fast, and allowing also high-throughput rates are a must. Several novel means of assay can be found in the literature, yet most research programs still persist in using older procedures for assay of proteases (Beynon & Bond 2001).

Spectrophotometric techniques are probably the most widely accepted methods of assay for proteolytic activity; they rely on differences in molar absorptivity between substrate(s) and product(s). The range of

spectrophotometric assay conditions reported in the literature for peptidases of interest to textile and agro-industrial-related endeavors is apparent in Table 1.1.4. Besides the wide diversity of substrates, note that the range of assay conditions for a given substrate also varies greatly – i.e. the wavelength can range from 280 to 660 nm, the temperature from 30 to 60 ºC, and the reaction time from 10 min to 1 h (when casein is used as substrate). In what specifically concerns assays for keratinase activity, keratin azure is widely accepted as substrate – and the most suited wavelength is 595 nm (Sane et al. 2002; al-Cai et al. 2008; Mabrouk 2008; Tatineni et al. 2008; Jaouadi et al. 2009; Syed et al. 2009; Elíades et al. 2010).

1.1.6. Production of proteases

One of the major constraints in the industrial application of enzymes (and other metabolites, for that matter) from microbial sources is the low productivity that is typical of fermentation processes. To enhance the rate of production of proteases, concerted efforts have focused on the physiology of the microorganisms, or on the design of the bioreactors and bioprocesses – aimed at improving growth and secondary metabolism (Burton et al. 2002); the key approaches encompass continuous fermentation bioprocesses, specific medium, innovative bioreactor implementation and overproduction in mesophilic hosts (Schiraldi & de Rosa 2002). It is beyond the scope of this review of the state of the art to explore in detail the latter two approaches, so an emphasis will be placed on improvement of bioprocesses and medium engineering.

1.1.6.1 Fermentation processes

Several commercially available enzymes are relatively non-expensive, but the most interesting are typically too costly for wide application (Beilen & Li 2002); since reduced costs of enzyme production may considerably expand the range and intensity of their applications as catalysts, efforts have been devoted to develop alternative technologies for peptidase production, especially solid-state fermentation (SSF) (Lazim et al. 2009; Rai et al. 2009; Vishwanatha et al. 2010).

SSF involves growth of microorganisms on moist solid substrate(s), in the absence of free-flowing water (Mukherjee et al. 2008); although peptidases are generally produced by submerged fermentation (SmF) (Gupta et al. 2002a), the use of SSF brings about a number of advantages over conventional SmF, viz. lower production costs, lower water and energy requirements, less extensive waste effluent generation, and increased product stability because of its lower dilution in the medium (Mukherjee et al. 2008; Lazim et al. 2009). This type of fermentation is still not broadly implemented on a commercial scale, yet advances in SSF technology are expected in the coming future – which will likely make peptidase production more accessible on the industrial scale.

1.1.6.2 Medium design

Peptidase production from bacterial sources is usually constitutive, or partially inducible in nature (Gupta et al. 2002b); and it is controlled by various complex mechanisms that operate during transition between the exponential growth and stationary phases – thus implying its strong dependence on medium

ingredients (Gupta et al. 2002a), especially nitrogen and carbon sources. Therefore, manipulation is needed to maximize growth, and associated protease yields via medium-based design strategies.

Optimization of the culture medium implies a large number of physiological and nutritional parameters, so medium composition is to be determined on a case-by-case basis (Brandelli et al. 2010). Besides such intrinsic bulk medium components as carbon and nitrogen, presence of easily metabolizable sugars and divalent metal ions is crucial – coupled with processing parameters, e.g. pH, temperature, degree of aeration, density of inoculum and rate of stirring (Kumar & Takagi 1999). Optimization of processing parameters (encompassing also medium components) by classical methods that involve change of "one-variable-at-a-time" is extremely time-consuming, so it is expensive when a large number of variables is to be tested (Rao et al. 2007). This approach also ignores interactions among parameters, yet it is still frequently used in bioprocess engineering to reach high yields of enzyme in fermentation systems (see Table 1.1.5). Said approach is useful chiefly at earlier stages of process optimization – when little is known about the factors that actually affect enzyme synthesis yield and rate. Consequently, preliminary studies ignoring mutual processing interactions are acceptable, but just to find whether or not one (or several) factor(s) under investigation affect protease production significantly – thus anticipating the most reasonable ranges for further optimization, which would already encompass such interactions (Myers & Montgomery 2002).

The latest decade has witnessed a great amount of R&D efforts pertaining to use of statistically validated methods to overcome the aforementioned

inability to pinpoint interactions among processing parameters (Table 1.1.5). Such improved screening methods include fractional factorial and Plackett-Burman designs; they are indeed often used in screening for key factors that affect the response when more than 3 factors are germane – and usually assume a suitable fractional form to prevent an excessively large number of experimental runs (Myers & Montgomery 2002).

Response surface methodologies (RSM) are more adequate in the final steps of medium optimization; in essence, they are a collection of statistical and mathematical techniques that are useful for developing, improving and optimizing processes – and have accordingly experienced extensive applications in industry (Myers & Montgomery 2002). RSM allow experimenters build polynomial models that can approximate the true response function within a vicinity of the optimum loci of factors for desirable responses (Puri et al. 2002). A number of selected examples of RSM applied in attempts to optimize fermentation media are listed also in Table 1.1.5.

1.1.7. Purification of proteases

Peptidases generally employed to commercial levels in the textile and agro-industries are crude extracts (Kumar & Takagi 1999); however, purification is needed before deepening knowledge on the operational features of any enzyme. There are no strict rules for purification of peptidases, but a general scheme for purification can be outlined: product recovery, isolation and purification, and eventual stabilization (Gupta et al. 2002a).

An ideal process of enzyme recovery involves a reduced number of downstream steps (Burton et al. 2002); the first steps typically encompass removal of cells, solids and colloids from the culture medium, and usually resort to filtration and centrifugation (Kumar & Takagi 1999). If the biocatalyst withstood high enough concentrations of a subsidiary product, a single isolation step without prior concentration would be possible (Burton et al. 2002). However, this is not what is normally found in practice – so a major constraint for many current processes is the large volume of water from which the product is to be removed, which creates the need for concentration steps, e.g. ultrafiltration, before isolation (Gupta et al. 2002a). To remove bulk protein and prepare the extract for subsequent chromatography, salting out brought about by solid ammonium sulfate, or solvent extraction using acetone and ethanol are often employed (Beynon & Bond 2001; Gupta et al. 2002a).

Following initial fractionation, the peptidase is either resuspended in, or dialyzed against the buffer to be used in the next step. To further purify the enzyme, a combination of column chromatography techniques is the usual routine (Gupta et al. 2002a). Commonly used chromatographic techniques include affinity chromatography, ion exchange chromatography, hydrophobic interaction chromatography, gel filtration and FPLC (Kumar & Takagi 1999; Gupta et al. 2002a). Ion exchange chromatography typically precedes size exclusion chromatography, because of the limitation of sample size in the latter (Beynon & Bond 2001). A few dedicated techniques for proteolytic enzymes, e.g. cysteine-type, trypsin-like and metallo-endopeptidases, have been described as well (Beynon & Bond 2001) – but it is crucial to carefully inspect all steps of the

purification protocol, in order to promptly pinpoint less efficient ones and thus conveniently design strategies for improvement.

Table 1.1.5 – Brief overview of optimization approaches for protease (including keratinase) activity improvement.

Approach	Methodology Design (variables)		Organism (Genera)	Yield gain	Software	Reference
Statistical	Placket-Burman RSM: CCD	(13) (4)	*Streptomyces*	4,96	Design Expert	Singh & Chhatpar 2010
Statistical	Placket-Burman Steepest ascent RSM: Box-Behnken	(10) (3) (3)	***Bacillus***	2,54	Design Expert	Liu et al. 2010
Statistical	Placket-Burman RSM: CCD	(7) (3)	***Bacillus***	2,6	SPSS	Romsomsa et al. 2010
Single-parameter		(20)	*Aspergillus*	n.a.		Kamath et al. 2010
1-at-a-time		(19)	*Pseudomonas*	n.a.		Kalaiarasi & Sunitha 2009
1-at-a-time Statistical	 RSM: Box-Behnken	(29) (4)	*Aspergillus*	n.a.	 Design Expert	Vishwanatha et al. 2010
1-at-a-time		(18)	*Penicillium*			Sindhu et al. 2009
1-at-a-time		(7)	***Bacillus***			Ul Qadar et al. 2009
Statistical	Placket-Burman RSM: CCD	(11) (4)	*Serratia*	n.a.	Design Expert	Venil & Lakshmanaperumalsamy 2009
1-at-a-time		(4)	***Bacillus***			Abusham et al. 2009
Statistical	Placket-Burman RSM: CCD	(4) (3)	*Laccocephalum*	8,14	SAS Matlab	Zhou et al. 2009
1-at-a-time		(29)	*Streptomyces*	n.a.		Lazim et al. 2009
Statistical	RSM: 2^2 factorial with 3 centre points	(2)	*Aspergillus* (commercial)	n.a.	Statistica	Peričin et al. 2009
1-at-a-time		(34)	Halophilic bacterium	n.a.		Joshi et al. 2008
Statistical	Placket-Burman RSM: CCD	(13) (4)	*Halobacterium*	3,9	Design Expert	Akolkar et al. 2009
Statistical	RSM: Box-Behnken	(5)	*Shewanella*	n.a.	Design Expert	Anbu et al. 2009
1-at-a-time		(25)	*Halobacillus*	n.a.		Karbalaei-Heidari et al. 2009
1-at-a-time		(6)	***Bacillus***	n.a.		Nadeem et al. 2008
1-at-a-time		(1)	***Bacillus***	1,9		Mukhtar & Ikram Ul 2007
1-at-a-time		(15)	*Streptomyces*	n.a.		Vonothini et al. 2008
Statistical	Genetic algoritm and particle swarm optimization		***Bacillus***	n.a.	Matlab	Skolpap et al. 2008
1-at-a-time		(3)	*Roseobacter*	n.a.		Shanmughapriya et al. 2008
1-at-a-time Statistical	 Placket-Burman RSM	(14) (11) (3)	*Aspergillus*	14,0	Design Expert SPSS Matlab	Hajji et al. 2008
Statistical	RSM: CRFD	(3)	Commercial	n.a.	Statistica	Bhaskar & Mahendrakar 2008
1-at-a-time		(25)	*Chryseobacterium*	n.a.		Wang et al. 2008b
Statistical	RSM: CCD	(5)	***Bacillus***	> 2,5	Matlab Feed-fwd network	Subba Rao et al. 2008
Statistical	RSM: CRFD	(4)	Commercial	n.a.	Statistica	Bhaskar et al. 2008
Statistical	RSM: FCCCD	(5)	***Bacillus***	1,5	Design Expert	Saran et al. 2007
1-at-a-time		(54)	*Aspergillus*	n.a.		Anandan et al. 2007
1-at-a-time		(22)	*Chromohalobacter*	n.a.		Vidyasagar et al. 2007
1-at-a-time		(10)	***Bacillus***	n.a.		Chu 2007
1-at-a-time Statistical	 Placket-Burman RSM	(19) (10) (3)	***Bacillus***	6,25	Design Expert	Tiwary & Gupta 2010

Table 1.1.5 – *continuation*

Approach	Methodology Design (variables)		Organism (Genera)	Yield gain	Software	Reference
1-at-a-time		(21)	***Bacillus***	n.a.		Kumar et al. 2010
Statistical	RSM: CCD RSM: CCD	(5) (3)	*Streptomyces*	3,5	Statistica	Tatineni et al. 2007
Statistical	RSM: CCD	(3)	***Bacillus***	3,0	Design Expert	Radha & Gunasekaran 2007
Statistical	RSM: Box-Behnken	(7)	*Scopulariopsis*	n.a.	Design Expert	Anbu et al. 2007
Statistical	RSM	(3)	***Bacillus***	n.a.	Design Expert Statistica	Ladeira et al. 2010
1-at-a-time Statistical	 RSM	(14) (3)	***Bacillus***	n.a.	Matlab Statistical softw.	Rai & Mukherjee 2010
1-at-a-time Statistical	 RSM: CCD	(15) (4)	***Bacillus***	n.a.	 Statistica	Huang et al. 2008
1-at-a-time		(5)	***Bacillus***	n.a.		Nascimento et al. 2007
Statistical	RSM: CCD	(3)	***Bacillus***	6,0	Statistical pack.	Oskouie et al. 2008
Statistical	Placket-Burman RSM: CCD	(8) (3)	***Bacillus***	2,3	Design Expert	Reddy et al. 2008
1-at-a-time Statistical	 RSM: 2^4 CCD	(24) (4)	*Colwellia*	n.a.	 Design Expert	Wang et al. 2008a
1-at-a-time Statistical	 FFD RSM: CCD	(14) (6) (2)	***Bacillus***	1,7	Design Expert	Cai & Zheng 2009

1.1.8. Industrial impact of enzyme technology

Enzyme technology is a truly interdisciplinary field – and more and more widely recognized as an important component towards white biotechnology. In agriculture, use of biocatalysts as feed additives has had positive effects upon environment, animal health and metabolic efficiency; in cleaning, enzymes are used as ingredients of detergents, thus increasing stain removal at competitive prices; and new enzymes from extremophiles also find applications in paper and textile industries, thus reducing the environmental burden associated therewith (Beilen & Li 2002).

1.1.8.1 Textile processing and agro-industrial applications

Textile processing and, more recently, also agro-industrial processing have benefited greatly from the use of proteolytic enzymes, in terms of both environmental impact and product quality (Kumar & Takagi 1999; Araújo et al. 2008). Peptidases remain the dominant hydrolytic type of enzymes, because of their extensive use in detergent and dairy industries (Kirk et al. 2002); they may contribute indeed to development of high value-added products.

In the latest decades, a vast set of reviews collected and extensively discussed information on the applications of proteolytic enzymes in those fields (Anwar & Saleemuddin 1998; Rao et al. 1998; Horikoshi 1999; Kumar & Takagi 1999; Gupta et al. 2002b; Kirk et al. 2002; Haki & Rakshit 2003; Maurer 2004; Saeki et al. 2007; Araújo et al. 2008; Kumar et al. 2008); more recently, some authors have devoted their attention specifically to applications of keratinases (Beilen & Li 2002; Gupta & Ramnani 2006; Brandelli 2008; Brandelli et al. 2010). A brief overview of current knowledge in this regard is presented below.

1.1.8.2 Protease-based wool finishing and hide-dehairing

Wool is a complex proteinaceous matrix; it exhibits several unique properties among all natural fibers – the surface scales of that fiber account indeed for the distinctive felting and shrinking properties of wool upon wetting. Since consumers have more and more eagerly looked for machine washability and sustained soft touch, the market value of wool has steadily decreased. In attempts to regain its (once leading) position within the textile and clothing industry, innovation in wool artifacts and processing is urged as a basis for

competitiveness – so alternatives both in quality of product and sophistication of process are in order. Applications of enzymes to wool may thus convey the possibility to bring about a greater added value; since wool fibers consist mainly of proteins and lipids, proteases and lipases will likely account for a major opportunity in processing that animal fiber.

The potential of proteolytic enzymes has recently been assessed with regard to removal of wool fiber scales – with consequent improvement of the anti-felting behavior of wool (Montazer & Ramin 2010; Raja & Thilagavathi 2010; Cai et al. 2011). However, an industrial process for plain enzymatic wool-finishing has not yet been put to work, because of technological difficulties in handling and control – coupled with relatively poor knowledge of these enzymes when acting on such a substrate fiber. Moreover, the proteolytic enzyme molecules are able to penetrate the fiber cortex owing to their small size – where they may compromise the inner parts of the wool structure (Araújo et al. 2008). Efforts have been made to increase the size of the enzyme (so as to reduce penetration) by chemical cross-linking or by attaching synthetic polymers thereto (Schroeder et al. 2004; Silva et al. 2004; Schroeder et al. 2006).

Alkaline proteases – and especially keratinases, without collagenolytic activity but presenting mild elastolytic activities, offer the possibility of an effective biotreatment of leather, particularly in terms of dehairing and bating of skins and hides; hence, they represent a suitable alternative to conventional tannery processes that resort to sulfide (Brandelli et al. 2010). In the traditional process, hair is gelatinized and converted into a pulp, whereas hair remains intact in the enzymatic process. Proteolytic enzymes able to meet these specifications should help breakdown the keratin tissue in the follicle, thereby

pulling intact hairs out without affecting the tensile strength of leather (Gupta & Ramnani 2006); this would result in production of higher-quality leather, and would also lead to improvement of wastewater quality, thus reducing pollution. Studies carried out elsewhere have met with success in using proteolytic enzymes for leather tanning (Riffel et al. 2003b; Macedo et al. 2005; Giongo et al. 2007; Jaouadi et al. 2009).

1.1.8.3 Protease-based laundry detergents

The use of proteases as detergent additives still represents the largest single use of industrial enzymes (Kirk et al. 2002). Over several years, subtilisins have proven suitable detergent proteases, because they efficiently carry out hydrolysis of insoluble protein-based stains in alkaline, thermophilic environments; therefore, several improvements have resulted from use of various forms of enzyme technology (Maurer 2004). Alkaline, thermophilic microorganisms are preferred sources of proteolytic enzymes for this purpose, since their thermophilic enzymes are claimed to hasten the hydrolysis process and diminish the risk of contamination – while withstanding harsh washing conditions (e.g. agitation, and presence of surfactants and oxidizing agents); however, mesophilic ones are less energy-consuming (Brandelli et al. 2010).

In the coming future, the detergent market is expected to redirect toward cooler washing steps, which will decrease the efficiency of traditional ingredients – so enzymes from psychrophilic microorganisms will be in particular demand for detergent formulation (Burg 2003). Extensive research has been conducted on the use of alkaline thermostable proteases, yet cold-active ones lag far behind in terms of characterization (Wang et al. 2008a; Yang et al. 2010); this calls for

further understanding prior to functionalization of such enzymes, when looking at effective commercial applications.

1.1.8.4 Protease-based processing of keratin-rich wastes

Microbial keratinases have attracted a great deal of attention in the past decade, partially owing to their ability to improve several industrial processes. However, as compared to other proteolytic enzymes, industrial applications of keratinases are scarce: these enzymes were already found useful in processing keratin residues, e.g. production of feed hydrolysates, feed supplements and nitrogen fertilizers (Brandelli et al. 2010).

Of particular interest are spent feathers: recently, they were converted to feather meal under high temperature and pressure, and then used as animal feed supplement (Gupta & Ramnani 2006). However, this is an expensive approach – and their poor digestibility and low nutritional value have urged use of keratinases to previously hydrolyze feather into a better nutritional ingredient (Onifade et al. 1998). The protein-rich hydrolysate generated from poultry can be useful in the preparation of nitrogen fertilizers or soil amendments (Brandelli 2008); and the potential conversion of keratinous wastes into biodegradable films and glues, for compostable packaging or edible film applications, has been reported as well (Gupta & Ramnani 2006).

1.1.9. Constraints on large-scale applicability of proteases

Despite extensive research efforts on several aspects of proteases, there are still numerous gaps in our understanding of these enzymes – so there is

room to improve their properties, in attempts to respond to demand by the textile and agro-industries (Rao et al. 1998). The stability of proteases remains a critical issue; both storage and operational stabilities affect the usefulness of enzymes as processing aids (O'Fagain 2003). Protein engineering, chemical modification and addition of stabilizing compounds are the main techniques employed at present for enzyme stabilization.

Immobilization may lead to enhanced stability gains, but it is generally undertaken to prevent loss of a biocatalyst or to improve bioreactor operation (O'Fagain 2003). Biocatalyst-specific constraints imposed on the process encompass the pH range over which optimal activity and stability of the biocatalyst is observed; the temperature range for optimal activity and stability; and the concentration range of reactants/products that can be tolerated without significant inhibition or saturation (Burton et al. 2002). Furthermore, prevention of autoproteolytic inactivation, change of substrate specificity and improvement of yield are important issues to be addressed (Rao et al. 1998).

Factors affecting extremozyme stability include also a number of ion pairs, besides reduction in the size of loops and in the number of cavities of the enzyme molecule itself, reduced ratio of molecular surface area to volume, changes in specific amino acid residues, increased hydrophobic interaction at subunit interfaces, changes in solvent-exposed surface areas, increase in the extent of secondary structure formation, and truncated amino acid and carboxyl termini; most of these potential stabilization mechanisms have been discussed elsewhere to some length (Demirjian et al. 2001).

1.1.10. Final considerations

Many research studies have unfolded (beyond doubt) the potential role of proteolytic enzymes in textile and agro-industrial applications. Nevertheless, commercial applications (and subsequent industrial market demand) of such enzymes are still in their infancy, chiefly because of scale-up and downstream processing constraints. Since proteases featuring unique physicochemical characteristics play already important roles in industry at large, research efforts toward development of more robust proteases (especially keratinases) are definitely in order.

Acknowledgments

A.C. Queiroga acknowledges a PhD fellowship (ref.: SFRH/BD/19121/2004), granted by Fundação para a Ciência e a Tecnologia (Portugal) and supervised by F.X. Malcata.

1.1.11. References

Abusham RA, Rahman R, Salleh AB, Basri M. 2009. Optimization of physical factors affecting the production of thermo-stable organic solvent-tolerant protease from a newly isolated halo tolerant *Bacillus subtilis* strain Rand. Microb Cell Fact 8:20-28.

Adıgüzel A, Bitlisli B, Yaşa İ, Eriksen N. 2009. Sequential secretion of collagenolytic, elastolytic, and keratinolytic proteases in peptide-limited cultures of two *Bacillus cereus* strains isolated from wool. J Appl Microbiol 107:226-234.

Akolkar A, Bharambe N, Trivedi S, Desai A. 2009. Statistical optimization of medium components for extracellular protease production by an extreme haloarchaeon, *Halobacterium* sp. SP1(1). Lett Appl Microbiol 48:77-83.

Alcaraz LD, Moreno-Hagelsieb G, Eguiarte LE, Souza V, Herrera-Estrella L, Olmedo G. 2010. Understanding the evolutionary relationships and major traits of *Bacillus* through comparative genomics. BMC Genomics 11:332-348.

Anandan D, Marmer W, Dudley R. 2007. Isolation, characterization and optimization of culture parameters for production of an alkaline protease isolated from *Aspergillus tamarii*. J Ind Microbiol Biotechnol 34:339-347.

Anbu P, Annadurai G, Lee J-F, Hur B-K. 2009. Optimization of alkaline protease production from *Shewanella oneidensis* MR-1 by response surface methodology. J Chem Technol Biotechnol 84:54-62.

Anbu P, Gopinath SCB, Hilda A, Lakshmipriya T, Annadurai G. 2007. Optimization of extracellular keratinase production by poultry farm isolate *Scopulariopsis brevicaulis*. Bioresource Technol 98:1298-1303.

Anbu P, Gopinath SCB, Hilda A, Priya TL, Annadurai G. 2005. Purification of keratinase from poultry farm isolate – *Scopulariopsis brevicaulis* and statistical optimization of enzyme activity. Enzyme Microb Technol 36:639-647.

Antão CM, Malcata FX. 2005. Plant serine proteases: biochemical, physiological and molecular features. Plant Physiol Biochem 43:637-650.

Anwar A, Saleemuddin M. 1998. Alkaline proteases: A review. Bioresource Technol 64:175-183.

Araújo R, Casal M, Cavaco-Paulo A. 2008. Application of enzymes for textile fibers processing. Biocatal Biotransfor 26:332-349.

Arnold FH, Volkov AA. 1999. Directed evolution of biocatalysts. Curr Opin Chem Biol 3:54-59.

Banik RM, Prakash M. 2004. Laundry detergent compatibility of the alkaline protease from *Bacillus cereus*. Microbiol Res 159:135-140.

Beilen JBv, Li Z. 2002. Enzyme technology: An overview. Curr Opin Biotechnol 13:338-344.

Benucci I, Liburdi K, Garzillo AMV, Esti M. 2011. Bromelain from pineapple stem in alcoholic-acidic buffers for wine application. Food Chem 124:1349-1353.

Beynon RJ, Bond JS. 2001. Proteolytic Enzymes: A Practical Approach. Oxford: Oxford University Press.

Bhaskar N, Benila T, Radha C, Lalitha RG. 2008. Optimization of enzymatic hydrolysis of visceral waste proteins of Catla (*Catla catla*) for preparing protein hydrolysate using a commercial protease. Bioresource Technol 99:335-343.

Bhaskar N, Mahendrakar NS. 2008. Protein hydrolysate from visceral waste proteins of Catla (*Catla catla*): Optimization of hydrolysis conditions for a commercial neutral protease. Bioresource Technol 99:4105-4111.

Brandelli A. 2008. Bacterial keratinases: useful enzymes for bioprocessing agroindustrial wastes and beyond. Food Bioprocess Technol 1:105-116.

Brandelli A, Daroit DJ, Riffel A. 2010. Biochemical features of microbial keratinases and their production and applications. Appl Microbiol Biotechnol 85:1735-1750.

Breithaupt H. 2001. The hunt for living gold. EMBO Reports 2:968-071.

Burg BVd. 2003. Extremophiles as a source for novel enzymes. Curr Opinion Microbiol 6:213-218.

Burton SG, Cowan DA, Woodley JM. 2002. The search for the ideal biocatalyst. Nature Biotechnol 20:37-45.

Cai CG, Chen JS, Qi JJ, Yin Y, Zheng XD. 2008. Purification and characterization of keratinase from a new *Bacillus subtilis* strain. J Zhejiang Univ-SCI B 9:713-720.

Cai CG, Zheng XD. 2009. Medium optimization for keratinase production in hair substrate by a new *Bacillus subtilis* KD-N2 using response surface methodology. J Ind Microbiol Biotechnol 36:875-883.

Cai S-B, Huang Z-H, Zhang X-Q, Cao Z-J, Zhou M-H, Hong F. 2011. Identification of a keratinase-producing bacterial strain and enzymatic study for its improvement on shrink resistance and tensile strength of wool- and polyester-blended fabric. Appl Biochem Biotech 163:112-126.

Chen BY, Wang HT. 2008. Utility of enzymes from *Fibrobacter succinogenes* and *Prevotella ruminicola* as detergent additives. J Ind Microbiol Biotechnol 35:923-930.

Chen K-N, Huang J-C, Chung C-I, Kuo W-Y, Chen M-J. 2011. Identification and characterization of H10 enzymes isolated from *Bacillus cereus* H10 with keratinolytic and proteolytic activities. World J Microbiol Biotechnol 27:349-358.

Cheng K, Lu FP, Li M, Liu LL, Liang XM. 2010. Purification and biochemical characterization of a serine alkaline protease TC4 from a new isolated *Bacillus alcalophilus* TCCC11004 in detergent formulations. Afr J Biotechnol 9:4942-4953.

Chu W-H. 2007. Optimization of extracellular alkaline protease production from species of *Bacillus*. J Ind Microbiol Biotechnol 34:241-245.

Demirjian DC, Morís-Varas F, Cassidy CS. 2001. Enzymes from extremophiles. Curr Opin Chem Biol 5:144-151.

Devaraj KB, Kumar PR, Prakash V. 2008. Purification, characterization, and solvent-induced thermal stabilization of ficin from *Ficus carica*. J Agric Food Chem 56:11417-11423.

Devi NKA, Balakrishnan K, Gopal R, Padmavathy S. 2008. *Bacillus clausii* MB9 from the east coast regions of India: isolation, biochemical characterization and antimicrobial potentials. Curr Sci 95:627-636.

Dienes D, Borjesson J, Hagglund P, Tjerneld F, Liden G, Reczey K, Stalbrand H. 2007. Identification of a trypsin-like serine protease from *Trichoderma reesei* QM9414. Enzyme Microb Technol 40:1087-1094.

Dubey VK, Pande M, Singh BK, Jagannadham MV. 2007. Papain-like proteases: applications of their inhibitors. Afr J Biotechnol 6:1077-1086.

Elíades L, Cabello M, Voget C, Galarza B, Saparrat M. 2010. Screening for alkaline keratinolytic activity in fungi isolated from soils of the biosphere reserve "Parque Costero del Sur" (Argentina). World J Microbiol Biotechnol 26:2105-2111.

Esposito TS, Amaral IPG, Buarque DS, Oliveira GB, Carvalho LB, Bezerra RS. 2009. Fish processing waste as a source of alkaline proteases for laundry detergent. Food Chem 112:125-130.

Fang Y, Liu S, Wang S, Lv M. 2009. Isolation and screening of a novel extracellular organic solvent-stable protease producer. Biochem Eng J 43:212-215.

Frazier WC, Rupp P. 1928. Studies on the proteolytic bacteria of milk I. A medium for the direct isolation of caseolytic milk bacteria. J Bacteriol 16:57-63.

Fujinami S, Fujisawa M. 2010. Industrial applications of alkaliphiles and their enzymes – past, present and future. Environ Technol 31:845 - 856.

Garcia-Kirchner O, Bautista-Ramirez M, Segura-Granados M. 1998. Submerged culture screening of two strains of *Streptomyces* sp. with high keratinolytic activity. Appl Biochem Biotech 70-72:277-284.

Genckal H, Tari C. 2006. Alkaline protease production from alkalophilic *Bacillus* sp. isolated from natural habitats. Enzyme Microb Technol 39:703-710.

Geok LP, Razak CNA, Rahman RNZ. 2003. Isolation and screening of an extracellular organic solvent-tolerant protease producer. Biochem Eng J 13:73-77.

Giongo J, Lucas F, Casarin F, Heeb P, Brandelli A. 2007. Keratinolytic proteases of *Bacillus* species isolated from the Amazon basin showing remarkable de-hairing activity. World J Microbiol Biotechnol 23:375-382.

Gupta A, Roy I, Patel RK, Singh SP, Khare SK, Gupta MN. 2005. One-step purification and characterization of an alkaline protease from haloalkaliphilic *Bacillus* sp. J Chromatogr A 1075:103-108.

Gupta R, Beg Q, Khan S, Chauhan B. 2002a. An overview on fermentation, downstream processing and properties of microbial alkaline proteases. Appl Microbiol Biotechnol 60:381-395.

Gupta R, Beg Q, Lorenz P. 2002b. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 59:15-32.

Gupta R, Ramnani P. 2006. Microbial keratinases and their prospective applications: an overview. Appl Microbiol Biotechnol 70:21-33.

Haddar A, Agrebi R, Bougatef A, Hmidet N, Sellami-Kamoun A, Nasri M. 2009. Two detergent stable alkaline serine-proteases from *Bacillus mojavensis* A21: purification, characterization and potential application as a laundry detergent additive. Bioresource Technol 100:3366-3373.

Hajji M, Rebai A, Gharsallah N, Nasri M. 2008. Optimization of alkaline protease production by *Aspergillus clavatus* ES1 in *Mirabilis jalapa* tuber powder using statistical experimental design. Appl Microbiol Biotechnol 79:915-923.

Haki GD, Rakshit SK. 2003. Developments in industrially important thermostable enzymes: a review. Bioresource Technol 89:17-34.

Horikoshi K. 1999. Alkaliphiles: some applications of their products for biotechnology. Microbiol Mol Biol Res 63:735-750.

Huang GR, Dai DH, Hu WL, Jiang JX. 2008. Optimization of medium composition for thermostable protease production by *Bacillus* sp HS08 with a statistical method. Afr J Biotechnol 7:1115-1122.

Infante I, Morel M, Ubalde M, Martínez-Rosales C, Belvisi S, Castro-Sowinski S. 2010. Wool-degrading *Bacillus* isolates: extracellular protease production for microbial processing of fabrics. World J Microbiol Biotechnol 26:1047-1052.

Jaouadi B, Abdelmalek B, Fodil D, Ferradji FZ, Rekik H, Zarai N, Bejar S. 2010. Purification and characterization of a thermostable keratinolytic serine alkaline proteinase from *Streptomyces* sp. strain AB1 with high stability in organic solvents. Bioresource Technol 101:8361-8369.

Jaouadi B, Ellouz-Chaabouni S, Ali M, Messaoud E, Naili B, Dhouib A, Bejar S. 2009. Excellent laundry detergent compatibility and high dehairing ability of the *Bacillus pumilus* CBS alkaline proteinase (SAPB). Biotechnol Bioproc Eng 14:503-512.

Joshi R, Dodia M, Singh S. 2008. Production and optimization of a commercially viable alkaline protease from a haloalkaliphilic bacterium. Biotechnol Bioproc Eng 13:552-559.

Kainoor PS, Naik GR. 2010. Production and characterization of feather degrading keratinase from *Bacillus* sp. JB 99. Indian J Biotechnol 9:384-390.

Kalaiarasi K, Sunitha PU. 2009. Optimization of alkaline protease production from *Pseudomonas fluorescens* isolated from meat waste contaminated soil. Afr J Biotechnol 8:7035-7041.

Kamal M, Hoog JO, Kaiser R, Shafqat J, Razzaki T, Zaidi ZH, Jornvall H. 1995. Isolation, characterization and structure of subtilisin from a thermostable *Bacillus subtilis* isolate. FEBS Letters 374:363-366.

Kamath P, Subrahmanyam VM, Rao JV, Raj PV. 2010. Optimization of cultural conditions for protease production by a fungal species. Indian J Pharm Sci 72:161-166.

Kanekar PP, Nilegaonkar SS, Sarnaik SS, Kelkar AS. 2002. Optimization of protease activity of alkaliphilic bacteria isolated from an alkaline lake in India. Bioresource Technol 85:87-93.

Karbalaei-Heidari H, Amoozegar M, Hajighasemi M, Ziaee A-A, Ventosa A. 2009. Production, optimization and purification of a novel extracellular protease from the moderately halophilic bacterium *Halobacillus karajensis*. J Ind Microbiol Biotechnol 36:21-27.

Kim CM, Kang SM, Jeon HJ, Shin SH. 2007. Production of *Vibrio vulnificus* metalloprotease VvpE begins during the early growth phase: usefulness of gelatin-zymography. J Microbiol Meth 70:96-102.

Kirk O, Borchet TV, Fuglsang CC. 2002. Industrial enzyme applications. Curr Opin Biotechnol 13:345-351.

Klomklao S. 2008. Digestive proteinases from marine organisms and their applications. Songklanakarin J Sci Technol 30:37-46.

Kumar CG, Takagi H. 1999. Microbial alkaline proteases: from a bioindustrial viewpoint. Biotechnol Adv 17:561-594.

Kumar D, Savitri N, Thakur R, Verma TCB. 2008. Microbial proteases and application as laundry detergent additive. Res J Microbiol 3:661-672.

Kumar R, Balaji S, Uma T, Mandal A, Sehgal P. 2010. Optimization of influential parameters for extracellular keratinase production by *Bacillus subtilis* (MTCC9102) in solid state fermentation using horn meal — a biowaste management. Appl Biochem Biotechnol 160:30-39.

Ladeira SA, Andrade MVV, Delatorre AB, Perez VH, Martins MLL. 2010. Utilização de resíduos agroindustriais para a produção de proteases pelo termofílico *Bacillus* sp. em fermentação submersa: optimização do meio de cultura usando a técnica de planejamento experimental. Química Nova 33:5.

Lazim H, Mankai H, Slama N, Barkallah I, Limam F. 2009. Production and optimization of thermophilic alkaline protease in solid-state fermentation by *Streptomyces* sp. CN902. J Ind Microbiol Biotechnol 36:531-537.

Leber TM, Balkwill FR. 1997. Zymography: a single-step staining method for quantitation of proteolytic activity on substrate gels. Anal Biochem 249:24-28.

Li C, Yu H, Liu S, Xing R, Guo Z, Li P. 2005. Factors affecting the protease activity of venom from jellyfish *Rhopilema esculentum* Kishinouye. Bioorg Med Chem Lett 15:5370-5374.

Li W, Zhou X, Lu P. 2004. Bottlenecks in the expression and secretion of heterologous proteins in *Bacillus subtilis*. Res Microbiol 155:605-610.

Liu S, Fang Y, Lv M, Wang S, Chen L. 2010. Optimization of the production of organic solvent-stable protease by *Bacillus sphaericus* DS11 with response surface methodology. Bioresource Technol 101:7924-7929.

Mabrouk MEM. 2008. Feather degradation by a new keratinolytic *Streptomyces* sp. MS-2. World J Microbiol Biotechnol 24:2331-2338.

Macedo AJ, da Silva WOB, Gava R, Driemeier D, Henriques JAP, Termignoni C. 2005. Novel keratinase from *Bacillus subtilis* S14 exhibiting remarkable dehairing capabilities. Appl Environ Microbiol 71:594-596.

Mala M, Srividya S. 2010. Partial purification and properties of a laundry detergent compatible alkaline protease from a newly isolated *Bacillus* species Y. Indian J Microbiol 50:309-317.

Manczinger L, Rozs M, Vágvölgyi C, Kevei F. 2003. Isolation and characterization of a new keratinolytic *Bacillus licheniformis* strain. World J Microbiol Biotechnol 19:35-39.

Manni L, Jellouli K, Ghorbel-Bellaaj O, Agrebi R, Haddar A, Sellami-Kamoun A, Nasri M. 2010. An oxidant- and solvent-stable protease produced by *Bacillus cereus* SV1: application in the deproteinization of shrimp wastes and as a laundry detergent additive. Appl Biochem Biotechnol 160:2308-2321.

Mansfeld J, Ulbrich-Hofmann R. 2007. The stability of engineered thermostable neutral proteases from *Bacillus stearothermophilus* in organic solvents and detergents. Biotechnol Bioeng 97:672-679.

Marshall WA. 1998. Aerial transport of keratinaceous substrate and distribution of the fungus *Geomyces pannorum* in Antarctic soils. Microb Ecol 36:212-219.

Martley FG, Jayashankar SR, Lawrence RC. 1970. An improved agar medium for the detection of proteolytic organisms in total bacterial counts. J Appl Microbiol 33:363-370.

Matta H, Punj V. 1998. Isolation and partial characterization of a thermostable extracellular protease of *Bacillus polymyxa* B-17. Int J Food Microbiol 42:139-145.

Maurer K-H. 2004. Detergent proteases. Curr Opinion Biotechnol 15:1-5.

Mehrotra S, Pandey PK, Gaur R, Darmwal NS. 1999. The production of alkaline protease by a *Bacillus* species isolate. Bioresource Technol 67:201-203.

Mendes CM, Brito MA, Porto TS, Porto ALF, Bezerra RS, Carvalho LB, Caneiro-Leao AMA, Carneiro-da-Cunha MG. 2009. Aquaculture by-product: a source of proteolytic enzymes for detergent additives. Chem Papers 63:662-669.

Meza JC, Auria R, Lomascolo A, Sigoillot JC, Casalot L. 2007. Role of ethanol on growth, laccase production and protease activity in *Pycnoporus cinnabarinus* ss3. Enzyme Microb Technol 41:162-168.

Montazer M, Ramin A. 2010. Influences of proteases and trans-glutaminases on wool. Fibres Text East Eur 18:98-102.

Moradian F, Khajeh K, Naderi-Manesh H, Sadeghizadeh M. 2009. Isolation, purification and characterization of a surfactants-, laundry detergents- and organic solvents-resistant alkaline protease from *Bacillus* sp. HR-08. Appl Biochem Biotechnol 159:33-45.

Moreira KA, Porto TS, Teixeira MFS, Porto ALF, Lima-Filho JL. 2003. New alkaline protease from *Nocardiopsis* sp.: partial purification and characterization. Process Biochem 39:67-72.

Mukherjee AK, Adhikari H, Rai SK. 2008. Production of alkaline protease by a thermophilic *Bacillus subtilis* under solid-state fermentation (SSF) condition using *Imperata cylindrica* grass and potato peel as low-cost medium: characterization and application of enzyme in detergent formulation. Biochem Eng J 39:353-361.

Mukhtar H, Ikram Ul H. 2007. Optimization of volume of fermentation medium for the production of alkaline protease by an EMS mutant strain of *Bacillus subtillis* IH-72. Pakist J Bot 39:2705-2715.

Myers RH, Montgomery DC. 2002. Response Surface Methodology: Process and Product Optimization Using Designed Experiments. New York, NY: Wiley Interscience.

Nadeem M, Qazi JI, Baig S, Syed QUA. 2007. Studies on commercially important alkaline protease from *Bacillus licheniformis* N-2 isolated from decaying organic soil. Turk J Biochem 32:171-177.

Nadeem M, Qazi JI, Syed QUA, Baig S. 2008. Optimization of process parameters for alkaline protease production by *Bacillus licheniformis* N-2 and kinetics studies in batch fermentation. Turk J Biol 32:243-251.

Najafi MF, Deobagkar DN, Mehrvarz M, Deobagkar DD. 2006. Enzymatic properties of a novel highly active and chelator resistant protease from a *Pseudomonas aeruginosa* PD100. Enzyme Microb Technol 39:1433-1440.

Nascimento WCA, Rocha da Silva RVC, Martins MLL. 2007. Otimização de um meio de cultura para a produção de proteases por um *Bacillus* sp. termofílico. Ciênc Tecnol Aliment 27:5.

Niehaus F, Bertoldo C, Kähler M, Antranikian G. 1999. Extremophiles as a source of novel enzymes for industrial application. Appl Microbiol Biotechnol 51:711-729.

O'Fagain C. 2003. Enzyme stabilization – recent experimental progress. Enzyme Microb Technol 33:137-149.

Olivera N, Sequeiros C, Sineriz F, Breccia JD. 2006. Characterization of alkaline proteases from a novel alkali-tolerant bacterium *Bacillus patagoniensis*. World J Microbiol Biotechnol 22:737-743.

Onifade AA, al-Sane NA, al-Musallam AA, al-Zarban S. 1998. A review: potentials for biotechnological applications of keratin-degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources. Bioresource Technol 66:1-11.

Oskouie SFG, Tabandeh F, Yakhchali B, Eftekhar F. 2008. Response surface optimization of medium composition for alkaline protease production by *Bacillus clausii*. Biochem Eng J 39:37-42.

Peričin D, Mađarev-Popović S, Radulović-Popović L. 2009. Optimization of conditions for acid protease partitioning and purification in aqueous two-phase systems using response surface methodology. Biotechnol Lett 31:43-47.

Pillai P, Archana G. 2008. Hide depilation and feather disintegration studies with keratinolytic serine protease from a novel *Bacillus subtilis* isolate. Appl Microbiol Biotechnol 78:643-650.

Prakash M, Banik R, Koch-Brandt C. 2005. Purification and characterization of *Bacillus cereus* protease suitable for detergent industry. Appl Biochem Biotechnol 127:143-155.

Puri S, Beg QK, Gupta R. 2002. Optimization of alkaline protease production from *Bacillus* sp. by response surface methodology. Curr Microbiol 44:286-290.

ul-Qadar SA, Shireen E, Iqbal S, Anwar A. 2009. Optimization of protease production from newly isolated strain of *Bacillus* sp. PCSIR EA-3. Indian J Biotechnol 8:286-290.

Quesada AR, Fajardo I, Rodríguez-Agudo D, Pachón JM, Medina MÂ. 1996. Zymography of extracellular matrix proteases. Biochem Educ 24:170-171.

Rachadech W, Navacharoen A, Ruangsit W, Pongtharangkul T, Vangnai A. 2010. An organic solvent-, detergent-, and thermostable alkaline protease from the mesophilic, organic solvent-tolerant *Bacillus licheniformis* 3C5. Microbiology 79:620-629.

Radha S, Gunasekaran P. 2007. Cloning and expression of keratinase gene in *Bacillus megaterium* and optimization of fermentation conditions for the production of keratinase by recombinant strain. J Appl Microbiol 103:1301-1310.

Rahman RNZRA, Geok LP, Basri M, Salleh AB. 2005. An organic solvent-tolerant protease from *Pseudomonas aeruginosa* strain K: nutritional factors affecting protease production. Enzyme Microb Technol 36:749-757.

Rai SK, Konwarh R, Mukherjee AK. 2009. Purification, characterization and biotechnological application of an alkaline β-keratinase produced by *Bacillus subtilis* RM-01 in solid-state fermentation using chicken-feather as substrate. Biochem Eng J 45:218-225.

Rai SK, Mukherjee AK. 2010. Statistical optimization of production, purification and industrial application of a laundry detergent and organic solvent-stable subtilisin-like serine protease (Alzwiprase) from *Bacillus subtilis* DM-04. Biochem Eng J 48:173-180.

Raja ASM, Thilagavathi G. 2010. Comparative study on the effect of acid and alkaline protease enzyme treatments on wool for improving handle and shrink resistance. J Text Inst 101:823-834.

Rao MB, Tanksale MSG, Deshpande VV. 1998. Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Res 62:597-635.

Rao YK, Tsay KJ, Wu WS, Tzeng YM. 2007. Medium optimization of carbon and nitrogen sources for the production of spores from *Bacillus amyloliquefaciens* B128 using response surface methodology. Process Biochem 42:535-541.

Rawlings ND, Barrett AJ, Bateman A. 2010. MEROPS: the peptidase database. Nucleic Acids Research 38:D227-D233.

Reddy LVA, Wee YJ, Ryu HW. 2008a. Purification and characterization of an organic solvent and detergent-tolerant novel protease produced by *Bacillus* sp. RKY3. J Chem Technol Biotechnol 83:1526-1533.

Reddy LVA, Wee YJ, Yun JS, Ryu HW. 2008b. Optimization of alkaline protease production by batch culture of *Bacillus* sp. RKY3 through Plackett-Burman and response surface methodological approaches. Bioresource Technol 99:2242-2249.

Riessen S, Antranikian G. 2001. Isolation of *Thermoanaerobacter keratinophilus* sp. nov., a novel thermophilic, anaerobic bacterium with keratinolytic activity. Extremophiles 5:399-408.

Riffel A, Lucas Fo, Heeb P, Brandelli A. 2003a. Characterization of a new keratinolytic bacterium that completely degrades native feather keratin. Arch Microbiol 179:258-265.

Riffel A, Ortolan S, Brandelli A. 2003b. De-hairing activity of extracellular proteases produced by keratinolytic bacteria. J Chem Technol Biotechnol 78:855-859.

Romsomsa N, Chim-Anagae P, Jangchud A. 2010. Optimization of silk degumming protease production from *Bacillus subtilis* C4 using Plackett-Burman design and response surface methodology. Sci Asia 36:118-124.

Saeki K, Ozaki K, Kobayashi T, Ito S. 2007. Detergent alkaline proteases: enzymatic properties, genes, and crystal structures. J Biosci Bioeng 103:501-508.

Šafařík I, Šafaříková M. 1994. A modified procedure for the detection of microbial producers of extracellular proteolytic enzymes. Biotechnol Tech 8:627-628.

al-Sane NA, al-Musallam AA, Onifade AA. 2002. The isolation of keratin degrading microorganisms from Kuwaiti soil: production and characterization of their keratinases. Kuwait J Sci Eng 29:125-138.

Saran S, Isar J, Saxena R. 2007a. Statistical optimization of conditions for protease production from *Bacillus* sp. and its scale-up in a bioreactor. Appl Biochem Biotechnol 141:229-239.

Saran S, Isar J, Saxena RK. 2007b. A modified method for the detection of microbial proteases on agar plates using tannic acid. J Biochem Biophys Meth 70:697-699.

Schiraldi C, de Rosa M. 2002. The production of biocatalysts and biomolecules from extremophiles. Trends Biotechnol 20:515-521.

Schroeder M, Lenting H, Kandelbauer A, Silva C, Cavaco-Paulo A, Guebitz G. 2006. Restricting detergent protease action to surface of protein fibers by chemical modification. Appl Microbiol Biotechnol 72:738-744.

Schroeder M, Schweitzer M, Lenting HBM, Guebitz GM. 2004. Chemical modification of proteases for wool cuticle scale removal. Biocatal Biotransf 22:299-305.

Shanmughapriya S, Krishnaveni J, Selvin J, Gandhimathi R, Arunkumar M, Thangavelu T, Kiran G, Natarajaseenivasan K. 2008. Optimization of extracellular thermotolerant alkaline protease produced by marine *Roseobacter* sp. (MMD040). Bioproc Biosyst Eng 31:427-433.

Shrinivas D, Naik GR. 2011. Characterization of alkaline thermostable keratinolytic protease from thermoalkalophilic *Bacillus halodurans* JB 99 exhibiting dehairing activity. Int Biodeter Biodegr 65:29-35.

Silva CJSM, Sousa F, Guebitz G, Cavaco-Paulo A. 2004. Chemical modifications on proteins using glutaraldehyde. Food Technol Biotechnol 42:51-56.

Sindhu R, Suprabha GN, Shashidhar S. 2009. Optimization of process parameters for the production of alkaline protease from *Penicillium godlewskii* SBSS 25 and its application in detergent industry. Afr J Microbiol Res 3:515-522.

Singh AK, Chhatpar HS. 2010. Optimization of protease production by *Streptomyces* sp. A6 using statistical approach for reclamation of shellfish waste. World J Microbiol Biotechnol 26:1631-1639.

Singh J, Vohra RM, Sahoo DK. 1999. Alkaline protease from a new obligate alkalophilic isolate of *Bacillus sphaericus*. Biotechnol Lett 21:921-924.

Skolpap W, Nuchprayoon S, Scharer JM, Grisdanurak N, Douglas PL, Moo-Young M. 2008. Fed-batch optimization of α-amylase and protease-producing *Bacillus subtilis* using genetic algorithm and particle swarm optimization. Chem Eng Sci 63:4090-4099.

Subba-Rao C, Sathish T, Mahalaxmi M, Suvarna Laxmi G, Sreenivas-Rao R, Prakasham RS. 2008. Modelling and optimization of fermentation factors for enhancement of alkaline protease production by isolated *Bacillus circulans* using feed-forward neural network and genetic algorithm. J Appl Microbiol 104:889-898.

Syed DG, Lee JC, Li WJ, Kim CJ, Agasar D. 2009. Production, characterization and application of keratinase from *Streptomyces gulbargensis*. Bioresource Technol 100:1868-1871.

Tang XY, Pan Y, Li S, He BF. 2008. Screening and isolation of an organic solvent-tolerant bacterium for high-yield production of organic solvent-stable protease. Bioresource Technol 99:7388-7392.

Tari C, Genckal H, Tokatli F. 2006. Optimization of a growth medium using a statistical approach for the production of an alkaline protease from a newly isolated *Bacillus* sp. L21. Process Biochem 41:659-665.

Tatineni R, Doddapaneni K, Potumarthi R, Mangamoori L. 2007. Optimization of keratinase production and enzyme activity using response surface methodology with *Streptomyces* sp7. Appl Biochem Biotechnol 141:187-201.

Tatineni R, Doddapaneni KK, Potumarthi RC, Vellanki RN, Kandathil MT, Kolli N, Mangamoori LN. 2008. Purification and characterization of an alkaline keratinase from *Streptomyces* sp. Bioresource Technol 99:1596-1602.

Thys RCS, Guzzon SO, Cladera-Olivera F, Brandelli A. 2006. Optimization of protease production by *Microbacterium* sp. in feather meal using response surface methodology. Process Biochem 41:67-73.

Tiwary E, Gupta R. 2010. Medium optimization for a novel 58 kDa dimeric keratinase from *Bacillus licheniformis* ER-15: biochemical characterization and application in feather degradation and dehairing of hides. Bioresource Technol 101:6103-6110.

Vavrová L, Muchová K, Barák I. 2010. Comparison of different *Bacillus subtilis* expression systems. Res Microbiol 161:791-797.

Venil CK, Lakshmanaperumalsamy P. 2009. Application of response surface methodology in medium optimization for protease production by the new strain of *Serratia marcescens* SB08. Pol J Microbiol 58:117-124.

Vidyasagar M, Prakash S, Jayalakshmi S, Sreeramulu K. 2007. Optimization of culture conditions for the production of halothermophilic

protease from halophilic bacterium *Chromohalobacter* sp. TVSP101. World J Microbiol Biotechnol 23:655-662.

Vishwanatha K, Rao A, Singh S. 2010. Acid protease production by solid-state fermentation using *Aspergillus oryzae* MTCC 5341: optimization of process parameters. J Ind Microbiol Biotechnol 37:129-138.

Vonothini G, Murugan M, Sivakumar K, Sudha S. 2008. Optimization of protease production by an *Actinomycete* strain, PS-18A isolated from an estuarine shrimp pond. Afr J Biotechnol 7:3225-3230.

Wahler D, Reymond J-L. 2001. High-throughput screening for biocatalysts. Curr Opinion Biotechnol 12:535-544.

Wang Q, Hou Y, Xu Z, Miao J, Li G. 2008a. Optimization of cold-active protease production by the psychrophilic bacterium *Colwellia* sp. NJ341 with response surface methodology. Bioresource Technol 99:1926-1931.

Wang S-L, Yang C-H, Liang T-W, Yen Y-H. 2008b. Optimization of conditions for protease production by *Chryseobacterium taeanense* TKU001. Bioresource Technol 99:3700-3707.

Wang SL, Chio YH, Yen YH, Wang CL. 2007. Two novel surfactant-stable alkaline proteases from *Vibrio fluvialis* TKU005 and their applications. Enzyme Microb Technol 40:1213-1220.

Westers L, Westers H, Quax WJ. 2004. *Bacillus subtilis* as cell factory for pharmaceutical proteins: a biotechnological approach to optimize the host organism. BBA Mol Cell Res 1694:299-310.

Xu B, Zhong QF, Tang XH, Yang YJ, Huang ZX. 2009. Isolation and characterization of a new keratinolytic bacterium that exhibits significant feather-degrading capability. Afr J Biotechnol 8:4590-4596.

Yallop CA, Edwards C, Williams ST. 1997. Isolation and growth physiology of novel thermoactinomycetes. J Appl Microbiol 83:685-692.

Yang CY, Wang F, Hao JH, Zhang K, Yuan N, Sun M. 2010. Identification of a proteolytic bacterium, HW08, and characterization of its extracellular cold-active alkaline metalloprotease Ps5. Biosci Biotech Biochnol 74:1220-1225.

Zhang B, Jiang DD, Zhou WW, Hao HK, Niu TG. 2009. Isolation and characterization of a new *Bacillus* sp. 50-3 with highly alkaline keratinase activity from *Calotes versicolor* faeces. World J Microbiol Biotechnol 25:583-590.

Zhang Q, Smith E, Shen J, Bishop D. 2006. An ethoxylated alkyl phosphate (anionic surfactant) for the promotion of activities of proteases and its potential use in the enzymatic processing of wool. Biotechnol Lett 28:717-723.

Zhou LH, Zhang YQ, Wang RJ, Shen XL, Li YQ, Guan WJ. 2009. Optimization of mycelial biomass and protease production by *Laccocephalum mylittae* in submerged fermentation. Afr J Biotechnol 8:1591-1601.

Zweers JC, Barak I, Becher D, Driessen AJM, Hecker M, Kontinen VP, Saller MJ, Vavrova L, van Dijl JM. 2008. Towards the development of *Bacillus subtilis* as a cell factory for membrane proteins and protein complexes. Microb Cell Fact 7:10-29.

Chapter 2

Screening for bacteria that produce suitable protease

Section 2.1

Isolation, selection and identification of a bacterial isolate from Portuguese Merino wool with high proteolytic activity

2.1.1. Abstract

Fleece samples were collected from Merino raw wool – a Portuguese ewe breed, on three distinct areas of the body, from animals exhibiting no symptoms or signs of abnormalities; they were then subjected to enumeration and isolation of 158 bacterial strains. Said isolates were screened for protease activity, using the spot technique, on Calcium Caseinate Agar containing 1 %(w/v) skim milk. The 36 isolates displaying the highest protease activity underwent a more refined assessment of enzymatic performance – by examining their cell-free supernatant extracts using casein as substrate. Two *Bacillus* isolates were eventually chosen owing to their highest proteolytic activities (24.6 and 15.9 U/mL), and identified using molecular biology tools.

2.1.2. Introduction

Proteases are ubiquitous enzymes in nature, and are essential for cell growth and differentiation (Gupta et al. 2002b). Extracellular proteases produced by microorganisms are of a particular commercial interest because they can easily be isolated from the growth medium without the need for expensive cell disruption and additional separation techniques. Examples of industrial scale applications of proteases encompass food and feed industries, detergent manufacture, peptide synthesis, leather processing, waste management, photography, medical applications, and textile uses – particularly silk degumming and wool finishing (Gupta & Ramnani 2006).

Owing to their biological and economic significance among hydrolytic enzymes, proteases from microbial sources have been extensively studied – and still remain a topic of current interest. Despite the various microbial sources of proteases available, only those that can release substantial amounts of extracellular enzymes are of industrial interest – so a market demand exists for extra nonconventional sources that may entail proteolytic activities high enough to lead to profitable commercial exploitation. Unusual micro-ecosystems have been the focus of several screening efforts, in attempts to characterize their specific adaptations to those local environments – which may, in addition, provide catalysts possessing an environment-friendly character suitable for modern industrial processes (Schumacher et al. 2001).

Microbial proteases can be assigned to various groups on the basis of the environmental conditions under which they best exert their activity – either acidic, neutral or alkaline; but also on the basis of the characteristics exhibited by

the catalytic group at the enzyme active site – either serine, aspartyl, thiol or metallo-proteases (Gupta et al. 2002b). Keratinases, in particular, encompass large serine- or metallo-proteases that are capable of degrading the highly rigid, strongly cross-linked structural polypeptide keratin – which is a major component of the epidermis and its appendages, viz. hair, feather, nails, horns, hoofs, scales and wool (Gupta & Ramnani 2006).

The skin (and related appendages) of animals usually hosts a wide and rich microflora – and ewes illustrate well this realization. Sheep fleece is indeed a complex dynamic environment, which provides microflora with nutrients from the skin and the secretory glands that open onto it – further to a number of exogenous and ill-defined nutrients from the outer environment (Halliday 2002). A few genera isolated previously (Jackson et al. 2002) are found only in association with mammals, while others are contributed by the environment (e.g. soil, pasture and dung) but are capable to survive and multiply in the fleece; although other microorganisms may also be found, they do not typically undergo significant multiplication. Most sheep harbor large numbers of bacteria in their fleece that do not damage the wool (Jackson et al. 2002), but may cause fleece and skin problems – and thus financial losses (London et al. 1984; Akhurst et al. 1997; el-Sukhon 2002; Norris et al. 2008). On the other hand, such microorganisms can synthesize alternative proteases that may be used to treat woolen textiles without harming the keratinous structure of the wool fiber itself, thus replacing classic harsh chemical treatments.

Nevertheless, few studies have to date focused on the microbial ecology of sheep fleece (Lyness et al. 1994; Jackson et al. 2002) – despite the possible detrimental interactions within this habitat (London & Griffith 1984; el-Sukhon

2002; Norris et al. 2008), or the positive interactions that may eventually result in improved fleece quality or else provide better tools for downstream wool treatment (Lyness et al. 1994).

Hence, the research effort described in this chapter was focused on the isolation and characterization of mesophilic and thermophilic bacteria exhibiting proteolytic activity, using a Portuguese native sheep breed as feedstock source.

2.1.3. Materials and Methods

2.1.3.1. Sampling of fleece

Raw wool samples were collected from three distinct body areas – head, flank and rear, of Portuguese *Merino* sheep grown in Vila Nova de Cerveira (Portugal). All tools used for cutting and storing fleece samples were autoclaved and thoroughly cleaned by alcohol dipping, so as to minimize cross-contamination. Samples were immediately refrigerated, and kept as such until arrival at our laboratory.

Small pieces of fleece were teased from the staples, and subsamples were obtained by adding 10 g of teased fleece to 95 ml of sterile deionized water. These samples were homogenized for 15 min at 260 rpm, using a Stomacher® Lab Blender (Seward, UK), and used as mother solution for further enumeration and isolation of microorganisms.

The pH of wool samples was measured after homogenizing 1 g of teased fleece in 9 ml of a 10 mM $CaCl_2$ solution, for 5 min at 260 rpm, using a Stomacher® Lab Blender.

2.1.3.2. Enumeration and isolation of bacteria

Serial decimal dilutions of the aforementioned mother solution were plated, in quadruplicate, onto Plate Count Agar – PCA (Merck, Germany) and Bacillus cereus Medium Agar – BCM (Lab M, UK); these were then incubated at 37 or 50 ºC. BCM was chosen for direct screening among members of the *Bacillus* genus – since several studies in the field have claimed them to be present and act as prolific protease producers (Giongo et al. 2007; Rai & Mukherjee 2009); PCA was used for broad enumeration and isolation of wool-associated bacteria. With regard to incubation temperature, an additional set of criteria was followed to explore two distinct strategies: 50 ºC was chosen to screen for thermophiles, owing to their strongly interesting molecular machinery – based on realization that sheep's fleece may easily reach 45 ºC by mid-Spring time (Eyal 1963); on the other hand, environment-friendly wool processing typically requires processes to take place at mild temperatures, so as to minimize energy consumption – hence, mesophilic microorganisms were targeted by incubation at 37 ºC. Plates were thus incubated for 3 d at both temperatures; the resulting colonies were then counted to estimate the viable and culturable bacterial population of wool – expressed as colony forming units per gram of wool (CFU/g) as depicted in Figure 2.1.1.

The subsamples, made of teased fleece diluted in deionized water, were also submitted to an additional treatment at 85 ºC for 10 min, and subsequently plated on PCA and incubated at 37 and 50 ºC for 3 d; these procedures were aimed at searching for spore-forming bacteria.

Colonies showing distinct morphological characteristics were pinpointed, for eventual isolation of strains.

2.1.3.3. Purification and preliminary characterization of isolates

Selected colonies were recovered in Nutrient Broth – NB (Lab M, UK), and isolated in Nutrient Agar – NA (Lab M, UK). After purification, via sequential steps of plating on NA and culturing on NB, strains were kept at -80 ºC in Tryptone Soy Broth (Amersham, UK), supplemented with 15 %(v/v) glycerol, until further use.

Purified cultures were characterized by Gram-staining, as well as presence of catalase and endospores, to establish groups of isolates suitable for further differential analysis and identification.

2.1.3.4. Preliminary assay for proteolytic activity

Screening procedures based on agar plate assays have been in use for long, aiming at detection of extracellular proteolytic activity in microorganisms (Frazier & Rupp 1928; Šafařík & Šafaříková 1994). This detection is sometimes difficult, due to the low contrast between un-hydrolyzed and hydrolyzed areas produced on the agar plate. Therefore, overlay of a suitable precipitating agent has been recommended to improve resolution, even though some damage may result upon the colonies (Šafařík & Šafaříková 1994; Saran et al. 2007). Since the goal of our study was not only to find protease producers, but also to isolate them for *a posteriori* studies, the possibility of using a precipitating agent was not pursued.

However, the need still remained to optimize a medium formulation to specifically detect proteolytic microorganisms without compromising the colonies; hence, several medium formulations based on data reported elsewhere (Frazier & Rupp 1928; Martley et al. 1970; Vermelho et al. 1996; Saran et al. 2007) were tested against different concentrations of a commercial protease (Protex Multiplus L, Genencor, USA), as follows: a) Skim Milk Agar – SMA, added with 0.5, 1.0 or 10 %(w/v) skim milk ; b) SMA, added with 0.2 g/l $CaCl_2$ – SMAC, and 0.5, 1.0 or 10 %(w/v) skim milk; and c) Calcium Caseinate Agar (Merck, Germany) – CCA, prepared according to Frazier and Rupp (Frazier & Rupp 1928), and added with 0, 0.5, 1.0 and 10 %(w/v) skim milk (SM). SMA was in turn composed of: NA supplemented with skim milk powder (Oxoid, UK).

Once the medium with the best performance for detection of proteolytic activity using a commercial protease was found (see Figure 2.1.2), it was tested against control microorganisms (viz. *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*), at 37 ºC, to validate the analytical method.

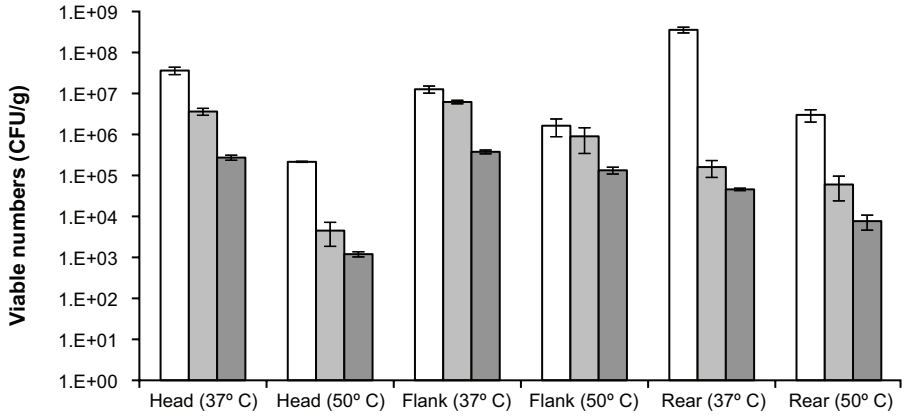


Figure 2.1.1 – Viable numbers (average ± standard deviation) of wool-associated bacterial population by body part, incubation temperature and growth medium: PCA – Plate Count Agar (white bars), BCM – Bacillus Cereus Medium (light grey bars) and PCA-HS – Plate Count Agar after heat-shock treatment (dark grey bars).

2.1.3.5. Screening for best protease producers

From every pure isolate, 10 µl-spots of an overnight culture, characterized by an OD_{600} of 0.6, were plated (with a total of 156 isolates), in quadruplicate, on the optimum growth medium described in the previous subsection, and used to screen for proteolytic activity at the corresponding growth temperatures. The fractions of non-proteolytic and proteolytic isolates from the wool-associated bacterial population with distinct colony morphology are depicted in Figure 2.1.3. The diameters of the digestion halos and of the colony sizes were recorded from 1 to 5 d. The isolates with the highest protease activity – i.e. those leading to the largest clearance ratios ([clearance extent]=[digestion diameter]-[colony diameter]), of at least 5 mm by 1 d and at least 15 mm by 5 d, were selected for a more refined measurement of enzyme activity.

Upon this isolation, the selected isolates were further cultured in NB for additional estimation of protease activity in liquid medium, using casein as substrate. The cell-free supernatants were used for colorimetric assessment of extracellular protease activity released during 1 d. One unit (U) of proteolytic activity was defined as the amount of enzyme able to hydrolyze casein so as to produce an absorbance equivalent to that of 1.0 µmol of tyrosine per min, at pH 7.5 and 37 ºC.

Aliquots were collected for total protein determination, which was achieved using the BCA™ Protein Assay Kit (Pierce, USA) – according to supplier's instructions.

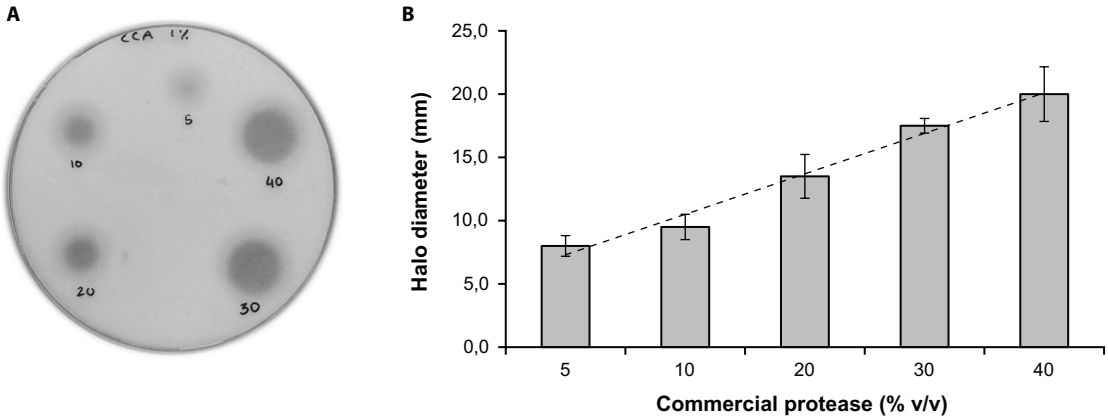


Figure 2.1.2 - Optimization of protease semi-quantitative plate assay: a) photograph of digestion halos obtained for various dilutions of commercial enzymes on a petri dish of CCA + 1% SM; and b) halo diameter produced by sequential dilution of commercial enzymes.

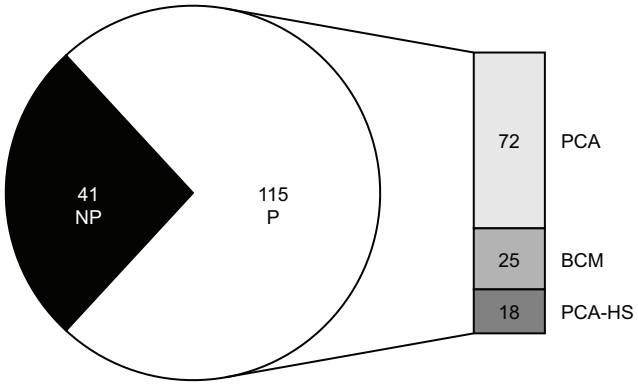


Figure 2.1.3 - Fraction of non-proteolytic (NP) and proteolytic (P) isolates (n=156) from the wool-associated bacterial population with distinct colony morphology, and distribution of proteolytic microorganisms of the latter using three culture media (PCA – Plate Count Agar, BCM – Bacillus cereus Medium and PCA-HS – Plate Count Agar after heat-shock treatment).

2.1.3.6. Molecular characterization of selected isolates

Cells from the two isolates showing the highest proteolytic activity, i.e. strains HTS102 and HTS119A, were harvested by centrifugation, and DNA was extracted using the Maxwell® 16 System, coupled with the Cell DNA Purification Kit (from Promega Corporation, Madison WI, USA) – according to manufacturer's instructions.

PCR amplification was carried out in a thermal cycler MyCycler™ (from Bio-Rad, Hercules CA, USA), using the bacterial primers 27f (5'-

AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3'). The profiles employed were: 5 min at 94 ºC, followed by 40 cycles of amplification of 1 min at 94 ºC, 1 min at 50 ºC and 1.5 min at 72 ºC, and complemented with a final extension of 7 min at 72 ºC.

Agarose gel electrophoresis was performed according to standard protocols using 1x TAE buffer (Sambrook & Russell 2001), and the DNA fragments were isolated from gels using the GFX™ PCR DNA and Gel Band Purification Kit (from GE Healthcare, Buckinghamshire, UK). Purified DNA fragments were finally sequenced at STAB Vida (Lisbon, Portugal).

Computer-assisted sequence analysis was performed using Vector NTI Advance™ 10 Software (from Invitrogen, Carlsbad CA, USA), and comparison of the sequences obtained was carried out using the BLAST software (from NCBI) (http://www.ncbi.nlm.nih.gov/).

The novel sequences associated with this study were deposited in GenBank, with the following accession numbers: HQ698269 and HQ698270.

2.1.3.7. Phylogenetic analysis of selected isolates

To assess the position of strains HTS102 and HTS119A within the scope of related research efforts aiming at biotechnological applications of proteases on keratinous substrates, a phylogenetic analysis was performed. To that goal, the 16S rRNA partial gene sequences, previously obtained for those two strains, were compared with the sequences for the same gene from other microorganisms described recently as protease producers – either (i) isolated from keratinous substrates or (ii) tested for industrial applications related to those substrates. To obtain the sequences, a series of queries were performed on

the ISI Web of Knowledge database (December, 2010), using the following keyword sets: “Bacillus + anti-felting” (0 results); “Bacillus + wool finishing” (0 results); “Bacillus + wool” (47 results, 0 relevant); “Bacillus + textile” (88 results, 2 relevant); “Bacillus + detergent” (203 results, 20 relevant); and “Bacillus + feather degrading” (59 results, 18 relevant). Bibliographic results were considered relevant when one, or both of the previous propositions were valid, and an accession number was provided. A total of 40 organisms were thus obtained, and their sequences retrieved from GenBank.

A multiple alignment of the 42 sequences (the 40 sequences mentioned above plus those from strains HTS102 and HTS119A) was performed using Clustal X2 software (Thompson et al. 1997) with default parameters, and visually inspected. The same software was used to compute the Neighbor-Joining tree (Saitou & Nei 1987) – for which the robustness of the nodes was assessed by doing 10000 bootstrap resamplings. The resulting phylogenetic tree was visualized using NJPLOT (Perrière & M. 1996).

2.1.4. Results

2.1.4.1. Enumeration and isolation of bacteria

The viable numbers of the bacterial population on wool varied from $2.1x10^5$ to $3.6x10^8$ CFU/g, depending on the bodily site of sampling (Figure 2.1.1). Although these numbers remained relatively unchanged over the 3 d of culture (as expected), the colonies exhibited large increases in size – and even an invasive growth, in a few cases. The most abundant population was obtained

from the Rear – with a total bacterial population of $3.6x10^8$ CFU/g at 37 ºC, and $3.0x10^6$ CFU/g at 50 ºC (Figure 2.1.1). Conversely, the highest bacterial diversity – i.e. the total number of colonies exhibiting distinct morphological characteristics, was found in the Head (Figure 2.1.1 and Figure 2.1.4): a total of 91 putatively different isolates was obtained here, followed by the Rear (51) and the Flank (14).

The viable numbers per gram of wool obtained via incubation at 50 ºC were relatively high – but they still ranked 1-3 log cycles lower than their 37 ºC counterparts. A total of 32 morphologically distinct colonies could be pinpointed from PCA plates, for samples submitted to heat-shock treatment (PCA-HS) – thus contrasting with the 93 isolated from PCA plates. Many of the colonies were pigmented with colors ranging from white and yellow to orange, on both PCA and PCA-HS. On BCM plates, the colonies were morphologically less distinct (as expected), due to medium selectivity – and the majority showed a pink or yellow pigmentation. Despite the lower biodiversity associated with this culture medium, the relatively large bacterial counts demonstrated the significant presence of species from the genus *Bacillus* associated with healthy wool samples.

Gram staining of isolates purified from morphologically distinct colonies indicated that wool-associated bacteria in the collected samples were either Gram-positive or -negative rods – together with very few Gram-positive cocci; Gram-positive microorganisms were predominant (62%) over Gram-negative ones (Figure 2.1.4). The Flank led to the fewest Gram-negative isolates with morphologically distinct colonies – just 2. On the other hand, the Head produced the highest numbers of Gram-negative rods, with a total of 50. Morphologically

distinct colonies, isolated on different media, were predominantly constituted by Gram-positive rods (69% and 58%, for PCA and BCM, respectively), except for the isolates from PCA-HS – which stained predominantly as Gram-negative (56%).

All samples possessed an alkaline pH: Head samples had a pH of ca. 9.5, Flank of ca. 8.7 and Rear of ca. 8.8, thus supporting the claim that alkalophiles and halotolerant microbial species are likely isolated from these micro-ecosystems.

2.1.4.2. Preliminary assay for proteolytic activity

All media tested showed a good performance in terms of detection of proteolytic activity – with no significant changes between the clearance extensions observed. One potential disadvantage of the SMA formulations tested is that acid-forming bacteria can produce clearance zones on such medium that are not caused by proteolysis proper (Martley et al. 1970). Consequently, CCA supplemented with 1 %(w/v) SM was used instead, because it permits a good visualization of the digestion halos (see Figure 2.1.2), whereas its opaque carbohydrate-free casein agar does not allow clearing specifically due to acid production. This medium formulation was thus further used to screen for protease producers among our wool-associated bacteria.

2.1.4.3. Screening for best protease producers

Among the 156 isolates obtained in this screening effort, 115 exhibited proteolytic activity – measured as clearance extent on CCA + 1 %(w/v) SM medium (Figure 2.1.3). The wool-associated proteolytic bacteria isolated here

could be tentatively organized into four groups – using the clearance extent as criterion: low proteolytic producers (**P**$^{-}$) – with clearance extent < 5 mm by 1 d, or < 15 mm by 5 d; moderately low proteolytic producers (**P**$^{+}$) – with clearance extent > 5 mm by 1 d, and < 15 mm by 5 d; moderately high proteolytic producers (**P**$^{++}$) – with clearance extent < 5 mm by 1 d, and > 15 mm by 5 d; and strongly proteolytic producers (**P**$^{+++}$) – with clearance extent > 5 mm by 1 d, and > 15 mm by 5 d (Figure 2.1.4). When considering the distribution of protease producers by body location, the results were highest for the Flank (100%), followed by the Rear (98%) and the Head (56%) (Figure 2.1.4).

The strains isolated at 50 ºC exhibited proteolytic activity – except only 2 strains out of 47, for which proteolytic activity was not detected. At 37 ºC, a total of 39 isolates had no proteolytic activity – all of which had been collected from the Head: 25 out of 50 Gram-negative rods isolated were indeed non-proteolytic. Nevertheless, it was from the same body area that the largest number of P^{+++} strains was recovered. With regard to the 115 isolates exhibiting proteolytic activity on CCA + 1 %(w/v) SM plates, 72 (i.e. 62%) were isolated from PCA plates, whereas 25 (i.e. 22%) and 18 (i.e. 16%) were isolated from BCM and PCA-HS, respectively (Figure 2.1.3). In addition, a significant fraction (i.e. 56%) of the highly proteolytic isolates were detected in BCM, when taking into account the within-sampling medium ratio between highly proteolytic and total proteolytic isolates; this compared with 36% and 17%, for PCA and PCA-HS, respectively. Therefore, most strongly proteolytic microorganisms were isolated from PCA, even though the highest ratio between highly proteolytic microorganisms and total proteolytic ones was obtained with BCM.

Based on the zone dimensions caused by hydrolysis, a total of 19 isolates were selected from the Head, 6 from the Flank and 8 from the Rear – and were further cultured in NB, for protease activity assessment. The results from this assay (summarized in Figure 2.1.5) revealed that HTS102 was the isolate bearing the highest proteolytic activity in liquid medium, when casein was used as substrate. The 2 isolates showing the highest proteolytic activity, viz. HTS102 and HTS119A, isolated from PCA and BCM, respectively, were further cultured for DNA extraction, PCR amplification and rRNA sequence analysis – as described in the following subsection.

2.1.4.4. Molecular characterization of best isolates

Among the extracellular protease producers, isolates HTS102 and HTS119A were selected for molecular identification – and could be assigned to the genus *Bacillus*, based on genetic similarity of their partial 16S rRNA gene sequence.

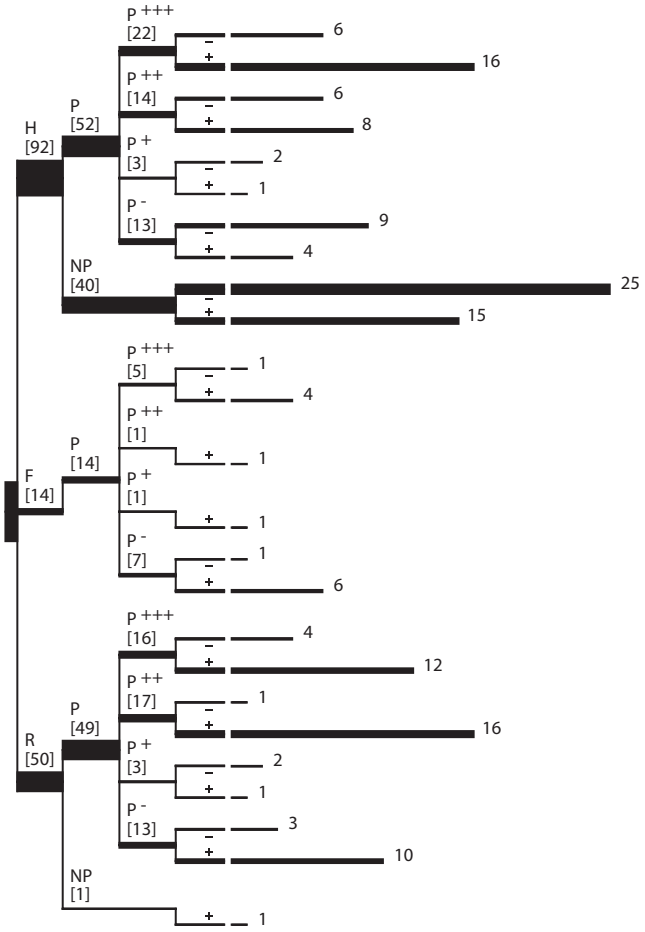


Figure 2.1.4 – Distribution of protease producers among the wool-associated bacterial population, ranked by proteolytic power (P – proteolytic; NP – non-proteolytic; P^{+++} – strongly proteolytic; P^{++} – moderately high proteolytic; P^{+} – moderately low proteolytic; and P^{-} – low proteolytic) and type of Gram-staining (+ – Gram-positive; and - – Gram-negative). H – Head; F – Flank; R – Rear.

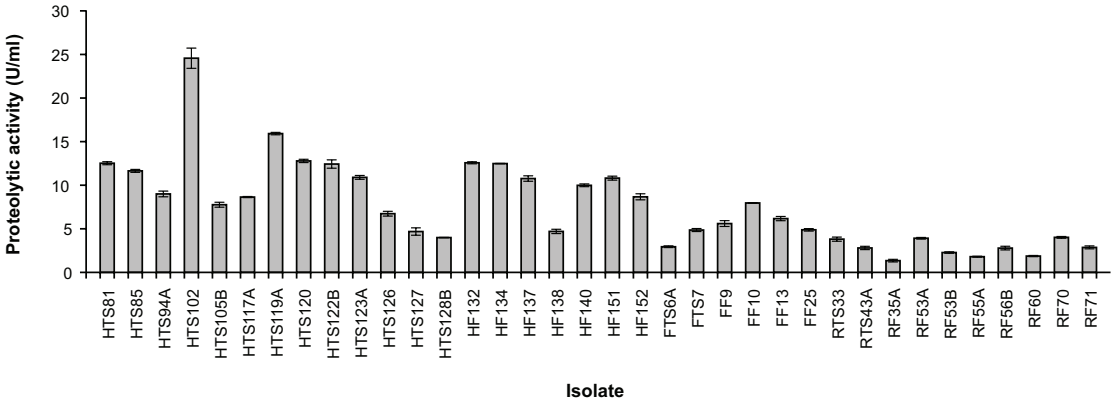


Figure 2.1.5 – Proteolytic activity (average ± standard deviation) of the strongest protease producers from the wool-associated bacterial population – using casein as substrate, incubated for 1 d.

2.1.4.5. Phylogenetic analysis of selected isolates

The organisms selected for the phylogenetic analysis included representatives of the phylum Firmicutes, Actinobacteria, Bacteroides and Proteobacteria, with organisms belonging to the families Bacillaceae, Streptomycetaceae, Pseudomonaceae, Flavobacteriaceae, Alcaligenaceae, Xanthomonadaceae, Paenibacillaceae and Vibrionaceae; this unfolded a great diversity within the best protease-producing bacteria. Regarding the origin of the isolates, three main sources could be identified: 16 organisms were isolated from soil and terrestrial (or aquatic) sediments, 9 from feather or wool matrices, and 8 from several kinds of effluents; only 5 organisms were isolated from other environments. As for the industrial applications described in the literature for each of the selected organisms, proteases from 16 isolates were already tested for application in detergents, 25 for keratin-degrading applications (mainly feather degradation and de-hairing) and 4 for fabric treatments.

Multiple alignment and phylogenetic reconstructions were performed using the new isolates HTS102 and HTS119A, as well as the remainder 40 sequences. The Neighbor-Joining tree topology computed (Figure 2.1.6) indicated that strains HTS102 and HTS119A clearly fell within the larger group, composed predominantly by *Bacillus* spp.

2.1.5. Discussion

The bacterial enumeration of the wool samples obtained from various parts of the fleece indicated that bacteria are present throughout the sheep whole body surface (Figure 2.1.1), but follow a non-uniform distribution. Recall

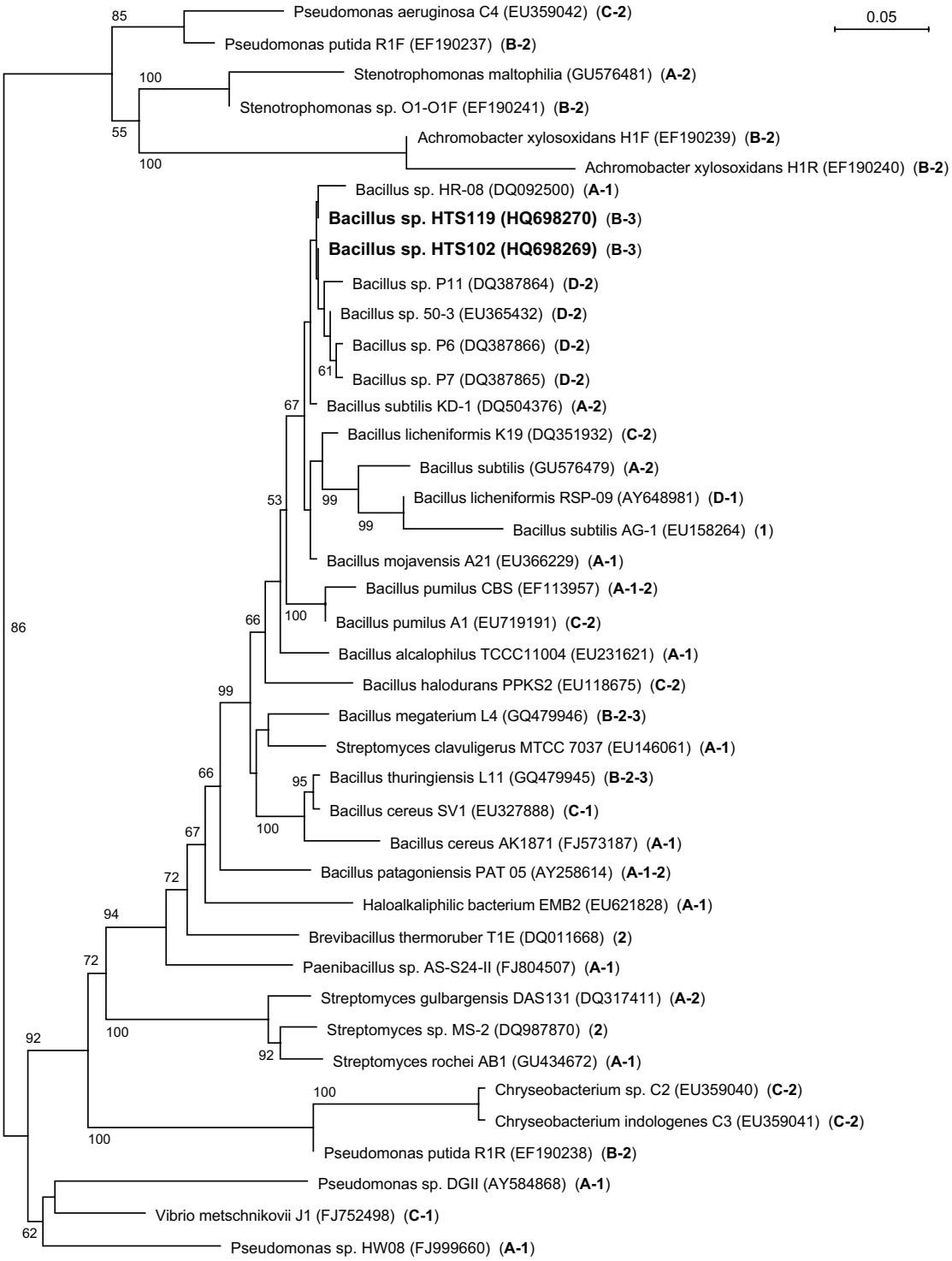


Figure 2.1.6 - Phylogenetic tree of 16S rRNA gene sequences of the strongest protease producers from the wool-associated bacterial population (i.e. strains HTS102 and HTS119A, shown in bold) and a set of other proteolytic microorganisms isolated from keratinous substrates (or tested for applications encompassing such substrates). The tree was generated by NJ analysis. Percentages of bootstrap values resulting from 10000 resamplings are indicated beside the nodes, whenever they were not below 50%. Each taxon is identified by the name and accession number (brackets). Isolation sources: A – soil or sediments, B – feather or wool, C – effluents and D – others. Tested applications: 1 – detergents, 2 – keratin degradation and 3 – fabric treatment.

that the sheep fleece is a highly absorbent matrix that is prone to accumulation of soil and fecal matter – both from the environment and the animal itself. It is, therefore, expected that the most abundant bacterial populations will be present in the ewe's areas that are more exposed to such contamination sources – especially the rear, the flank and the belly (Jackson et al. 2002). Our studies corroborate this assumption, since the viable numbers obtained for the population isolated from the Rear were well above those from the Flank and the Head sites, both at 37 and 50 ºC (Figure 2.1.1). Similar results were also reported for Australian and New Zealander fleece (Lyness et al. 1994; Jackson et al. 2002) – even knowing that environmental conditions prevailing locally influence the total bacterial numbers, and thus account for the (small) differences observed.

However, a distinction ought to be made between abundance and diversity. As shown here, diversity was found to be higher on the Head zone, as assessed by the number of distinct colonies on agar plates (Figure 2.1.4) – unlike happened with abundance. This fact indicates that wool microflora sources vary along the sheep body, with abundance of the bacterial population being higher and diversity lower wherever the outer environment is the primary source of bacteria. On the other hand, the bacteria present in the fleece are preferentially associated with sheep in the body parts less exposed to environmental contamination.

It is interesting to notice that more than 60% of the cultured bacteria formed pigmented colonies on PCA, PCA-HS and BCM plates; colors ranged from yellowish to orange, with only a small fraction of microorganisms producing cream, white or colorless colonies – which are the typical pigmentations associated with soil and fecal bacteria (Jackson et al. 2002). These findings are

once again in agreement with the claim that exogenous contamination is not the primary determinant of the total bacterial counts on the fleece. Furthermore, such a pigmentation has been reported to convey resistance to ultra-violet radiation in outer bacteria (Sundin & Jacobs 1999) – which is a necessary feature for bacteria to successfully colonize the fleece (Jackson et al. 2002). The range of pigmentation colors (and other morphologically distinctive characteristics) observed among colonies entail the existence of a wide bacterial biodiversity within the fleece; besides not being *per se* harmful to the final wool quality, such a biodiversity can even turn essential in suppressing fleece disorders that are detrimental to wool (Lyness et al. 1994) – a fact that deserves further study.

The viable numbers obtained when incubation was performed at 50 ºC were relatively high, but this realization should not come as a surprise. Skin and fleece surface temperatures of unshorn sheep may in fact reach up to 45 ºC, and the midway between the fleece surface and the skin of sheep often reaches 55 ºC when exposed to direct sunlight – and may topically exceed 60 ºC (Eyal 1963). This allows many thermophilic organisms to, at least, linger within the fleece, thus leading to viable numbers after incubation at 50 ºC that are only ca. 1-3 log cycles below those measured at 37 ºC.

As expected, the heat-treated sample unfolded a less abundant and diverse bacterial population (Figure 2.1.1 and 2.1.3). The weather prevailing by the time the fleece was sampled was relatively warm, so mesophilic and thermophilic bacteria had rather favorable surroundings to grow. Accordingly, the viable numbers of the bacterial population, obtained both at 37 º and 50 ºC, were associated with a higher ratio than between those obtained in PCA or PCA-HS (Figure 2.1.1).

The predominant bacteria isolated were Gram-positive rods – which contrasts with data reported elsewhere, which indicated prevalence of Gram-positive cocci (Jackson et al. 2002). A high proteolytic activity (P^{+++}) – measured by plate assay with CCA + 1 %(w/v) SM, was detected in 43 out of the 115 proteolytic isolates – which is a rather high score, likely accounted for by the abundance of proteinaceous substrates available on the sheep fleece, and the high ecological pressure raised on the wild microflora surviving thereon.

Results from previous experience of proteolytic activity were taken advantage of, in attempts to generate a final batch of candidates for the quantitative proteolytic assay. The "double-screening" strategy employed here – i.e. starting with a large pool of isolates, screening the whole pool for proteolytic activity using either of two distinct methodologies, and finally constituting a group with the best performers by either methodology, was rationalized on the basis that sometimes isolates may start (or stop) exhibiting certain characteristics after repeated culture maintenance cycles. Results from such a quantitative proteolytic assay encompassing the 36 selected isolates are depicted in Figure 2.1.5. It is important to emphasize that a few isolates were very good protease producers in liquid culture, despite displaying only a relatively small clearance zone on CCA + 1 %(w/v) SM plates. It has likewise been reported that some members of the *Bacillus* genus produce very narrow zones of hydrolysis on casein-agar, even realizing that they are very good protease producers in liquid culture (Kumar & Takagi 1999). An illustrative example of this situation pertains to isolate HF138: it belongs to the P^{+} group, yet it produced significantly higher amounts of proteolytic activity ($P<0.05$) than HTS128A, RF70 or RTS43A – all of which belong to the P^{+++} group. Isolates

HTS102, HTS119A, HTS120, HF132 and HF134 exhibited, in turn, significantly higher proteolytic activities ($P<0.05$) than all other isolates in the P^{+++} group.

Despite the complete match of the partial 16S rRNA gene sequences obtained for isolates HTS102 and HTS119A, differences were found in terms of colony morphology and amount of enzyme produced ($P<0.01$). Regardless of the several reports published on alkaline proteases from thermophilic *Bacillus* spp., scarce information is available on proteases produced by microorganisms growing below 60 ºC (Kumar & Takagi 1999). This means that isolates HTS102 and HTS119A might be of great interest for alternative applications in the wool industry, since they grow and release protease at 37 ºC – while operating well in the alkaline pH range.

In order to evaluate the taxonomic position of isolates HTS102 and HTS119A within the pool of relevant keratinolytic protease-producing microorganisms, a phylogenetic tree was computed – as depicted in Figure 2.1.6. Both isolates belong to the *Bacillus* genus, so databases were searched for combinations of relevant keywords and "*Bacillus*"; hence, a bias towards this genus was expected – and actually observed, in the set of organisms selected for the phylogenetic analysis. Isolates HTS102 and HTS119A were eventually included in a main group composed mostly by strains close to *Bacillus subtilis* and *Bacillus licheniformis*.

Concerning the origin of the selected microorganisms, a large portion (i.e. 25 in 41) was isolated from sources directly related to the hosts' bodies and whereabouts – viz. soil and sediments, feather and wool. No particular pattern could be perceived in the phylogenetic tree regarding the origin of said

microorganisms, with closely related organisms often being isolated from different – yet environmentally related sources.

With regard to the industrial applications for which the proteases were tested, no clear pattern arose from the phylogenetic tree; however, most microorganisms were tested for keratin degrading applications (25 in 41), thus reflecting a larger research effort towards waste reutilization.

The absence of a clear link between the source of the microorganisms, the applications for which their proteases were tested, and the phylogenetic relationships among them may be explained by the nature of the environments from which they were collected. In this context, being able to degrade keratinous compounds provides a competitive advantage, as an alternative nutritional source will be available that may allow the microorganism to ultimately survive. Hence, most of the microflora in these environments is expected to possess the required machinery to uptake available keratin. In addition, most research in this field appears to be "application-driven" – with target applications being the trigger for efforts to isolate novel microorganisms. Since most of the genera represented in this phylogenetic tree are known to be prolific protease-producers, it is reasonable to rationalize a link between the applications tested and the phylogenetic relationships depicted in Figure 2.1.6 – as a consequence of the microorganisms chosen being able to perform in all such applications. Therefore, a considerable amount of research effort should be shifted from novel microorganism prospection to screening for alternative applications of already existing (and characterized) microbial isolates.

Acknowledgments

A.C. Queiroga received a Ph.D. fellowship (ref. SFRH/BD/19212/2004), granted by Fundação para a Ciência e a Tecnologia (Portugal) and supervised by F.X. Malcata. Partial funding to cover research expenses was via projects "GoBlue" (POCTI 13-02-03-SDR-01254), granted by Agência de Inovação (Portugal), and "BioTex" (POCI/CTM/58312/2004), granted by Fundação para a Ciência e a Tecnologia (Portugal). Authors acknowledge Ângela Brito and Paula Tamagnini for assistance in obtaining 16S rRNA gene sequences.

2.1.6. References

Akhurst RJ, Lyness EW, Zhang QY, Cooper DJ, Pinnock DE. 1997. A 16S rRNA gene oligonucleotide probe for identification of *Bacillus thuringiensis* isolates from sheep fleece. J Invertebr Pathol 69:24-30.

Eyal E. 1963. Shorn and unshorn Awassi sheep. 4. Skin temperature and changes in temperature and humidity in fleece and its surface. J Agric Sci 60:183-193.

Frazier WC, Rupp P. 1928. Studies on the proteolytic bacteria of milk I. A medium for the direct isolation of caseolytic milk bacteria. J Bacteriol 16:57-63.

Giongo J, Lucas F, Casarin F, Heeb P, Brandelli A. 2007. Keratinolytic proteases of *Bacillus* species isolated from the Amazon basin showing remarkable de-hairing activity. World J Microbiol Biotechnol 23:375-382.

Gupta R, Beg Q, Lorenz P. 2002b. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 59:15-32.

Gupta R, Ramnani P. 2006. Microbial keratinases and their prospective applications: an overview. Appl Microbiol Biotechnol 70:21-33.

Halliday LA. 2002. Woolscouring, carbonising and effluent treatment. In: Simpson WS, Crawshaw GH, Editors. Woodhead Textile Series. Cambridge, U.K.: Woodhead Publishing. pp. 21-22.

Jackson TA, Pearson JF, Young SD, Armstrong J, O'Callaghan M. 2002. Abundance and distribution of microbial populations in sheep fleece. New Zeal J Agr Res 45:49-55.

Kumar CG, Takagi H. 1999. Microbial alkaline proteases: from a bioindustrial viewpoint. Biotechnol Adv 17:561-594.

London CJ, Griffith IP. 1984. Characterization of Pseudomonads isolated from diseased fleece. Appl Environ Microbiol 47 993-997.

London CJ, Griffith IP, Kortt AA. 1984. Proteinases produced by Pseudomonads isolated from sheep fleece. Appl Environ Microbiol 47:75-79.

Lyness EW, Pinnock DE, Cooper DJ, Milner R. 1994. Microbial ecology of sheep fleece. Agric Ecosyst Environ 49:103-112.

Martley FG, Jayashankar SR, Lawrence RC. 1970. An improved agar medium for the detection of proteolytic organisms in total bacterial counts. J Appl Microbiol 33:363-370.

Norris BJ, Colditz IG, Dixon TJ. 2008. Fleece rot and dermatophilosis in sheep. Vet Microbiol 128:217-230.

Perrière G, M. G. 1996. WWW-Query: An on-line retrieval system for biological sequence banks. Biochimie 78:364-369.

Rai SK, Mukherjee AK. 2009. Ecological significance and some biotechnological application of an organic solvent stable alkaline serine protease from *Bacillus subtilis* strain DM-04. Bioresource Technol 100:2642-2645.

Šafařík I, Šafaříková M. 1994. A modified procedure for the detection of microbial producers of extracellular proteolytic enzymes. Biotechnol Tech 8:627-628.

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-425.

Sambrook J, Russell DW. 2001. Molecular Cloning: A Laboratory Manual. New York, NY, USA: Cold Spring Harbor Laboratory Press.

Saran S, Isar J, Saxena RK. 2007. A modified method for the detection of microbial proteases on agar plates using tannic acid. J Biochem Biophys Meth 70:697-699.

Schumacher K, Heine E, Hicker H. 2001. Extremozymes for improving wool properties. J Biotechnol 89:281-288.

el-Sukhon SN. 2002. Isolation and characterization of *Pseudomonas aeruginosa* from sheep with fleece rot in northern and middle Jordan. Vet Dermatol 13:247-251.

Sundin GW, Jacobs JL. 1999. Ultraviolet radiation (UVR) sensitivity analysis and UVR survival strategies of a bacterial community from the phyllosphere of field-grown peanut (*Arachis hypogeae* L.). Microb Ecol 38:27-38.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acid Res 24:4876-4882.

Vermelho AB, Meirelles MNL, Lopes A, Petinate SDG, Chaia AA, Branquinha MH. 1996. Detection of extracellular proteases from microorganisms on agar plates. Mem Inst Oswaldo Cruz 91:755-760.

Section 2.2

Preliminary assessment of enzyme applicability for wool processing

2.2.1. Abstract

A total of 158 microbial strains, previously isolated from raw wool samples of Portuguese *Merino* breed of sheep, were screened for extracellular protease activity. The 12 isolates with the highest overall activity were further tested via incubation in nutrient broth, and assay of cell-free supernatants used casein as substrate protein. The cell-free supernatants of the three isolates exhibiting the best performance were finally tested on knitted wool using bursting strength and area shrinkage as quantitative parameters, and microstructure using scanning electron microscopy as qualitative parameter – to conclude on their putative role upon the fiber features. The aforementioned three isolates produced lower weight loss and area shrinkage than those brought about by a commercial reference enzyme under similar operating conditions, without significantly loosing bursting strength.

2.2.2. Introduction

Most biotechnological tools currently in use for textile processing are still inspired on approaches that have been applied for more than 2000 years (Gübitz et al. 2001); in fact, few innovations have been meanwhile developed that feature mainly enzymes. Enzymes can indeed be applied to virtually all-manufacturing steps in the textile industry, from fiber and fabric processing, to laundry detergents and effluent treatment. The most widely employed enzymes are hydrolases (e.g. cellulases and amylases) and oxidoreductases (e.g. laccases, peroxidases and catalases).

Recall that enzymes catalyze chemical reactions, and typically possess a great specificity and lead to major rate enhancements; this realization provides paramount opportunities for industrial applications thereof, aimed at more efficient and economic conversions (van Beilen et al. 2002). Furthermore, advances in biotechnology at large, and particularly in such areas as protein and genetic engineering, have made available enzymes especially tailored for specific applications (Kirk et al. 2002). So far, research on enzyme applications in textile processing has aimed at a balance between beneficial effects (mainly on texturization) and potential mechanical losses (mainly of strength) (Gübitz et al. 2001). Furthermore, additional contributions to sustainability encompassing energy and raw material consumption, waste production and stability/safety of product (Schmid et al. 2002), have also played a role as driving forces for said research efforts.

Wool is a complex proteinaceous matrix, which exhibits several unique properties among the natural fiber world. The surface scales of that fiber account

for the distinctive felting and shrinking properties of wool upon wetting. Since consumers have placed increasingly higher demands on machine washability and sustained soft handle touch, the market value of wool has been steadily decreasing. In attempts to regain its (once leading) position within the European textile and clothing industry, innovation is urged as a basis for competitiveness, via alternatives both in quality of product and sophistication of process. Applications of enzymes to wool may thus contain the potential to bring about a greater added value; since wool fibers consist mainly of proteins and lipids, proteases and lipases will likely account for a major opportunity in processing of that fiber.

At present, applications of the aforementioned enzymes in wool processing are rather limited, not only because of technological difficulties in handling and control, but also due to poor knowledge of these enzymes when acting on such a substrate fiber. Note, in particular, that proteases are degradation enzymes that catalyze hydrolysis of proteins (Rao et al. 1998); they have been employed for over 80 years in industrial treatment of wool, in attempts to impart such desirable properties as better handle features and higher shrink-resistance (Cortez et al. 2004). However, current enzymatic processes are difficult to implement and control on the industrial level, given that such pre-set specifications as penetration of enzyme into the fiber can hardly be controlled – thus causing excessive damage to the fiber cuticle, with consequent high degrees of weight and strength losses (Cortez et al. 2004). For this reason, application of proteases in wool processing remains a challenging task; there are a number of commercial proteases available, but their

performance has not yet been found fully satisfactory in inducing low shrinkage at low levels of strength loss.

The goal of this research effort was thus to screen for (wild) bacterial sources of extracellular proteases that are able to act specifically on wool. To said purpose, a total of 158 strains (previously isolated from raw wool samples of the Portuguese *Merino* breed of sheep) were screened for protease activity, via the spot technique on agar plates; the 12 best performers were then subjected to refined characterization of their enzymatic activities. Their potential applicability in wool finishing was finally demonstrated for the very best in terms of such parameters as bursting strength and area shrinkage, as well as surface micro-morphology.

2.2.3. Materials and Methods

2.2.3.1. Microorganism sources

Raw wool samples were collected from three distinct parts (viz. head, flank, and rear) of Portuguese *Merino* sheep. Solutions of each sample (10 g of wool + 95 mL of sterile water) were homogenized for 15 min at 260 rpm, using a Stomacher® Lab Blender (Seward, UK), and cultured in quadruplicate on Plate Count Agar (Merck, Germany) and Bacillus cereus Medium Agar (Lab M, UK), at 37 and 50 ºC – for eventual isolation of strains, which were tentatively pinpointed as those colonies bearing distinct morphological characteristics. After purification via sequential steps of plating on Nutrient agar plates and culturing

on Nutrient broth, strains were kept at −80 ºC with glycerol (30%, w/v) until further use.

2.2.3.2. Wool source

Knitted, 100% *Merino* wool fabrics (with an average fiber diameter of 19.5 μm) were kindly supplied by Orfama, Organização Fabril de Malhas (Portugal). Pieces of 12 cm x 12 cm were cut therefrom and duly cast off, and a square of 10 cm x 10 cm was marked on the fabrics using a pen. All fabrics were pre-treated with 2.5 %(v/v) chlorine, in order to modify the scale surface, and thus make the fibers more susceptible to enzyme attack (Cardamone et al. 2004).

2.2.3.3. Protein content assay

Throughout the period of protease activity monitoring (as described below in detail), aliquots were withdrawn for total protein determination (by absorbance at 660 nm), using the Total Protein Kit, Micro-Lowry, with modifications (Sigma–Aldrich, USA).

2.2.3.4. Proteolytic activity assays

Qualitative assays

All strains isolated from *Merino* wool samples (as described above) were screened for protease activity, via the spot technique, on Calcium Caseinate Agar (Merck) containing 1 %(w/v) skim milk (Oxoid, UK) (Gupta et al. 2002a); results were recorded every day for a 5 day-period.

The isolates exhibiting the highest protease activity, detected as largest clearance diameter (at least 5 mm by 1 day, and at least 20 mm by 5 days), were selected for further (refined) estimation of enzymatic activity.

Quantitative assays

Upon isolation as described above, the selected isolates were further cultured in Nutrient Broth E (Lab M, UK), for alternative estimation of protease activity, using casein as substrate. The activity of each strain was evaluated for 5 days, via monitoring the proteolytic activity of cell-free supernatants (sterilized by filtration through a 0.45 μm-filter) using colorimetric determination (at 660 nm, with the Folin–Ciocalteu's reagent) of the extent of casein breakdown, according to manufacturer's instructions (Sigma–Aldrich, USA). One unit (U) of proteolytic activity was defined as the amount of enzyme able to hydrolyze casein, so as to produce an absorbance equivalent to that produced by 1.0 μmol of tyrosine per min, at pH 7.5 and 37 ºC.

2.2.3.5. Wool finishing assays

Enzyme source

The neutral alkaline protease for textiles, Protex Multiplus L™, from *Bacillus lentus*, was kindly supplied by Genencor International (Rochester, NY, USA). Before enzymatic treatment of wool fabrics was in order, its activity in solutions of 0.75 and 1 g/L (in 10 mM sodium carbonate buffer, pH 9) was determined, using also casein as substrate.

Those strains exhibiting proteolytic activities on casein larger than that exhibited by the higher concentration of the commercial protease used as

reference were selected for supplementary assaying directly on wool fabrics. Hence, the culture supernatants of those strains were filtered through 0.45 μm sterile membranes, and applied as (crude) enzyme solutions on knitted wool fabrics (as described below).

Protease processing

Each pre-treated fabric was subjected (in triplicate) to processing by eight different enzyme combinations, for two reaction times (15 and 30 min), and plain air-dried (for treatments with odd numbers) or tumble-dried (for treatments with even numbers) after home laundering, as depicted in Table 2.2.1; a total of 32 independent experiments were thus carried out. In addition, the fabrics were subjected to two control treatments, using only 10 mM phosphate buffer (pH 7) or 10 mM sodium carbonate buffer (pH 9) for the same two reaction times, and two drying methods described above – thus totaling 8 control experiments. All those 40 different treatments were performed at 60 ºC, using a Washtester WT (Werner Mathis, Germany), at a weight ratio of ca. 1:20. Quenching of the enzyme was via rapidly raising the temperature to 75 ºC, and washing for 5 min. The fabrics were afterwards cooled, drained and rinsed.

Quantitative assays

The bursting strength and shrinkage extent of enzyme-treated wool fabrics were monitored during three subsequent household wash and dry cycles, using a model WFL 1300 washing machine (Bosch, Germany); the program for wool (35 min wash, at 30 ºC) was used, with one measure of Woolite® fabric wash, and a total wash load of 1 kg (dry mass). After each wash cycle, the fabrics

Table 2.2.1 Experimental design encompassing treatment conditions

Processing Conditions	Experimental Runs																				
	1 - 2	3 - 4	5 - 6	7 - 8	9 - 10	11 - 12	13 - 14	15 - 16	17 - 18	19 - 20	21 - 22	23 - 24	25 - 26	27 - 28	29 - 30	31 - 32	33 - 34	35 - 36	37 - 38	39 - 40
Buffer at pH:	7	7	9	9																
Protex Multiplus L (g/L)					0.75	0.75	1	1												
Crude extract of isolate **102** at pH:									7.9	7.9	9	9								
Crude extract of isolate **151** at pH:													7.7	7.7	9	9				
Crude extract of isolate **152** at pH:																	7.7	7.7	9	9
Reaction time (min)	15	30	15	30	15	30	15	30	15	30	15	30	15	30	15	30	15	30	15	30

were air- or tumble-dried using a WTL 4310 tumble-drying machine (Bosch), with the program F (60 ºC) for 50 min.

Bursting strength was assessed on 7.3 cm^2 pieces of wool fabrics, after handling (washing and drying) as described above, according to ISO 13938-2 except in number of replicates (which was only 3 instead of 5, owing to lack of sample size), using a Tru-Burst 610.

Shrinkage extent was calculated based on the original size (length and width) of the fabric, prior to enzyme treatment (Schroeder et al. 2004). Measurements in each direction were made on the specimens after handling (washing and drying) as described above, and the average dimensional change (% DC) was calculated via % DC= (A−B)/Bx100, where A and B denote the area of fabric after and before, respectively, the three subsequent household wash and dry cycles (Anon. 2006).

Qualitative assays

The putative fiber damage caused by the experimental enzymatic treatment was ascertained via scanning electron microscopy. Toward this purpose, 2.5 cm x 2.5 cm pieces were cut off from the 12 cm x 12 cm knitted wool fabrics, glued to aluminum specimen studs (with double-sided tape), and coated with a thin layer of gold in a sputter coater (for 60 s, at 2 kV and 22 mA). Imaging was obtained in a JSM-5600LV scanning electron microscope (Jeol, Japan), operating at 15 kV.

2.2.3.6. Statistical analysis

All results shown (unless otherwise stated) are the arithmetic means of at least 3 specimens of each sample, with the corresponding standard deviations. The means of enzyme-treated samples were compared with the corresponding controls, using one-way ANOVA at a significance level of 5% – with the supplementary multiple comparison tests LSD and Tukey-HSD, to identify which pairs of means were statistically different at that significance level.

2.2.4. Results and Discussion

Bacterial enzymes, employed by the textile industry for some time, include amylases that can act at boiling temperatures, and proteases that can stand the alkaline range; both are obtained from extremophiles, and account for ca. 70% of the overall market in our area of interest. Enzymes from non-extremophiles are not used so often; hence, enzymes from wild strains that populate natural environments (as is actual wool on the living animal) are in principle interesting, and were thus considered.

The 158 bacterial isolates, screened for protease activity, produced the results tabulated in Table 2.2.2. Of the isolates tested, 125 out of 158 (i.e. 79.1%) exhibited some degree of protease activity on agar plates, as apparent by the presence of a clear zone surrounding the corresponding colonies. Furthermore, 58 out of 158 (i.e. 36.7%) showed a considerable degree of protease activity, as indicated by a diameter of the clearance zone around the colony greater than 20 mm by 5 days. Only 12 out of 158 isolates (i.e. 7.6%) exhibited halos greater than 5 mm by 1 day, and greater than 20 mm by 5 days.

Table 2.2.2 Semi-quantitative estimation of protease activity by isolates from Portuguese raw *Merino* wool samples, on caseinate agar plates incubated at two temperatures for two periods

Isolates tested (number)	**Isolates within each clearance diameter range** (number)							
	No proteolysis		**≥ 5 mm halo (1 d)**		**< 20 mm halo (5 d)**		**≥ 20 mm halo (5 d)**	
	37 ºC	50 ºC	37 ºC	50 ºC	37 ºC	50 ºC	37 ºC	50 ºC
158	32	1	22	22	38	29	41	17

The latter 12 isolates were further monitored for production of protease, using 5 days of incubation, as plotted in Figure 2.2.1; solutions of 0.75 and 1.0 g/L of the commercial reference protease exhibited 36.4 and 54.5 U/mL, respectively. To quantitatively assay for protease activity of those isolates, the substrate chosen was casein – because it is considered as a standard, commercially available substrate suitable for accurate analysis and wide comparison.

By 1 day of growth, all strains exhibited an activity of less than 36.4 U/mL, which is the activity equivalent to the minimum recommended by the supplier for application of the commercial protease; a special mention is deserved by strains 9 and 102. By 5 days of growth, 6 out of the 12 isolates (i.e. 33, 102, 123A, 140, 151 and 152) exhibited protease activity above 36.4 U/mL. On the other hand, 3 out of those 6 isolates (i.e. 102, 151 and 152) actually showed proteolytic activities greater than 54.5 U/mL. Furthermore, those 3 isolates yielded the greatest specific activities (4.4, 2.6 and 2.1 U/g of protein). Hence, only isolates 102, 151 and 152 were selected for preliminary studies of action upon wool fabrics, including weight loss. This parameter provides information about the activity over the wool fiber, but not about where the enzyme acts in/on the fiber; therefore, data on the burst strength were generated as well.

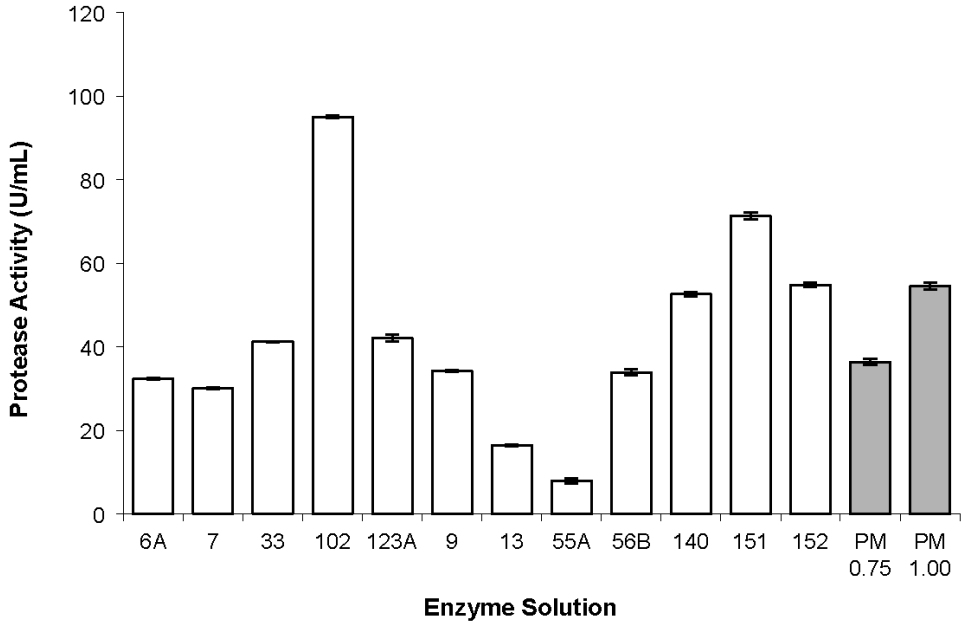


Figure 2.2.1 Quantitative estimation of protease activity by the best performant 12 isolates from Portuguese raw *Merino* wool samples (white bars) and by commercial protease (grey bars), on casein by 5 days of incubation (average± S.D.).

Statistical analyses of our results unfolded statistically significant differences between the levels of area shrinkage among the samples, and between their levels of bursting strength. However, one-way ANOVA does not allow one to conclude which treatment(s) entail(s) bursting strengths and area shrinkages significantly different from the others; a multiple comparison test is further required in this particular, as was accordingly applied.

Data on dimensional changes, specifically on percent area shrinkage, are plotted in Figure 2.2.2. The data pertaining to surface area measurements following wash-drying cycles suggest that shrinkage was chiefly reduced by enzymatic treatment for 30 min with the enzyme solution from isolate 102 (pH 9), followed by air-drying (see assay 23), with an area shrinkage of only 3.95±0.27%. However, Tukey-HSD test indicated that the result from assay 23 did not statistically differ from those from assays 13, 17, 19, 21, 25, 29, 33 and 35

– all encompassing treatments with novel enzymes, except assay 13 that encompasses use of 1 g/L of commercial protease for 15 min (*p*-value of 0.097).

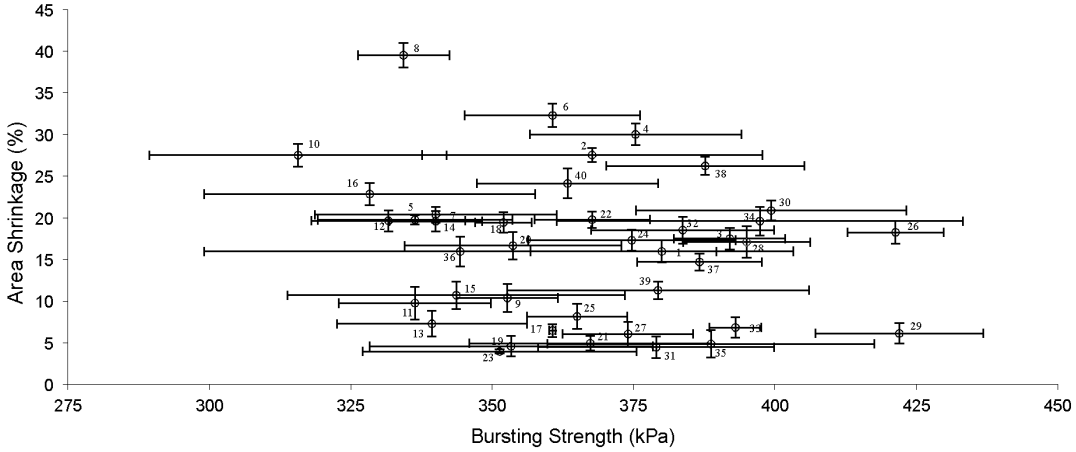


Figure 2.2.2. Area shrinkage (average ± S.D.) and bursting strength (average ± S.D.) for each treatment condition combination of enzyme, pH, reaction time and drying method, after three washing cycles, of samples of knitted wool fabrics (as detailed in Table 2.2.1). The samples treated with buffer solutions (at pH 7 or 9, assays 1–8) instead of enzyme, whether commercial or newly isolated, were used as controls in this experiment.

Inspection of Figure 2.2.2 further indicates that tumble-drying causes unacceptable dimensional changes on wool fabrics, even when proteases were employed at values ranging from 15.95 to 39.52%. For all types of treatment, one finds that the percent area shrinkage was smaller when samples were treated for longer reaction times (30 instead of 15 min), except when plain buffer solutions (pH 7 or pH 9) were considered (assays 1–9, Figure 2.2.2). Treatment of knitted wool with plain buffer solutions produced in fact the greatest percent area shrinkage, thus indicating that presence of said solutions could not protect the fabric against shrinkage as much as enzymatic treatments did. Air dried samples treated with enzyme from isolate 102 exhibited smaller shrinkages at pH 9 (assays 21 and 23) than at pH 7.9 (assays 17 and 19); however, both treatments originated area shrinkages below 7%, which is acceptable for an "easy care"-labeled woolen product.

On the other hand, samples treated with enzyme from isolate 151 led to area shrinkage, viz. 7.68%, larger than at pH 9 (assays 29 and 31) – which were found to be unacceptable dimensional changes for a commercial woolen product. Another conclusion that can be withdrawn from Figure 2.2.2 is that enzymes from isolates 102, 151 and 152 protected the samples from shrinkage better than the commercial enzyme tested did. All enzymatic treatments that caused a dimensional change below 8% (viz. assays 25 < 31 < 19 < 35 < 21 < 27 < 29 < 17 < 33) are not statistically different from each other, but are significantly lower than those produced by treatment with commercial enzyme – except, again, with regard to run 13. Considering only the air dried samples, all treatments with enzymes from new isolates presented a great potential for anti-shrinkage of wool fabrics, as indicated by the associated values of area shrinkage below said 8%.

Data on bursting strength are also presented in Figure 2.2.2. There unfolded no significant changes, as given by Tukey-HSD test, in the bursting strengths of wool samples subjected to the same enzymatic treatment, but to different drying methods. Hence, tumble-drying significantly promotes dimensional changes on wool samples, yet the drying method plays no significant role on bursting strength thereof. When samples were treated for longer reaction times (30 instead of 15 min), no significant differences could be pinpointed in bursting strength; this observation might indicate that the enzyme acts only on the surface (as made apparent by the smaller percent area shrinkage), thus not damaging the fiber itself.

Analysis of the results obtained following enzymatic treatments leading to dimensional changes below 8%, one finds somewhat unexpected results in terms

of bursting strength: the smaller the percent area shrinkage, the smaller the bursting strength. Globally, the treatment leading to the highest bursting strength was assay 29 (422±15 kPa) – which entails use of enzyme from isolate 151, at pH 9, for 15 min and subjected to air-drying; however, the bursting strengths obtained in experimental runs 21, 27, 31 and 35 are not significantly different from that obtained in assay 29 (according to a Tukey-HSD test, which yielded a *p*-value of 0.117).

A good enzyme for wool finishing should induce low dimensional shrinkage and low strength loss (or, equivalently high bursting strength) – i.e. one should focus on the lower right corner in Figure 2.2.2. Since all those enzymatic treatments produced no significantly different dimensional changes, one may then choose the treatment yielding the highest bursting strength coupled with economic and environment considerations. The choice would then be of process 29, which encompasses use of enzyme from isolate 151, at pH 9, for 15 min and air-drying, even though isolate 102 yielded the highest protease activity. The rationale underlying this choice is a major issue, if industrial applications are sought.

Selected micrographs, obtained via scanning electron microscopy, are displayed in Figure 2.2.3. No significant differences could be pinpointed, in terms of surface appearance, between all enzyme-treated and control samples – except regarding samples treated with commercial protease at 1 g/L for 30 min (assay 15), which caused fibers to break (as apparent in Figure 2.2.3a). One can also conclude that treatment with enzyme from isolate 151 did not cause perceptible fiber damage, even though it greatly reduced the scales on the surface relative to

the control sample – which encompassed only chlorine pre-treatment (see Figure 2.2.3b and c).

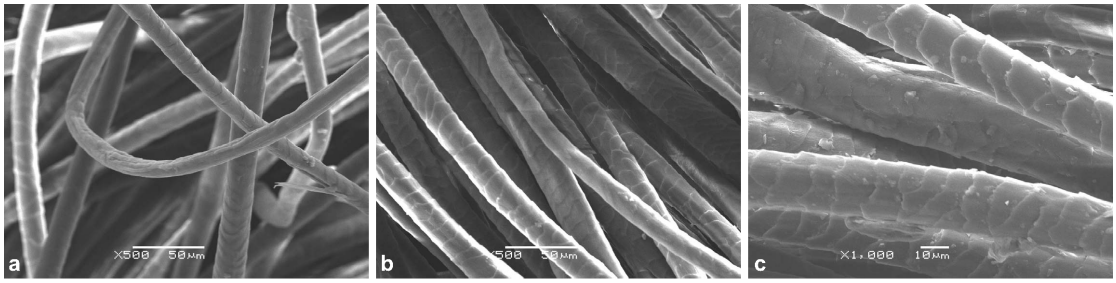


Figure 2.2.3. Scanning electron micrographs of samples of knitted wool fabrics, treated with: (a) commercial protease (1 g/L) for 30 min and air dried, after pre-treatment with 2.5 %(v/v) chlorine; (b) crude enzyme solution from isolate 151 for 15 min, at pH 9, after pre-treatment with 2.5 %(v/v) chlorine; (c) none, after pre-treatment with 2.5 %(v/v) chlorine.

2.2.5. Conclusions

Use of our novel microbial proteases in handling of clothes does not lead to increased damage of wool fabric, as compared to conventional industrial conditions, i.e. plain chlorination treatment; this realization entails bursting strength, area shrinkage and microstructural parameters. However, statistically significant decreases in area shrinkage after laundering were observed, without significant losses of bursting strength. Although the best anti-shrinkage performance is obtained with the enzyme from isolate 151 – at pH 9, for 15 min and subjected to air-drying, employment of similar treatment conditions for smaller periods of time is a viable alternative, since it leads to a very good bursting strength with no significant difference in area shrinkage.

Acknowledgments

A.C. Queiroga received a Ph.D. fellowship (ref. SFRH/BD/19212/2004), granted by Fundação para a Ciência e Tecnologia (Portugal) and supervised by F. X. Malcata. Partial funding to cover research expenses was received via projects "GoBlue" (POCTI 13-02-03-SDR-01254), granted by Agência de Inovação (Portugal) and "BioTex" (POCI/CTM/58312/2004), granted by Fundação o para a Ciência e Tecnologia (Portugal). The authors are grateful to Ms. Ana Ferreira for technical support in sample preparation and imaging by SEM.

2.2.6. References

2006. AATCC Testing Method 135-2004, Dimensional changes of fabrics after home laundering. In American Association of Textile Chemists and Colorists. pp. 231–234.

Beilen JBv, Li Z. 2002. Enzyme technology: An overview. Curr Opin Biotechnol 13:338-344

Cardamone JM, Yao J, Nunez A. 2004. DCCA shrinkproofing of wool: Part I: Importance of antichlorination. Textile Res J 74:555-560.

Cortez J, Bonner PLR, Griffin M. 2004. Application of transglutaminases in the modification of wool textiles. Enzyme Microb Technol 34:64-72.

Gubitz GM, Cavaco-Paulo A. 2001. Biotechnology in the textile industry - perspectives for the new millennium. J Biotechnol 89:89-90.

Gupta R, Beg Q, Khan S, Chauhan B. 2002a. An overview on fermentation, downstream processing and properties of microbial alkaline proteases. Appl Microbiol Biotechnol 60:381-395.

Kirk O, Borchet TV, Fuglsang CC. 2002. Industrial enzyme applications. Curr Opin Biotechnol 13:345-351.

Rao MB, Tanksale MSG, Deshpande VV. 1998. Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol R 62:597-635.

Schmid A, Hollmann F, Park JB, Buhler B. 2002. The use of enzymes in the chemical industry in Europe. Curr Opin Biotechnol 13:359-366.

Schroeder M, Schweitzer M, Lenting HBM, Guebitz GM. 2004. Chemical modification of proteases for wool cuticle scale removal. Biocatal Biotransfor 22:299-305.

Chapter 3

Optimization of protease production and activity

Section 3.1

Nutritional and processing factors affecting protease productivity by *Bacillus* sp. HTS 102

3.1.1. Abstract

The synthesis of an extracellular protease by *Bacillus* sp. HTS102 – a wild strain recently isolated from the wool of Portuguese Merino ewes, was optimized. Following a preliminary screening for the most relevant processing factors, a fractional factorial design (2_{VI}^{6-1}) was applied to ascertain the effects of six relevant parameters – viz. yeast extract concentration, peptone level, inoculum size, stirring rate, temperature and pH. The concentrations of yeast extract and peptone, as well as the incubation temperature and pH were found to play significant roles; and the 2-factor interaction between yeast extract level and pH was also significant. A 2.2-fold increase in the overall level of protease synthesis was eventually attained, with the improved medium relative to the basal medium.

3.1.2. Introduction

Use of enzymes for industrial processing has received considerable attention in recent years (Ibrahim & al-Salamah 2009), owing mainly to environmental concerns; and proteases on their own already account for ca. 40% of the total enzyme sales worldwide (Gupta et al. 2002b). Proteases are indeed essential for cell growth and differentiation, so they are ubiquitously found in living organisms (Navaneeth et al. 2009) – with *Alcaligenes faecalis*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Bacillus cereus, Bacillus licheniformis*, *Bacillus lentus*, *Clostridium* sp. and *Aeromonas hydrophilica* accounting for some of the highest rates of synthesis thereof (Gupta et al. 2002b; Shafee et al. 2005). Extracellular proteases are particularly convenient from a production standpoint – and large productivities have accordingly been observed for several species of the *Bacillus* genus (Navaneeth et al. 2009).

Despite the large array of commercial proteases available at present, an ever-growing portfolio of uses for them, coupled with a consequent by higher and higher demand have promoted comprehensive screening efforts for new strains of bacteria – which can produce proteases with better features and/or be susceptible to development of lower cost/ higher throughput industrial processes (Huang et al. 2008). It has for long been recognized that one major obstacle to successful biotechnological applications in the industry at large is the overall costs of enzyme production and downstream purification (Gupta et al. 2002b); 30-40% of the final cost of enzyme arises indeed from the culture medium used for fermentation of its source strain (Joo & Chang 2005), so

optimization of fermentation will assume a great relevance if economically feasible processes are sought.

A number of comprehensive efforts have been developed by biochemists and biochemical engineers to increase protease yields (Gupta et al. 2002b), yet no ideal and all-purpose medium for maximum microbial protease production has been formulated to date – since each microorganism has its own specific growth and enzyme synthesis/secretion constraints, and thus its unique physicochemical and nutritional requirements (Reddy et al. 2008b). Therefore, the qualitative and quantitative profiles of the culture medium, further to other operating factors, are to be optimized for each microorganism and desired metabolite. For instance, extracellular protease production is strongly influenced by such medium features as C/N ratio, presence/absence of readily metabolizable sugars and nitrogen sources, and availability of specific metal ions; however, such processing conditions as rate of aeration, inoculum density, pH, temperature and incubation time also play a crucial role (Gupta et al. 2002b).

Optimization of processing parameters (encompassing also medium components) by classical methods that involve change of "one-variable-at-a-time" is extremely time-consuming, and thus expensive when a large number of variables is to be considered (Rao et al. 2007). Despite ignoring the interactions among parameters, it is still the most frequently used strategy in bioprocess engineering to obtain high yields of enzyme in microbial systems (Puri et al. 2002). This is especially true at earlier stages of process optimization, when little is known about the factors that actually affect enzyme synthesis yield and rate. As a result, preliminary studies ignoring mutual processing interactions are acceptable just to find whether or not one (or several) factor(s) under

investigation affect protease production significantly – and thus anticipate the most reasonable ranges for further optimization encompassing such interactions (Myers & Montgomery 2002).

To overcome the aforementioned inability of the "one-at-a-time" approach to pinpoint interactions among the processing parameters, factorial designs have been proposed (Puri et al. 2002); these are in fact frequently used in screening for key factors that affect the response when more than 3 factors are at stake – and usually assume a suitable fractional form to avoid the need for an excessively large number of experiments (Myers & Montgomery 2002).

The major goal of the research effort described in this chapter was thus to find the best combination of processing factors aimed at improving protease production by the wool-associated *Bacillus* sp*.* strain HTS 102 – a novel strain recently isolated from a Portuguese autochthonous sheep breed. After conducting several comparative "one-factor-at-a-time" studies, a 2_{VI}^{6-1} fractional factorial design was applied to better elucidate the main effects and two-factor interactions associated with the most promising processing parameters – and eventually to increase the protease productivity of that microorganism to an extent sufficient to backup eventual industrial interest.

3.1.3. Materials and methods

3.1.3.1 Preliminary screening via "one-factor-at-a-time" approach

Reference culture medium

The Nutrient Broth (NB) medium was used as control, and was prepared according to the recipe provided by the manufacturer (Lab M, UK) – but using ingredients added separately, viz.: 2.0 g/L yeast extract (Biokar Diagnostics, France), 1.0 g/L beef extract (Merck, Germany), 5.0 g/L peptone (Sigma-Aldrich, USA) and 5.0 g/L NaCl (Carlo Erba, Italy).

Carbon/nitrogen source selection

The beef and yeast extracts, referred to in the previous section, were each one replaced with alternative carbon and nitrogen sources, respectively, at equivalent concentrations. Carbon sources tested were starch, glycerol, lactose, sucrose, glucose and fructose. Nitrogen sources considered were peptone, tryptone, ammonium chloride and ammonium sulfate.

Significant physicochemical parameter determination

A total of 10 physicochemical factors – that had been reported previously in the literature to influence protease production in a number of bacteria, were tested in attempts to find those critical for protease production by *Bacillus* sp. HTS 102. Such factors were metal ion concentration – 10 mM $FeSO_4$ and 0.1 g/L $MgSO_4$, inoculum density, pH value, incubation temperature, stirring rate, and presence of 0.8 %(w/v) Na_2CO_3, 10 mM $CaCl_2$, 0.02 %(v/v) Triton X-100 and 1.0 g/L K_2HPO_4.

Nutrient Broth constituent effects

Several medium formulations were prepared by removing "one-constituent-at-a-time", so as to ascertain the effect of each of the NB medium constituents upon protease production by the target bacterium. The media assayed for are tabulated in Table 3.1.1.

Table 3.1.1 Characterization of medium formulations, based on Nutrient Broth (NB), tested in attempts to enhance protease productivity

Formulation code	Nutrients (g/L)				Description
	Yeast Extract	Beef Extract	Peptone	NaCl	
NB-C	2	1	5	5	NB complete
NB-Y	0	1	5	5	NB without yeast extract
NB-B	2	0	5	5	NB without beef extract
NB-P	2	1	0	5	NB without peptone
NB-N	2	1	5	0	NB without NaCl
NB-YN	0	1	5	0	NB without yeast extract and NaCl

3.1.3.2 Optimization via 2_{VI}^{6-1} fractional factorial design approach

After selection of a total of 6 key factors for protease production by *Bacillus* sp. HTS102, levels of yeast extract and peptone, inoculum density, stirring rate, pH value and incubation temperature were optimized via a 2_{VI}^{6-1} fractional factorial, unreplicated design. The range of values for each factor was chosen based on information available in the literature, coupled with experience meanwhile gained during the "one-factor-at-a-time" comparative studies. The ranges of values used within such a fractional factorial design are depicted in Table 3.1.2.

Table 3.1.2 Definition of processing parameters, in original and coded format, within a 2_{VI}^{6-1} experimental design, tested in attempts to enhance protease productivity

Coded parameter	Alias	Description	Actual values corresponding to coded levels	
			-1	+1
X_1	1	Concentration of yeast extract (g/L)	5.0	10.0
X_2	2	Concentration of peptone (g/L)	2.0	4.0
X_3	3	Level of inoculum (%,v/v)	1.0	3.0
X_4	4	Stirring rate (rpm)	0	100
X_5	5	Temperature (ºC)	34	40
X_6	12345	pH	6.0	8.0

3.1.3.3 Fermentation conditions

Erlenmeyer flasks (250 ml) containing 25 ml of growth medium were inoculated with 1 %(v/v) fresh inoculum (0.6 $O.D._{600nm}$). The extracellular protease activity was quantitated after a 36 h-incubation period at 37 ºC, unless stated otherwise. The microorganism used in this study was *Bacillus* sp. HTS 102 (GenBank accession number HQ698269), isolated from Portuguese Merino wool – and formally deposited in a publicly accessible culture collection (LMG 26323, from BCCM/LMG Bacterial Culture Collection, Ghent, Belgium).

3.1.3.4 Protease activity assay

The cell-free supernatants (sterilized by filtration through a 0.45 μm filter) were assayed for protease activity using a colorimetric determination of casein breakdown extent (at 660 nm, and resorting to Folin-Ciocalteu's reagent), according to supplier's instructions (Sigma-Aldrich). One unit (U) of proteolytic activity was defined as the amount of enzyme able to hydrolyze casein so as to

produce an absorbance variation per min equal to that produced by 1.0 µmol of tyrosine, at pH 7.5 and 37 ºC.

3.1.3.5 Protein content assay

Throughout the 36 h-incubation period, aliquots were withdrawn for total protein assay (measured by absorbance at 562 nm), using the BCA™ Protein Assay Kit (Pierce, USA) – according again to supplier's instructions.

3.1.3.6 Data statistical analysis

Unless otherwise stated, all data presented here result from two independent experiments, each run in duplicate.

The means of each treatment following the "one-factor-at-a-time" approach were compared using one-way analysis of variance (ANOVA), at a significance level of 5 %; a supplementary multiple comparison test (Tukey-HSD) was employed to pinpoint which pairs of means were statistically different from each other, at that significance level.

The statistical software package Design-Expert®, v. 8.0.3 (Stat-Ease, Minneapolis MN, USA) was used to set up and analyze the results of the fractional factorial designs. The statistical significance of the underlying linear model equation and its terms was assessed via Fischer's *F*-tests. The quality-of-fit of said polynomial model equation was expressed by a coefficient of determination (R^2) and an adjusted R^2. The fitted polynomial equation was represented as contour plots for the two-factor interactions – to better illustrate the underlying relationships between response and level tested of each processing parameter.

3.1.4. Results

3.1.4.1 Carbon/nitrogen source selection

Of the several carbon sources tested, the NB medium formulation yielded the highest protease activity by far (Figure 3.1.1a). As for the nitrogen sources, all tested inorganic and organic compounds appeared to hamper enzyme production relative to that observed in the control using plain NB (Figure 3.1.1b). When yeast extract and peptone were replaced by plain peptone, the production of protease was remarkably reduced, thus suggesting that yeast extract and peptone may act synergistically.

3.1.4.2 Significant physicochemical parameter determination

To ascertain the effects of distinct ingredients (including trace metals) upon protease activity, the reference medium (NB) was enriched therewith on a "one-by-one" basis. All components tested led to lower protease production than using the reference medium (Figure 3.1.1c).

The effect of initial pH upon protease production was sought at pH values 4, 7 and 10 (Figure 3.1.1d). These results showed that *Bacillus* sp. HTS 102 was able to grow and release protease in a broad pH range.

The stirring rate affected protease production according to a "U-type" pattern (Figure 3.1.1e). In all 4 assays carried out (i.e. 2 independent experiments, each run in duplicate), horizontal orbital shaking of the culture at 50 rpm yielded less protease activity than any other rate tested. Surprisingly, no stirring at all produced essentially similar effects as stirring at rates above 50 rpm.

The maximum protease activity was attained at 37 ºC (Figure 3.1.1f), whereas dramatically lower levels were obtained at 30 and 55 ºC – i.e. 2.3 and 2.5 %, respectively, of the activity observed at 37 ºC.

The inoculum level affected growth to a considerable degree, but not protease synthesis (Figure 3.1.1g). The highest protease production was achieved at an inoculum size of 4 %, even though the difference between 2%- and 4%-inoculum levels was not statistically significant (P>0.05).

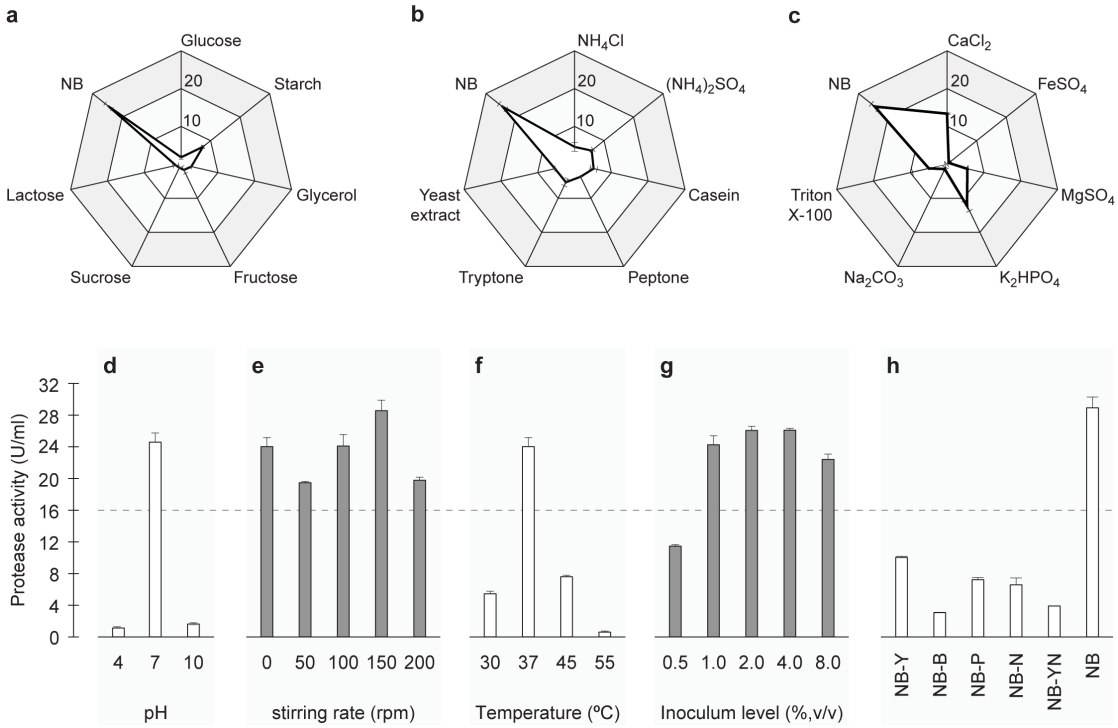


Figure 3.1.1 Results of trials following a preliminary "one-factor-at-a-time" approach, aimed at screening for relevant overall factors upon protease activity: effects of a) carbon sources (g/L), b) nitrogen sources (g/L) and c) salts and surfactants (g/L), using a star layout; and effect of d) pH, e) stirring, f) temperature, g) inoculum level and h) NB medium constituents.

3.1.4.3 Nutrient Broth constituent effects

The nutrient broth constituent that affected the most (P<0.05) the production of protease the most was beef extract (Figure 3.1.1h). Nevertheless, every other constituent of the base NB medium played a role upon production of

enzyme, because exclusion thereof "one-at-a-time" greatly reduced enzyme production. Furthermore, when yeast extract and NaCl were simultaneously excluded from the medium, a further decrease in protease production was observed – thus indicating that NaCl, despite not being a source of nitrogen or carbon, probably plays a role in assuring the appropriate ionic strength for protease synthesis.

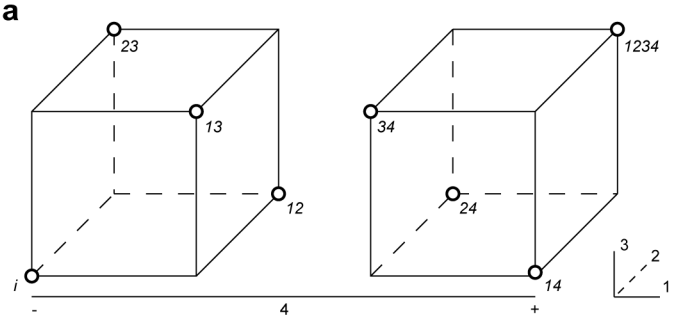
The design matrix of the 2_{IV}^{4-1} experiments, and the corresponding protease activity measurements and predictions by the underlying polynomial, are depicted in Figure 3.1.2. The data obtained from the 2_{IV}^{4-1} experiments were also subjected to analysis of variance (ANOVA). A half-normal probability plot of the estimates of the effects (Figure 3.1.3a) permitted selection of the significant terms only; a Fischer's *F*-test was used for this purpose (Gu et al. 2005). The ANOVA results indicated that the interactions between beef extract and yeast extract were significant (Figure 3.1.3b), whereas linear effects of beef extract and yeast extract were unexpectedly not significant; however, these terms could not be excluded from the model, because of the need to satisfy hierarchical conditions therein. The high overall *F*-value found (42.38) indicates that the postulated model provides a good fit – with a mere 0.17%-chance that such a large value occurs due to pure noise (Figure 3.1.3b).

Our experimental data were thus fitted to by the following two-factor interaction model equation:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j \qquad (3.1.1)$$

where Y is the predicted response, β_0 is the intercept term, β_i's are the linear effects, and β_{ij}'s are the interaction effects – with X_i and X_j being the processing variables.

a



b

Coded parameters	Alias	Description	Actual values corresponding to coded values	
			-1	+1
X_1	1	Beef extract (g/L)	0.5	1.5
X_2	2	Yeast extract (g/L)	2.5	7.5
X_3	3	NaCl (g/L)	1.0	3.0
X_4	123	Peptone (g/L)	1.0	3.0

c

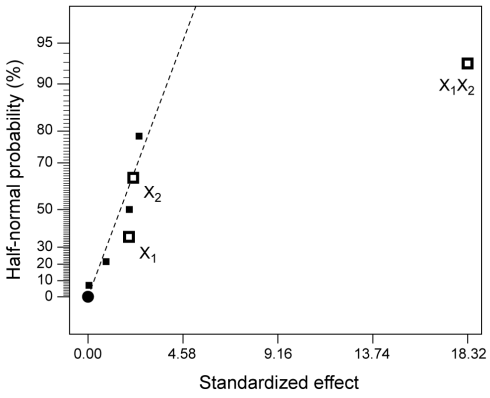
Exp.	Run order	Coded parameter				Alias	Protease activity (U/mL)	
		X_1	X_2	X_3	X_4		Observed	Predicted
1	4	- 1	- 1	- 1	- 1	i	20,50	20.27
2	3	+ 1	- 1	- 1	+ 1	14	41.27	40.57
3	8	- 1	+ 1	- 1	+ 1	24	43.42	40.78
4	1	+ 1	+ 1	- 1	- 1	12	22.62	24.43
5	6	- 1	- 1	+ 1	+ 1	34	20.05	20.27
6	5	+ 1	- 1	+ 1	- 1	13	39.88	40.57
7	2	- 1	+ 1	+ 1	- 1	23	38.13	40.78
8	7	+ 1	+ 1	+ 1	+ 1	1234	26.25	24.43

Figure 3.1.2 Results of trials following a 2_{IV}^{4-1} fractional factorial design aimed at elucidating the effects of key medium constituents upon protease activity: a) geometric representation of design, b) definition of processing parameters and testing range thereof, and c) design matrix with observed and predicted values

In order to determine the levels of each variable leading to maximum protease production, contour plots were constructed by representing the protease activity versus any two independent variables, while maintaining the

remaining variables at their optimum levels. As shown in Figure 3.1.4, an increase in protease activity was observed when the beef extract concentration increased and the yeast extract concentration decreased; a similar trend held when the concentration of beef extract decreased and the concentration of yeast extract increased – so these variables do indeed interact with each other.

a



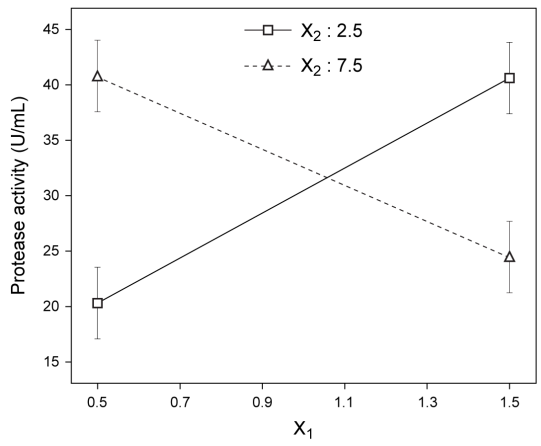
b

Source	SS	dF	MS	F	p
Model	688.76	3	229.59	42.38	0.0017
X_1	7.83	1	7.83	1.45	0.2955
X_2	9.52	1	9.52	1.76	0.2556
X_1X_2	671.41	1	671.41	123.93	0.0004
Residual	21.67	4	5.42		
Total	710.44	7			

SS, Sum of Squares; dF, degrees of Freedom; MS, Mean Square; F, F ratio; p, probability value.

Figure 3.1.3 Results of analysis of variance encompassing the parameters with lowest individual linear effects, but with the highest interaction effects upon protease activity: a) half-normal probability plot of effects (R^2=0.9695; adj R^2=0.9466; pred R^2=0.8780; adeq precision=12.458) and b) associated probabilities.

a



b

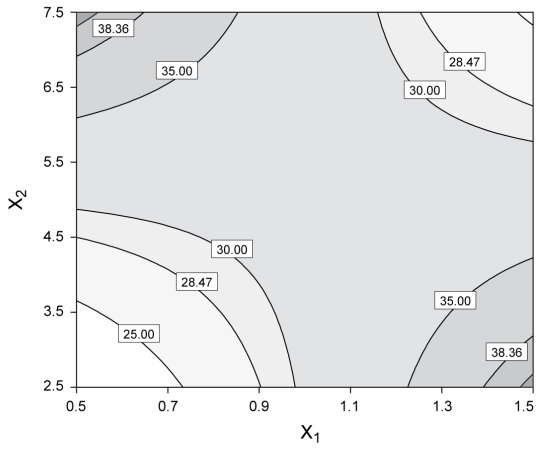


Figure 3.1.4 Best fit model plots: a) interaction plot and b) contour plot, associated with the significant X_1X_2 interaction.

3.1.4.4 Optimization via 2_{VI}^{6-1} fractional factorial design

The 2_{VI}^{6-1} fractional factorial experimental design was aimed at determining the ingredients that affected the most the rate of synthesis of protease. There were accordingly 6 factors – and each one was examined at a higher (+1) and a lower (-1) level; the design variables and their levels are provided in detail in Table 3.1.2. One half fraction of the full factorial design was adopted, which thus encompassed the 32 treatments shown in Figure 3.1.5.

The 2_{VI}^{6-1} fractional factorial design is characterized in Table 3.1.2, and the corresponding results are illustrated in Figure 3.1.5 and Figure 3.1.6. The protease activity measured varied widely from 7.40 and 54.64 U/mL, among the various combinations studied. From the results of the linear regression analysis based on the 2_{VI}^{6-1} fractional factorial design, one was led to the following simplified form:

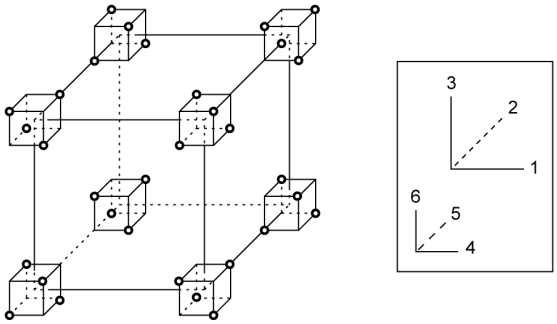
$$Y = -4.69 + 1.20X_1 - 0.36X_2 + 0.18X_5 + 0.80X_6 - 0.19X_1X_6 \qquad (3.1.2)$$

The associated regression and determination coefficients for the above model are given in Figure 3.1.6b – and unfold a high significance thereof, owing to the associated large *F*-value and R^2 value close to unity; furthermore, it held an "adequate precision" value of 22.038.

Using the whole information produced in this research effort, the maximum protease activity was reached under the following fermentation conditions: 10 g/L yeast extract, 2 g/L peptone, 3 %(v/v) inoculum, 100 rpm stirring rate, 40 ºC and pH 6. Furthermore, a less expensive fermentation medium could be attained by reducing the inoculum size to just 1 %(v/v) – and

less expensive operating conditions were feasible by eliminating stirring, as both of them would not significantly compromise our final goal.

a



b

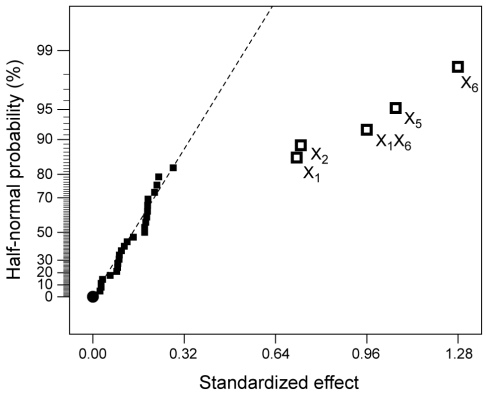
Exp.	Run order	Coded parameter						Alias	Protease activity (U/mL)	
		X_1	X_2	X_3	X_4	X_5	X_6		Observed	Predicted
1	10	- 1	- 1	- 1	- 1	- 1	- 1	i	29.80	31.81
2	26	+ 1	- 1	- 1	- 1	- 1	+ 1	16	15.51	13.32
3	12	- 1	+ 1	- 1	- 1	- 1	+ 1	26	18.26	21.07
4	8	+ 1	+ 1	- 1	- 1	- 1	- 1	12	27.22	26.63
5	2	- 1	- 1	+ 1	- 1	- 1	+ 1	36	25.84	28.30
6	1	+ 1	- 1	+ 1	- 1	- 1	- 1	13	37.21	34.69
7	9	- 1	+ 1	+ 1	- 1	- 1	- 1	23	22.76	24.11
8	23	+ 1	+ 1	+ 1	- 1	- 1	+ 1	1236	8.02	8.53
9	17	- 1	- 1	- 1	+ 1	- 1	+ 1	46	26.03	28.30
10	20	+ 1	- 1	- 1	+ 1	- 1	- 1	14	37.22	34.69
11	27	- 1	+ 1	- 1	+ 1	- 1	- 1	24	26.68	24.11
12	19	+ 1	+ 1	- 1	+ 1	- 1	+ 1	1246	7.40	8.53
13	3	- 1	- 1	+ 1	+ 1	- 1	- 1	34	36.94	31.81
14	14	+ 1	- 1	+ 1	+ 1	- 1	+ 1	1346	16.20	13.32
15	18	- 1	+ 1	+ 1	+ 1	- 1	+ 1	2346	18.12	21.07
16	16	+ 1	+ 1	+ 1	+ 1	- 1	- 1	1234	25.26	26.63
17	21	- 1	- 1	- 1	- 1	+ 1	+ 1	56	32.63	40.70
18	24	+ 1	- 1	- 1	- 1	+ 1	- 1	15	47.30	48.30
19	32	- 1	+ 1	- 1	- 1	+ 1	- 1	25	37.34	35.64
20	6	+ 1	+ 1	- 1	- 1	+ 1	+ 1	1256	12.29	15.84
21	31	- 1	- 1	+ 1	- 1	+ 1	- 1	35	37.67	44.89
22	7	+ 1	- 1	+ 1	- 1	+ 1	+ 1	1356	18.98	22.18
23	30	- 1	+ 1	+ 1	- 1	+ 1	+ 1	2356	36.26	31.92
24	13	+ 1	+ 1	+ 1	- 1	+ 1	- 1	1235	35.84	38.69
25	15	- 1	- 1	- 1	+ 1	+ 1	- 1	45	39.97	44.89
26	25	+ 1	- 1	- 1	+ 1	+ 1	+ 1	1456	20.33	22.18
27	11	- 1	+ 1	- 1	+ 1	+ 1	+ 1	2456	38.14	31.92
28	22	+ 1	+ 1	- 1	+ 1	+ 1	- 1	1245	32.71	38.69
29	28	- 1	- 1	+ 1	+ 1	+ 1	+ 1	3456	52.88	40.70
30	4	+ 1	- 1	+ 1	+ 1	+ 1	- 1	1345	54.64	48.30
31	29	- 1	+ 1	+ 1	+ 1	+ 1	- 1	2345	41.09	35.64
32	5	+ 1	+ 1	+ 1	+ 1	+ 1	+ 1	123456	21.32	15.84

Figure 3.1.5 Results of trials following a 2_{VI}^{6-1} design, aimed at elucidating the processing parameters considered upon protease activity: a) geometric representation of design; and b) design matrix with observed and associated predicted values.

3.1.5. Discussion

The first stage in the development of microbial-based industrial processes is to isolate a strain able to produce a target metabolite (or a related one) to sufficiently high yields (Kumar & Takagi 1999). This approach entails intensive screening, and thus testing of a large number of strains in attempts to pinpoint fast producers, or even alternative useful metabolites. The conventional practice pertaining to extracellular microbial products is to bring about growth on agar plate media, and then assess the microbial capacity of synthesis from the radius of the product zone diffusing away around the source colony; this strategy was considered in the previous chapter, when attempting to isolate protease-producing bacteria intended for novel biotechnological modifications of wool in the textile industry.

a



b

Source	SS	dF	MS	F	p
Model	37.81	5	7.56	42.41	< 0.0001
X_1	4.08	1	4.08	22.88	< 0.0001
X_2	4.25	1	4.25	23.84	< 0.0001
X_5	9.01	1	9.01	50.54	< 0.0001
X_6	13.10	1	13.10	73.45	< 0.0001
X_1X_6	7.37	1	7.37	41.34	< 0.0001
Residual	4.64	26	0.18		
Total	42.44	31			

SS, Sum of Squares; dF, degrees of Freedom; MS, Mean Square; F, F ratio; p, probability value.

Figure 3.1.6 Results of variance encompassing the parameters with highest individual linear effects upon protease activity: a) half-normal probability plot of effects (R^2=0.8908; adj R^2=0.8698; pred R^2=0.8345; adeq precision=22.038) and b) associated probabilities.

Due to the ever-increasing economic relevance of alkaline proteases, optimization of a large number of fermentation parameters was accordingly sought – including medium composition and culture conditions, using extracellular protease production by *Bacillus* sp. HTS102 as objective function.

After having isolated one (or more) strain(s) with promising features, one should then proceed to optimization of metabolite synthesis and secretion, in order to make its industrial use as feasible as possible. Recall that the optimization protocol followed here encompassed two sequential approaches: first, an "one-factor-at-a-time" set of experiments was performed using a total of 24 factors – so as to reduce such a long list to a much smaller and more tractable list of key factors toward our goal; and second, a 2_{VI}^{6-1} fractional factorial design was carried out, to test the effects of only the 6 key factors previously identified.

After the aforementioned comprehensive preliminary screening following a "one-factor-at-a-time" approach, six variables were further tested using a 2_{VI}^{6-1} fractional factorial design; yeast extract, peptone, temperature and pH were eventually found to affect protease production to a significant degree. This experimental approach proved adequate in statistical terms – and a significant improvement (viz. 2.2-fold) could successfully be accomplished in the level of production of said novel protease. More detailed discussion of the procedure followed and of the results generated is provided below.

3.1.5.1 Significant physicochemical parameters

None of the tested several carbon sources yielded as good a protease productivity as the NB medium formulation – thus indicating that presence of the original constituents in the medium instead of beef extract either inhibit protease production, or at least fail to promote it. Note that protease synthesis was reduced by ca. 93% in the presence of glucose, probably due to catabolite repression (Chi et al. 2007) – even though other authors have reported otherwise (Son et al. 2008).

Since the results encompassing protease production using yeast extract and peptone together suggested that they may act synergistically, both nutrients were selected for the subsequent optimization step. On the other hand, pH 7 seemed to be optimum, but it became apparent that pH is a significant factor upon enzyme production; hence, it was also considered for the 2_{VI}^{6-1} fractional factorial design afterwards.

It is known that stirring promotes oxygen solubility, disruption of air bubbles putatively formed and dispersion of cell clusters (Joo & Chang 2006) – which may favorably contribute to protease synthesis by facilitating access of cells to the nutrients in the medium, while avoiding inhibitory product concentration build-up. However, high stirring rates are also known to cause cell disruption – which poses a difficulty that might overcome the abovementioned advantages of stirring. The similar results obtained for protease production under high stirring rates and no stirring at all may instead be due to little oxygen availability and difficulty to access nutrients. Since agitation is an important factor when considering economically viable industrial processes, stirring rate was picked as a factor to proceed with.

While 37 ºC appeared to be optimum, it is clear that temperature is a significant factor upon protease production; hence, it was considered for the 2_{VI}^{6-1} fractional factorial design.

Inoculum density seemed to have some impact upon the enzyme production level, and it is surely an important parameter to be considered when economically viable industrial processes are envisaged; hence, this factor was also included in subsequent optimization studies.

After the "one-factor-at-a-time" studies on the effect of individual NB constituents, it was rather difficult to draw a conclusion on which nutrients should be selected for the next step of optimization. Therefore, a further assessment of their interaction when used together was carried out, via a fractional factorial design with four independent factors only. The decision on the concentration ranges (i.e. the lower, -1, and the upper, +1, levels) to be considered in this experiment was based on the results previously obtained in the screening experiments with basal medium (i.e. NB) as central point; in view of our purpose here, each was performed only once.

The response to beef extract (X_1) and yeast extract (X_2) levels, in terms of protease production (Y) by *Bacillus* sp. HTS 102, was according to:

$$Y = -9.29 + 38.62 X_1 + 7.76 X_2 - 7.33 X_1 X_2 \qquad (3.1.3)$$

This regression equation holds a good fit, as its multiple correlation coefficient (R^2) is 0.9695; recall that a value above 0.75 indicates an acceptable fit of the model to the experimental data (Reddy et al. 2008b). This coefficient is in fact an estimate of the fraction of the overall variation in the data that can be accounted for by the model, so our model was able to statistically explain 96.95% of the variation in the responses. The value of 0.9466 for the "adjusted R^2" further confirms significance of the above model, which is in reasonable agreement with the "predicted R^2" (i.e. 0.8780). The "adequate precision value" of our model is 12.46, thus suggesting that it can safely be used to "navigate" through the design space (Reddy et al. 2008b). The negative coefficient associated with the two-factor interaction term (X_1X_2) is also consistent with the results displayed in Figure 3.1.4.

Since there was no statistically significant difference between effects at the upper limit (+1) of any of the two variables, beef extract was excluded from the next step of optimization for economic reasons – as it is the more expensive nutrient. Consequently, further optimization studies resorted only to a single concentration of beef extract (0.5 g/L). Furthermore, the effects of NaCl and peptone upon protease activity were found below the *t*-value threshold, but the latter concentration was anyway selected as a factor for the next optimization step – since it appeared as important to further investigate its role upon enzyme production. On the other hand, NaCl was fixed at its lowest level (i.e. 1.0 g/L) since high levels can induce osmotic unbalance in bacteria, and thus raise the costs incurred in with medium formulation.

In view of the considerations above, the combined effects of yeast extract, peptone, inoculum, stirring, temperature and pH were picked up as key determinants for protease production, and were thus included in the next fractional factorial design.

3.1.5.2 Optimization via 2_{VI}^{6-1} fractional factorial design

Following normalization of the data by a square root transformation, the results of ANOVA shown in Figure 3.1.6 allowed four major conclusions to be drawn: i) variations in the levels of inoculum or stirring rate did not significantly affect protease production, so they were removed from the final ANOVA model; ii) protease production was significantly affected by yeast extract, peptone, temperature and pH, at a significance level of 0.1%; iii) the effect of temperature was positive, thus implying that its increase will promote protease production – whereas the effect of the linear model term for peptone was negative, so

protease production will be enhanced by lower levels thereof; and iv) interaction between yeast extract and pH produced a (negative) effect upon protease production that was stronger than both their corresponding linear effects (Figure 3.1.7a) – which is consistent with the claim (Liu et al. 2005) that extracellular secretion of proteases in *Bacillus* spp. is a manifestation of nitrogen limitation prevailing at the onset of the stationary phase.

Recall that an interaction between factors occurs when the overall response is different from their independent combination. Interaction plots, as is the case of Figure 3.1.7a, make it easy to interpret two-factor interactions, as they exhibit non-parallel lines whenever the effect of one factor depends on the level of the other; hence, the interaction between yeast extract and pH proved significant. The contour plot represented in Figure 3.1.7b is a two-dimensional representation of the response across the selected factors; it indicates that the surface is not symmetrical, and that no peak can be perceived – even though room exists for further attempts to fine tune the optimal loci associated with protease production.

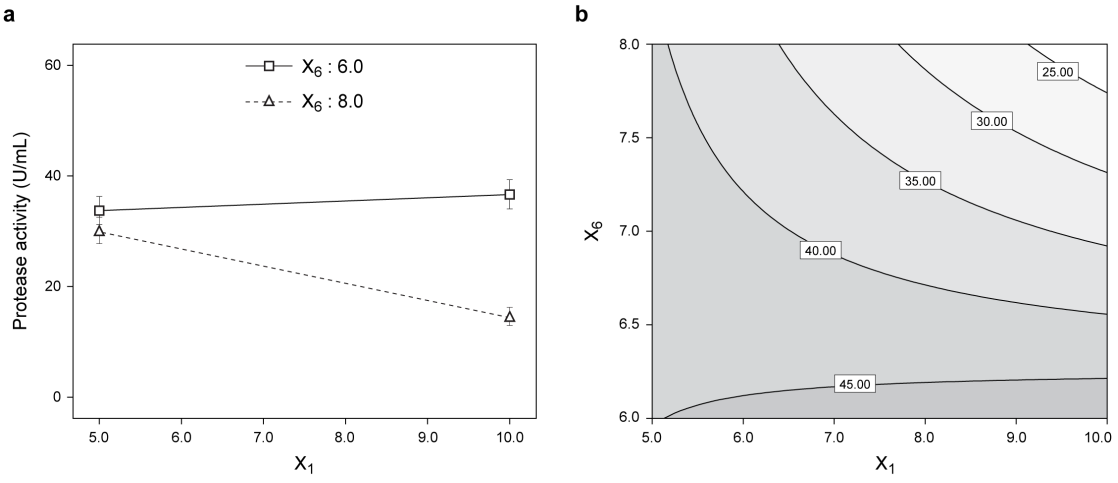


Figure 3.1.7 Best fit model plots: a) interaction plot and b) contour plot, associated with the significant X_1X_6 interaction.

Acknowledgments

A.C. Queiroga acknowledges a PhD fellowship (ref.: SFRH/BD/19121/2004), granted by Fundação para a Ciência e a Tecnologia (Portugal) and supervised by F.X. Malcata.

3.1.6. References

Chi Z, Ma C, Wang P, Li HF. 2007. Optimization of medium and cultivation conditions for alkaline protease production by the marine yeast *Aureobasidium pullulans*. Bioresource Technol 98:534-538.

Gu XB, Zheng ZM, Yu HQ, Wang J, Liang FL, Liu RL. 2005. Optimization of medium constituents for a novel lipopeptide production by *Bacillus subtilis* MO-01 by a response surface method. Process Biochem 40:3196-3201.

Gupta R, Beg Q, Lorenz P. 2002b. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 59:15-32.

Huang GR, Dai DH, Hu WL, Jiang JX. 2008. Optimization of medium composition for thermostable protease production by *Bacillus* sp. HS08 with a statistical method. Afr J Biotechnol 7:1115-1122.

Ibrahim ASS, al-Salamah AA. 2009. Optimization of media and cultivation conditions for alkaline protease production by alkaliphilic *Bacillus halodurans*. Res J Microbiol 4:251-259.

Joo H-S, Chang C-S. 2005. Production of protease from a new alkalophilic *Bacillus* sp. I-312 grown on soybean meal: optimization and some properties. Process Biochem 40:1263-1270.

Joo H-S, Chang C-S. 2006. Production of an oxidant and SDS-stable alkaline protease from an alkalophilic *Bacillus clausii* I-52 by submerged fermentation: feasibility as a laundry detergent additive. Enzyme Microb Technol 38:176-183.

Kumar CG, Takagi H. 1999. Microbial alkaline proteases: from a bioindustrial viewpoint. Biotechnol Adv 17:561-594.

Liu J, Xing J, Chang T, Ma Z, Liu H. 2005. Optimization of nutritional conditions for nattokinase production by *Bacillus natto* NLSSE using statistical experimental methods. Process Biochem 40:2757-2762.

Myers RH, Montgomery DC. 2002. Response Surface Methodology: Process and Product Optimization Using Designed Experiments. New York, NY: Wiley Interscience.

Navaneeth S, Bhuvanesh S, Bhaskar V, Kumar PV, Kandaswamy SKJ, Achary A. 2009. Optimization of medium for the production of subtilisin from *Bacillus subtilis* MTCC 441. Afr J Biotechnol 8:6327-6331.

Puri S, Beg QK, Gupta R. 2002. Optimization of alkaline protease production from *Bacillus* sp. by response surface methodology. Curr Microbiol 44:286-290.

Rao YK, Tsay KJ, Wu WS, Tzeng YM. 2007. Medium optimization of carbon and nitrogen sources for the production of spores from *Bacillus amyloliquefaciens* B128 using response surface methodology. Process Biochem 42:535-541.

Reddy LVA, Wee YJ, Yun JS, Ryu HW. 2008b. Optimization of alkaline protease production by batch culture of *Bacillus* sp. RKY3 through Plackett-Burman and response surface methodological approaches. Bioresource Technol 99:2242-2249.

Shafee N, Aris SN, Rahman RNZA, Basri M, Salleh AB. 2005. Optimization of environmental and nutritional conditions for the production of

alkaline protease by a newly isolated bacterium *Bacillus cereus* strain 146. J Appl Sci Res 1:1-8.

Son H-J, Park H-C, Kim H-S, Lee C-Y. 2008. Nutritional regulation of keratinolytic activity in *Bacillus pumilis*. Biotechnol Lett 30:461-465.

Section 3.2

Optimization of protease synthesis via central composite design and response surface methodology

3.2.1. Abstract

The influence of yeast extract, peptone, temperature and pH upon protease productivity by *Bacillus* sp. HTS102 – a novel wild strain isolated from wool of a Portuguese sheep breed (Merino), was investigated. A 2^4 full factorial, central composite design, together with a response surface methodology were used to carry out the experiments and aid in analysis of the results. Among the individual parameters tested, temperature and peptone produced significant effects upon the response. A high correlation coefficient (R^2=0.994, P<0.01) indicated that the empiric second-order polynomial model postulated was adequate to predict the protease productivity, and led to the following loci for the optima: temperature of 43 ºC, peptone content of 1.4 g/L, pH of 5.1 and yeast

extract concentration of 10.0 g/L. The maximum protease activity was 56.8 U/mL, which was more than twice that obtained with the basal medium.

3.2.2. Introduction

Proteolytic enzymes have been routinely used in the detergent industry; this large-scale application has stimulated their commercial development, thus promoting fundamental and applied research focused thereon (Reddy et al. 2008b). However, proteolytic enzymes have also found numerous applications in other industrial sectors, e.g. food and feed, leather, textile, pharmaceutical, fine chemistry, and effluent and waste treatment (Gupta et al. 2002b).

Proteases are ubiquitous in nature; however, proteases from microbial sources possess advantages over alternative sources because microorganisms can be cultured to high densities and exhibit short doubling times – besides the methods of fermentation being well-established; this has led to abundant and regular supply of said enzymes (Seong et al. 2004). From a production standpoint, microbial extracellular proteases are even more convenient owing to the ease of extraction and purification relative to their intracellular counterparts: large productivities have in particular been observed with several species of the *Bacillus* genus (Navaneeth et al. 2009). As a consequence, there has been a renewed interest on wild microorganisms that can synthesize and excrete proteases (Puri et al. 2002; Seong et al. 2004; Wang et al. 2008).

Hence, a growing array of proteases available commercially has prompted an expansion also of the portfolio of uses; at present, comprehensive screening for new strains of bacteria has aimed at production of proteases with better

properties, and more appropriate for industrial processes that feature lower costs and higher throughputs (Huang et al. 2008). Such efforts are necessarily complemented with optimization of processing conditions: significant differences in protease synthesis rates observed when a given microorganism is subjected to distinct culturing conditions indicate that usually a few essential nutrients are to be supplied, further to narrow ranges of processing conditions (Wilson 1930). On the other hand, the overall costs of enzyme production arising from downstream purification have remained a major obstacle to successful implementation of white biotechnology (Gupta et al. 2002b), so bioprocess optimization entailing extracellular enzymes is of central importance toward economically feasible processes (Reddy et al. 2008b).

In view of the above, many biochemists and process engineers have focused on how to improve protease yield. Classical approaches involving change of "one-variable-at-a-time" are extremely time-consuming, and thus expensive when many variables are considered altogether (Rao et al. 2007); furthermore, they are intrinsically unable to pinpoint interactions among processing parameters. Hence, more powerful statistical experimental designs have been proposed (Puri et al. 2002); one successful example is response surface methodology (RSM) (Myers & Montgomery 2002) – which allows model building that can accurately approximate the true response function within a region around the optima, using processing parameters as independent factors (Puri et al. 2002). Several studies are accordingly available of application of RSM to optimize fermentation medium composition and physical processing factors, namely toward maximizing protease productivity (Bhaskar et al. 2008; Oskouie et al. 2008; Reddy et al. 2008b; Wang et al. 2008; Cai & Zheng 2009).

The research effort described in this chapter was aimed at optimizing the protease production by the wool-associated *Bacillus* sp. strain HTS 102 – via a 2^4 full factorial, central composite design using the effect of yeast extract, peptone, temperature and pH as regressors. Said novel strain was the outcome of a comprehensive screening among 158 isolates from Portuguese Merino sheep.

3.2.3. Materials and methods

3.2.3.1 Curvature of starting polynomial model

Work described in previous chapters, using a 2_{VI}^{6-1} fractional factorial design unfolded two-factor interaction among factors – i.e. the existence of model curvature. Therefore, the original experimental design was expanded so as to also encompass 3 center points; the values of each factor at said center point were considered as the average setting of the design – and are depicted in Table 3.2.1.

3.2.3.2 Optimization via full factorial central composite design

Only 4 out of 24 parameters tentatively screened were previously proven to exhibit a significant effect upon protease productivity by the *Bacillus* sp. strain HTS102: yeast extract level, peptone content, temperature and pH. Hence, these were selected for optimization here via a 2^4 full factorial, central composite composite design: the levels of each factor used for the center points corresponded to those leading to the best response in the preliminary 2_{VI}^{6-1} fractional factorial design – and are depicted in Table 3.2.2. Said type of design

with 4 factors encompasses 30 runs (with 8 star points and 6 center points); the associated details are tabulated in Table 3.2.3.

Table 3.2.1 Expansion of 2_{IV}^{6-1} fractional factorial design with 3 center points, to check for process stability and possible curvature: definition of processing parameters (in original and coded format) and range tested.

Coded parameters	Description	Actual values corresponding to coded values		
		-1	0	+1
X_1	Yeast Extract (g/L)	5	7.5	10
X_2	Peptone (g/L)	2	3	4
X_3	Inoculum Level (%, v/v)	1	2	3
X_4	Agitation (rpm)	0	50	100
X_5	Temperature (ºC)	34	37	40
X_6	pH	6	7	8

Table 3.2.2 2^4 full factorial central composite design, to enhance protease productivity: definition of processing parameters (in original and coded format).

Coded parameters	Description	Actual values corresponding to coded values				
		-2	-1	0	+1	+2
X_1	Temperature (ºC)	30	35	40	45	50
X_2	Peptone (g/L)	1.0	1.5	2.0	2.5	3.0
X_3	pH	4	5	6	7	8
X_4	Yeast Extract (g/L)	8	9	10	11	12

3.2.3.3 Microorganism source and fermentation conditions

The strain employed was *Bacillus* sp. HTS102 (GenBank accession number HQ698269; reference LMG 26323, from BCCM/LMG Bacterial Culture Collection, Ghent, Belgium), a wool-associated microorganism with strong proteolytic features. The basal fermentation medium contained 0.5 g/L meat extract (Merck, Germany) and 1.0 g/L NaCl. Erlenmeyer flasks (250 ml) containing 25 ml of growth medium were inoculated with 1 %(v/v) fresh

inoculum (corresponding to an optical density of 0.6 at 600 nm). The extracellular protease activity was quantitated after a 36 h-incubation period without stirring.

3.2.3.4 Protease activity assay

The cell-free supernatants (sterilized by filtration through a 0.45 µm filter) were assayed for protease activity using colorimetry (660 nm), based on the extent of casein breakdown as assessed by Folin-Ciocalteu's reagent (Sigma-Aldrich). One unit (U) of proteolytic activity was defined as the amount of enzyme able to hydrolyze casein at pH 7.5 at 37 ºC, so as to produce an absorbance variation per min equal to that produced by 1.0 µmol of tyrosine.

3.2.3.5 Protein content assay

Throughout the 36 h-incubation period, aliquots were withdrawn for total protein assay (measured by absorbance at 562 nm), using the BCA™ Protein Assay Kit (Pierce, USA).

3.2.3.6 Statistical analyses

The statistical software package Design-Expert®, v. 8.0.3 (Stat-Ease, Minneapolis MN, USA) was used to set up and analyze the central composite design at stake. The statistical significance of the underlying model equation and corresponding terms was assessed via Fischer's *F*-tests; and its quality-of-fit was ascertained by the coefficient of determination (R^2) and an adjusted R^2. The fitted polynomial equation was represented as response surface plots, for the

two-factor interactions – to better illustrate their underlying relationships and effects upon the response.

Table 3.2.3 2^4 central composite design, aimed at optimizing protease productivity: design matrix (in coded format) and results of trials (observed and predicted).

Exp.	Run Order	Coded parameter				Protease activity (U/mL)	
		X_1	X_2	X_3	X_4	Observed	Predicted
1	15	-1	-1	-1	-1	21.98	22.56
2	10	1	-1	-1	-1	51.20	52.42
3	2	-1	1	-1	-1	16.69	17.56
4	17	1	1	-1	-1	29.21	28.20
5	3	-1	-1	1	-1	20.31	20.25
6	13	1	-1	1	-1	51.75	53.44
7	16	-1	1	1	-1	21.98	20.88
8	7	1	1	1	-1	36.45	36.24
9	5	-1	-1	-1	1	26.71	27.77
10	20	1	-1	-1	1	50.64	50.69
11	14	-1	1	-1	1	21.15	19.18
12	8	1	1	-1	1	22.82	23.72
13	1	-1	-1	1	1	23.65	23.52
14	4	1	-1	1	1	49.53	49.28
15	6	-1	1	1	1	21.15	21.16
16	19	1	1	1	1	31.16	29.27
17	9	0	0	0	0	3.34	3.46
18	1	0	0	0	0	26.71	26.63
19	12	0	0	0	0	44.52	42.12
20	18	0	0	0	0	16.97	18.66
21	25	-2	0	0	0	45.91	44.76
22	30	2	0	0	0	47.30	48.72
23	26	0	-2	0	0	38.67	37.82
24	23	0	2	0	0	35.61	36.72
25	21	0	0	-2	0	47.86	48.72
26	27	0	0	2	0	50.08	48.72
27	24	0	0	0	-2	49.53	48.72
28	28	0	0	0	2	47.30	48.72
29	29	0	0	0	0	48.41	48.72
30	22	0	0	0	0	48.97	48.72

3.2.4. Results and discussion

3.2.4.1 Parameter interaction and curvature

The design matrix, with observed and predicted values, that underlined the trials following the 2_{IV}^{6-1} fractional factorial design, expanded with 3 center point runs, is depicted in Table 3.2.4a. The *F*-value of 45.66 – associated with an R^2 value very close to unit and an adequate precision of 22.142 (see Table 3.2.4b), imply that there is no statistically significant reason to doubt the functional form of the model.

Based on examination of the results of the analysis of variance represented in Table 3.2.4b, one concluded that the "lack of fit" is significant – thus indicating the relevance of curvature; hence, the first-model equation (represented in terms of coded factors), given by

$$Y = 5.16 - 0.36X_1 - 0.36X_2 + 0.53X_5 - 0.64X_6 - 0.48X_1X_6 \qquad (3.2.1)$$

where X_1, X_2, X_5 and X_6 denote yeast extract level, peptone level, temperature and pH, respectively, was inadequate (Myers & Montgomery 2002). Addition of center point runs allowed the curvature to be tested, but did not permit estimation of the individual quadratic effects of each factor (Tamhane 2009). When neither the lack of fit or the curvature is significant, the optimization process should follow the path of steepest ascent – i.e. along the linear direction of response enhancement, until no further increase in response is detected, or when significant curvature (or lack of fit) is found . Since the lack of fit tested after adding center point runs proved significant, one had to expand the design with axial points – so that the response would be fitted to a second order model,

via estimation of the individual quadratic effects of the factors under a RSM approach.

Table 3.2.4 2_{IV}^{6-1} Fractional factorial design, to check for process stability and possible curvature: a) design matrix (in coded format) and results of trials (observed and predicted); and b) results of analysis of variance encompassing the parameters with highest individual linear effects, upon protease productivity (R^2=0.891; adj R^2=0.871; pred R^2=0.834; adeq precision=22.142).

a

Exp.	Run Order	Coded parameter						Protease activity (U/mL)	
		X_1	X_2	X_3	X_4	X_5	X_6	Observed	Predicted
1	10	-1	-1	-1	-1	-1	-1	29.81	31.81
2	26	1	-1	-1	-1	-1	1	15.52	13.32
3	12	-1	1	-1	-1	-1	1	18.23	21.07
4	8	1	1	-1	-1	-1	-1	27.25	26.63
5	2	-1	-1	1	-1	-1	1	25.81	28.30
6	1	1	-1	1	-1	-1	-1	37.21	34.69
7	9	-1	1	1	-1	-1	-1	22.75	24.11
8	23	1	1	1	-1	-1	1	8.01	8.53
9	17	-1	-1	-1	1	-1	1	26.01	28.30
10	20	1	-1	-1	1	-1	-1	37.21	34.69
11	27	-1	1	-1	1	-1	-1	26.73	24.11
12	19	1	1	-1	1	-1	1	7.40	8.53
13	3	-1	-1	1	1	-1	-1	36.97	31.81
14	14	1	-1	1	1	-1	1	16.24	13.32
15	18	-1	1	1	1	-1	1	18.15	21.07
16	16	1	1	1	1	-1	-1	25.30	26.63
17	21	-1	-1	-1	-1	1	1	32.60	40.70
18	24	1	-1	-1	-1	1	-1	47.33	48.30
19	32	-1	1	-1	-1	1	-1	37.33	35.64
20	6	1	1	-1	-1	1	1	12.32	15.84
21	31	-1	-1	1	-1	1	-1	37.70	44.89
22	7	1	-1	1	-1	1	1	19.01	22.18
23	30	-1	1	1	-1	1	1	36.24	31.92
24	13	1	1	1	-1	1	-1	35.88	38.69
25	15	-1	-1	-1	1	1	-1	39.94	44.89
26	25	1	-1	-1	1	1	1	20.34	22.18
27	11	-1	1	-1	1	1	1	38.19	31.92
28	22	1	1	-1	1	1	-1	32.72	38.69
29	28	-1	-1	1	1	1	1	52.85	40.70
30	4	1	-1	1	1	1	-1	54.61	48.30
31	29	-1	1	1	1	1	-1	41.09	35.64
32	5	1	1	1	1	1	1	21.34	15.84
33	33	0	0	0	0	0	0	25.10	25.30
34	35	0	0	0	0	0	0	25.50	25.30
35	34	0	0	0	0	0	0	25.30	25.30

b

Source	SS	df	MS	F	p	
Model	37.81	5	7.56	45.66	< 0.0001	*
X_1	4.08	1	4.08	24.64	< 0.0001	*
X_2	4.25	1	4.25	25.67	< 0.0001	*
X_5	9.01	1	9.01	54.42	< 0.0001	*
X_6	13.10	1	13.10	79.09	< 0.0001	*
X_1X_6	7.37	1	7.37	44.51	< 0.0001	*
Residual	4.64	28	0.17			
Lack of Fit	4.64	26	0.18	502.79	0.0020	*
Pure Error	7.093×10^{-4}	2	3.546×10^{-4}			
Cor Total	42.63	34				

SS, Sum of Squares; dF, degrees of Freedom; MS, Mean Square; F, F ratio; p, probability value

3.2.4.2 Response surface analysis

Recall that addition of axial points to a fractional (or full) factorial design containing center runs gives rise to a central composite design. For such a design with 6 factors (i.e. yeast extract level, peptone level, inoculum level, stirring rate, temperature and pH), the value of α for the axial points would be ± 2.378 – thus implying that their actual values would lie beyond the limits specified for the factor settings. On the other hand, the factors stirring rate and inoculum level were found not significant for protease productivity, so they were dropped out as variables; they were accordingly fixed at their lowest levels for economic considerations, i.e. further optimization experiments used an inoculum level of 1 %(v/v) and incubation without agitation.

In view of the considerations above, a 2^4 full factorial, central composite design was attempted – using the levels of yeast extract and peptone as nutritional factors, and temperature and pH as processing factors – as depicted in Table 3.2.3. This design contains an imbedded factorial design with center points, expanded with a group of axial (star) points; a total of 30 runs was thus

established – encompassing several combinations of temperature (X_1), peptone level (X_2), pH (X_3) and yeast extract level (X_4).

The optimal levels of the factors, and assessment of how interactions among them affect protease productivity, were determined based on the results of the experiments run according to the aforementioned central composite design. The values for protease activity varied widely – from 3.34 to 51.75 U/mL, among the various combinations under scrutiny (see Table 3.2.3). Following normalization of the data by a square root transformation, these results were analyzed by regular analysis of variance; the outcome of this analysis is depicted in Table 3.2.5. The production of protease was significantly affected by temperature (X_1) and peptone (X_2), with $P<0.001$; and also by pH (X_3), with $P<0.05$.

Therefore, a second-order polynomial function was thus fitted to the experimental results, viz.

$$\begin{aligned} Y = & -104.23 + 3.43X_1 + 10.34X_2 - 0.37X_3 + 6.19X_4 - 0.14X_1X_2 \\ & + 0.02X_1X_3 - 0.03X_1X_4 + 0.32X_2X_3 - 0.16X_2X_4 - 0.04X_3X_4 \\ & - 0.03X_1^2 - 1.57X_2^2 - 0.04X_3^2 - 0.22X_4^2 \end{aligned} \qquad (3.2.2)$$

Inspection of the magnitude of the parameters in Eq. (3.2.2), one concludes that peptone content produces the maximum linear positive effect ($P<0.01$) upon protease productivity – followed by the linear positive effect of yeast extract. The positive quadratic effects of temperature, peptone and yeast extract were all significant ($P<0.01$). Among the two-factor interactions, the negative effect of the temperature x peptone interaction is to be outlined ($P<0.01$); this means that the extent of the effect of temperature upon protease production depends also on the actual level of peptone used. The linear effect of yeast extract was negligible,

yet its quadratic effect was significant (P<0.01) – so a low level of yeast extract produces a marginal effect, which increases fast when higher levels are provided.

The regression and determination coefficients associated with said model are given in Table 3.2.5; they unfold a high significance, as derived from the large F-value and the R^2 value very close to unit; and the "adequate precision" as high as 56.06. The lack-of-fit test in Table 3.2.5 (P=0.053) indicates that the quadratic model postulated appears adequate.

Table 3.2.5 2^4 Central composite design to optimize protease productivity: results of analysis of variance (R^2=0.994; adj R^2=0.989; pred R^2=0.970; adeq precision=56.056).

Source	SS	dF	MS	F	p
Model	49.71	14	3.55	188.10	< 0.0001 *
X_1	16.31	1	16.31	864.30	< 0.0001 *
X_2	7.11	1	7.11	376.64	< 0.0001 *
X_3	0.12	1	0.12	6.51	0.0221
X_4	0.011	1	0.011	0.60	0.4520
X_1X_2	1.85	1	1.85	98.24	< 0.0001 *
X_1X_3	0.10	1	0.10	5.44	0.0341
X_1X_4	0.40	1	0.40	21.34	0.0003
X_2X_3	0.41	1	0.41	21.83	0.0003
X_2X_4	0.11	1	0.11	5.59	0.0319
X_3X_4	0.028	1	0.028	1.49	0.2411
X_1^2	20.63	1	20.63	1093.13	< 0.0001 *
X_2^2	4.23	1	4.23	224.24	< 0.0001 *
X_3^2	0.034	1	0.034	1.80	0.2001
X_4^2	1.31	1	1.31	69.42	< 0.0001 *
Residual	0.28	15	0.019		
Lack of Fit	0.26	10	0.026	4.59	0.0532
Pure Error	0.028	5	$5.562X10^{-3}$		
Cor Total	49.99	29			

SS, Sum of Squares; dF, degrees of Freedom; MS, Mean Square; F, F ratio; p, probability value

The geometric nature of the second-order model is displayed in Figure 3.2.1 – which unfolds the effect of every set of two variables upon protease productivity, while the remaining variables are held at their zero level (center point). These response surface plots make it easy to analyze the response surface curves of two-factor interactions – and to predict the response, or estimate the

mean response at a particular point in the process variable space (Myers & Montgomery 2002). Detection of the nature and location of any stationary point is also an important issue of such a second-order analysis (Myers & Montgomery 2002), which obviously depends on the signs and magnitudes of the coefficients in Eq. (3.2.2).

In Figures 3.2.1a, 3.2.1c and 3.2.1f, the center of the system (stationary point) corresponds to a point of maximum response. In Figures 3.2.1b, 3.2.1d and 3.2.1e, the type of surface defined by the second-order model is a "rising ridge", thus suggesting that extrapolation beyond the experimental region will, for precaution, require additional experimentation. In any case, several directions will lead to improvement, so there is no single (correct) direction; however, our fitted model may aid in identifying treatment combinations that would likely lead to better outcomes. The optimum levels of the four variables under study were estimated using the Solver function of Microsoft Excel; they corresponded to be a temperature of 43 ºC, a peptone level of 1.4 g/L, a pH of 5.1, and a yeast extract content of 10.0 g/L – with a predicted maximum activity of 56.76 U/mL, which is more than twice that obtained under the basal conditions. This realization emphasizes the need and usefulness of the optimization strategy developed.

Despite the bacterial strain selected for this study having been isolated from a moderately alkaline environment (pH 9.5), the optimum levels of its protease productivity were found at an acidic pH (pH 5.1). It is not at all uncommon that optimum processing conditions for growth differ from optimum processing conditions for specific metabolite production by a given microorganism; e.g. *Bacillus polymyxa* produces enzymes optimally at pH 5 (Castro et al. 1992),

whereas it is typically cultivated for growth purposes at pH values in the vicinity of 7.0.

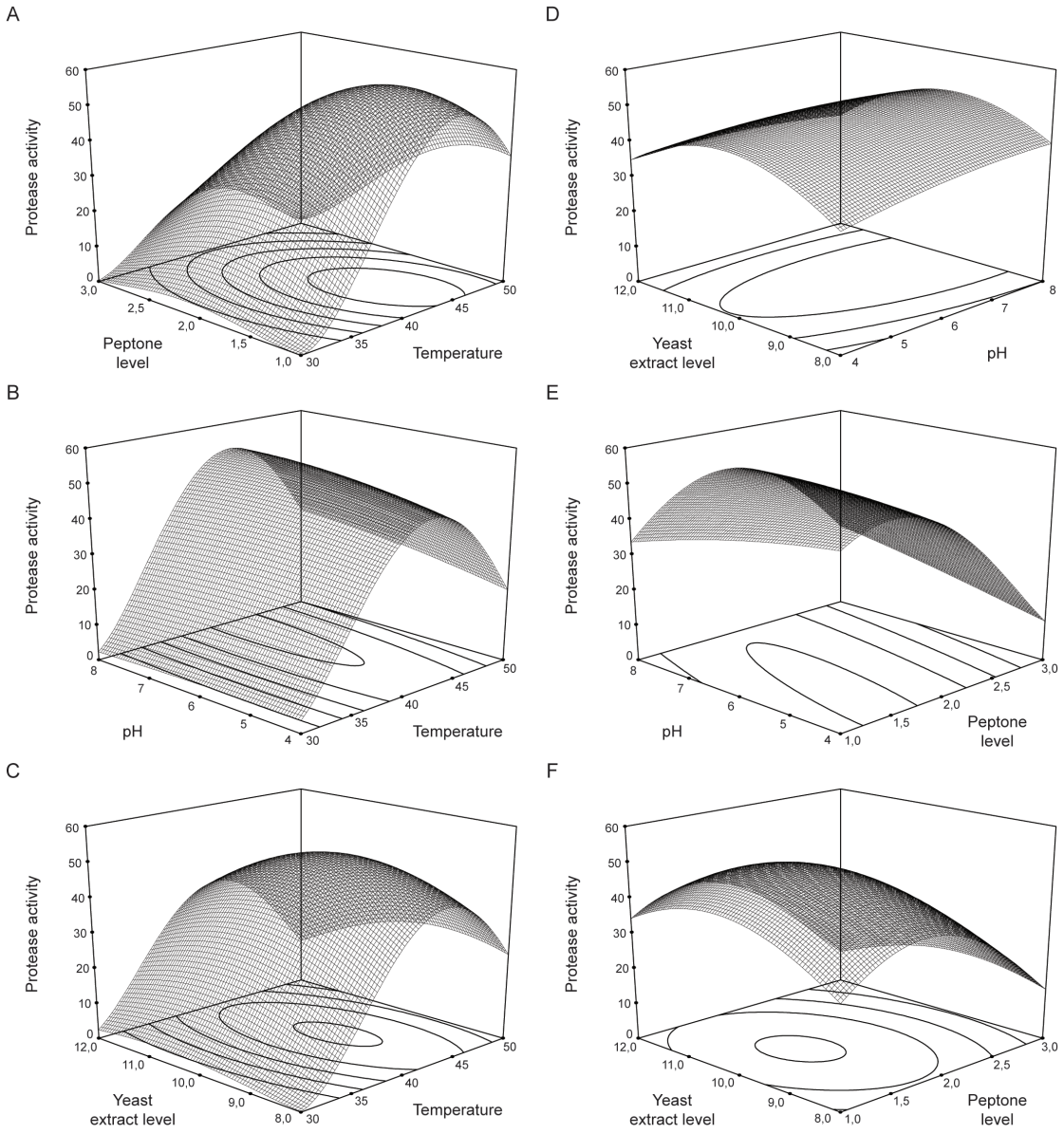


Figure 3.2.1 Response surfaces and corresponding contours of protease productivity by *Bacillus* sp. HTS 102, in terms of interaction between a) temperature and peptone content, b) temperature and pH, c) temperature and yeast extract level, d) pH and yeast extract level, e) peptone content and pH, and f) peptone content and yeast extract level.

3.2.4.3 Validation of model

The optimum conditions for protease production by *Bacillus* sp. HTS102, as predicted by the second order polynomial model, were experimentally

checked. The estimated protease activity was 54.57 U/mL, whereas the predicted value was 56.76 U/mL. This entails a prediction accuracy of our model above 95% – so, in general, it validates our approach.

3.2.5. Conclusions

Synthesis of an extracellular protease by *Bacillus* sp. HTS102 – a wild strain previously isolated from the wool of Portuguese Merino sheep, was optimized using statistical methods, based on response surface methodology encompassing 4 easily manipulated process parameters. This work is relevant toward eventual development of an economically feasible fermentation process encompassing that strain. The protease production was dependent chiefly on temperature and peptone level, and on pH and yeast extract level to a lesser degree; the optimum loci were 5.1 for pH, 43 ºC for temperature, 1.4 g/L for peptone level and 10.0 g/L for yeast extract content. Under the optimum conditions, a 2.3-fold increase in protease productivity was possible – using the basal medium as reference.

Acknowledgments

A.C. Queiroga received a PhD fellowship (ref. SFRH/BD/19121/2004), granted by Fundação para a Ciência e a Tecnologia (Portugal) and supervised by F.X. Malcata.

3.2.6. References

NIST/SEMTECH e-Handbook of Statistical Methods.

Bhaskar V, Raj JTR, Kandasamy SKJ, Vijaykumar P, Achary A. 2008. Optimization of production of subtilisin in solid substrate fermentation using response surface methodology. Afr J Biotechnol 7:2286-2291.

Cai CG, Zheng XD. 2009. Medium optimization for keratinase production in hair substrate by a new *Bacillus subtilis* KD-N2 using response surface methodology. J Ind Microbiol Biotechnol 36:875-883.

Castro G, Santopietro L, Siñeriz F. 1992. Acid pullulanase from *Bacillus polymyxa* MIR-23. Appl Biochem Biotechnol 37:227-233.

Gupta R, Beg Q, Lorenz P. 2002b. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 59:15-32.

Huang GR, Dai DH, Hu WL, Jiang JX. 2008. Optimization of medium composition for thermostable protease production by *Bacillus* sp. HS08 with a statistical method. Afr J Biotechnol 7:1115-1122.

Myers RH, Montgomery DC. 2002. Response Surface Methodology: Process and Product Optimization Using Designed Experiments. New York, NY: Wiley Interscience.

Navaneeth S, Bhuvanesh S, Bhaskar V, Kumar PV, Kandaswamy SKJ, Achary A. 2009. Optimization of medium for the production of subtilisin from *Bacillus subtilis* MTCC 441. Afr J Biotechnol 8:6327-6331.

Oskouie SFG, Tabandeh F, Yakhchali B, Eftekhar F. 2008. Response surface optimization of medium composition for alkaline protease production by *Bacillus clausii*. Biochem Eng J 39:37-42.

Puri S, Beg QK, Gupta R. 2002. Optimization of alkaline protease production from *Bacillus* sp. by response surface methodology. Curr Microbiol 44:286-290.

Rao YK, Tsay KJ, Wu WS, Tzeng YM. 2007. Medium optimization of carbon and nitrogen sources for the production of spores from *Bacillus amyloliquefaciens* B128 using response surface methodology. Process Biochem 42:535-541.

Reddy LVA, Wee YJ, Yun JS, Ryu HW. 2008b. Optimization of alkaline protease production by batch culture of *Bacillus* sp. RKY3 through Plackett-Burman and response surface methodological approaches. Bioresource Technol 99:2242-2249.

Seong CN, Jo JS, Choi SK, Kim SW, Kim SJ, Lee OH, Han JM, Yoo JC. 2004. Production, purification, and characterization of a novel thermostable serine protease from soil isolate, *Streptomyces tendae*. Biotechnol Lett 26:907-909.

Tamhane AC. 2009. Statistical Analysis of Designed Experiments: Theory and Applications. New Jersey, NJ: Wiley.

Wang Q, Hou Y, Xu Z, Miao J, Li G. 2008. Optimization of cold-active protease production by the psychrophilic bacterium *Colwellia* sp. NJ341 with response surface methodology. Bioresource Technol 99:1926-1931.

Wilson ED. 1930. Studies in bacterial proteases. I. The relation of protease production to the culture medium. J Bacteriol 20:41-59.

Chapter 4

Assessment of protease applicability in wool industry: laboratory-scale studies

Section 4.1

Potential applications of protease from *Bacillus* sp. HTS 102 in wool processing and laundry

4.1.1. Abstract

A novel wild strain, recently isolated from the wool of a Portuguese breed of sheep and tentatively identified as *Bacillus* sp. HTS 102, was selected for further study among several other isolates – because of its unusually high caseinolytic activity. This chapter describes the biochemical characterization of the (crude) extracellular protease synthesized by said strain – and, in particular, its assessment for detergent formulation, in attempts to increase the shrink-resistance of wool fabrics. Woven wool fabric samples treated with that enzyme exhibited indeed increased wettability and softness – associated with low area shrinkage and acceptable strength loss. The aforementioned protease proved stable over relatively wide pH and temperature ranges, and showed considerable tolerance to surfactants and oxidizing agents. Additionally, it was rather stable

and compatible with common laundry detergents – thus unfolding a potential for proteinaceous stain removal at commercial scale. Its apparent independence on such divalent cation as Ca^{2+} is also noteworthy, should industrial application as a detergent additive be considered.

4.1.2. Introduction

Wool is chiefly composed of (animal) proteins, which account for its unique structural and mechanical properties (Plowman 2003). Wool fibers also contain ca. 1.5 %(w/w) lipids that serve as major barrier against penetration of hydrophilic compounds (Fonollosa et al. 2004).

Among the several interesting and useful properties of wool amid the fiber world, one should outline those arising from its surface scales – which are responsible for distinctive felting and shrinkage features of wool fabrics upon wetting; these are probably the most important factors with an impact upon the final quality, processability and appearance of wool yarns. Shrinkage constitutes, however, a major drawback of wool fibers; hence, a number of chlorination processes have been in use to minimize it in commercial products (Cardamone et al. 2005). Chlorination degrades and partially dissolves the cuticle via oxidative breakdown of disulfide linkages, thus leading to scale smoothing or even removal – which is effective toward shrinkage control (Cardamone et al. 2004). The most successful industrial process of this kind is the Hercosett chlorine process; despite its advantages in anti-felting results – associated with low extent damage and weigh loss, it holds a significant environmental impact because of the absorbable organic halogens released (Araújo et al. 2008).

An ever-growing environmental awareness within the textile industry has promoted search for environmentally favorable alternatives of wool treatment. Many authors have accordingly suggested benign chemical processes to treat wool fibers (Galland-Irmouli et al. 1999; Molina et al. 2002), but almost all failed in providing effective shrink-resistance along with economic feasibility. Recently, microbial proteases – especially those of the subtilisin type, have been under scrutiny as eco-friendly, biotechnological alternatives to chemical pre-treatment of wool.

Recall that wool is a rather complex fiber; hence, application of proteases as specific catalysts in its processing constitutes a major opportunity for tailored fiber modification, and eventual improvement thereof. Furthermore, the use of such enzymes may dramatically reduce the rate of production of pollutants – while achieving essentially equivalent results in shrink-resistance, impurity removal and increased dye affinity to classical chemical processing (Araújo et al. 2008). An increased body of evidence has it that microbial proteases are particularly suitable for industrial utilization because they are easily produced to large scale, and stable to the alkaline and hot conditions prevailing in the textile industrial processing. However, a general large-scale process encompassing use of such biocatalysts is yet to be established – mainly due to the ability of the small-sized enzymes to penetrate the inner parts of the fiber, thus irreversibly damaging them. Furthermore, the offer of such enzymes in the market is still reduced – so screening for novel protease-producing microorganisms with higher specificity for cuticles is in order.

On the other hand, physical and mechanical properties of wool fabrics are greatly affected by the detergents used upon wetting (Chen et al. 2004).

Consequently, the composition of wool-care products – e.g. laundry detergents, is of great importance to abide to the consumer demand for machine washability and sustained soft handle touch, while retaining the ability to efficiently clean said wool products. Detergents (under several brand names) are commercially available containing different enzymes – chiefly hydrolases, e.g. as proteases and lipases. Once again, suitability of a protease for inclusion in a detergent depends on its compatibility with said detergents in a wide range of washing temperatures (Kumar et al. 2008); a recent thrust has occurred encompassing novel alkaline proteases that are active at low temperatures (Chauhan & Gupta 2004), in view of their performance toward energy savings during domestic washing.

In this research effort, the ability of a protease excreted by the novel *Bacillus* sp. strain HTS 102 – previously isolated from Portuguese Merino ewe's wool, to impart shrink-resistance to wool fabrics and for inclusion in laundry detergents was assessed.

4.1.3. Materials and methods

4.1.3.1 Source of microbial enzyme

The promising caseinolytic activity of the wool-associated bacterial strain HTS 102 – isolated in advance from the wool of healthy Portuguese Merino sheep, was described elsewhere in previous chapters; such preliminary data accounted for its selection, in attempts to impart shrink-resistance to wool fabrics and to integrate the formulation of laundry detergents. The

aforementioned strain HTS 102 belongs to the *Bacillus* genus, based on 16S rRNA partial gene sequence (GenBank accession number HQ698269); it was also already deposited in an accessible culture collection (reference LMG 26323, from BCCM/LMG Bacterial Culture Collection, Ghent, Belgium).

A neutral alkaline protease, Protex Multiplus L™, synthesized by *Bacillus lentus*, was kindly supplied by Genencor International (Rochester, USA), and used as reference in comparative studies of wool treatment.

4.1.3.2 Growth of, and enzyme production by microorganism

Previous work on optimization of fermentation conditions led to the following optimal combination of processing parameters for protease productivity: 1.4 g/L of peptone, 10.0 g/L of yeast extract, 0.5 g/L of meat extract, 1.0 g/L of NaCl and pH 5.1. Hence, Erlenmeyer flasks (250 ml) containing 25 ml of growth medium with the composition above were inoculated with 1 %(v/v) fresh inoculum (exhibiting an $O.D._{600nm}$ of 0.6), and incubated at 43 ºC for 36 h. The culture supernatants were then filtered through 0.45 μm sterile membranes, and applied on knitted wool fabrics – or else tested for their compatibility with laundry detergents, in terms of their performance in stain removal performance (as described below).

4.1.3.3 Assays for protease activity and protein content

The filter-sterilized samples were assayed for protease activity using a colorimetric determination of the extent of breakdown of standard casein – as described before (Queiroga et al. 2007). One unit (U) of proteolytic activity was defined as the amount of enzyme able to hydrolyze casein at a rate that produces

a variation in absorbance equal to that produced by 1.0 µmol of tyrosine, at pH 7.5 at 37 ºC.

Determination of the total protein was via the BCA™ Protein Assay Kit (Pierce, USA), according to supplier's instructions.

4.1.3.4 Effects of surfactants and oxidants upon protease stability

The effect of common surfactants (viz. SDS, Triton X-100 and Tween 20) and common oxidizing agents (viz. hydrogen peroxide and sodium perborate) upon enzyme stability was ascertained by incubating the enzyme in the presence of various concentrations of said compounds, for 1 h at room temperature (see Table 4.1.1). The residual activity was determined using casein as substrate, after setting the activity of the original enzyme, without surfactants or oxidizing agents, to 100%.

4.1.3.5 Effects of enzyme inhibitors and metal ions upon protease activity

The effect of two peptidase inhibitors upon enzyme activity was studied. The enzyme was accordingly pre-incubated with 1 or 2 mM of serine peptidase inhibitor AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride), and with 1 or 2 mM of EDTA, for 30 min at room temperature. The residual activity was measured using casein as substrate, after setting the activity of the original enzyme extract (without inhibitors) to 100%.

The influence of several metal ions upon enzyme activity was also assessed. The divalent metal cations Ba^{2+}, Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} were thus added (5 mM) to the enzyme solution, and the remaining activity after 30

min of incubation at room temperature was assayed for using casein as substrate – after setting the caseinolytic activity of the original extract, without metallic ions, to 100%.

4.1.3.6 Effects of pH and temperature upon protease activity and stability

The effect of pH upon enzyme activity was evaluated over the pH range 6–12, by incubating the enzyme at said pH values for 15 min. The effect of pH upon enzyme stability was evaluated over the same pH range, by once again incubating the enzyme at said pH values for 1 h – and measuring the residual activity using casein as substrate.

On the other hand, the effect of temperature upon enzyme activity was determined at temperatures between 25 and 70 ºC, by incubating the enzyme at said temperature for 15 min. Likewise, the effect of temperature upon enzyme stability was determined over the temperature range 30–55 ºC, by also incubating the enzyme at those temperatures up to 1 h and pH 8.0; the residual activity was measured using casein as substrate, and incubating at 35 ºC and pH 8 – after setting the activity of the original, non-heated enzyme extract to 100%.

4.1.3.7 Tests of protease in wool processing

Source of wool

Knitted, plain Merino wool fabrics (with an average fiber diameter of 19.5 μm) were kindly supplied by Orfama – Organização Fabril de Malhas, S.A. (Portugal). Pieces of 12 x 12 cm were cut therefrom and duly cast off, and a square of 10 x 10 cm was carefully marked on the fabrics using a pen. All fabrics

were pre-treated with 2.5 %(v/v) chlorine, to modify the scale surface and thus make the fibers more susceptible to enzyme attack (Cardamone et al. 2004).

Enzymatic treatment conditions

For protease treatment of the experimental wool fabrics, both enzyme extract and commercial enzyme were assayed (in triplicate) for their caseinolytic activity, and appropriately diluted to a final activity of 20 U mL^{-1} in the final reaction mixture. Treatment of said fabrics using only 10 mM of Tris-HCl buffer (pH 8.0) was taken as negative control. All said treatments were performed for 30 min at 35 ºC, using a Washtester WT (Werner Mathis, Germany) at a weight ratio of ca. 1:20. Quenching of the enzyme was via rapidly raising temperature to 75 ºC, and washing thereafter for 5 min. The fabrics were afterwards cooled, drained and rinsed with plenty of tap water.

Analytical methods

The wettability and softness degree of woven wool fabrics were assessed before and after enzymatic treatment.

The wettability, or degree of water absorption, was measured by placing the fabrics wide open on the surface of a glass plate, and letting 10 µL of deionized water drip on it from a height distance of 1 cm. The procedure was repeated 15 times, and the average time required for the droplet to completely penetrate the fabric was recorded.

A panel of 20 experienced judges assessed the perceived softness, via rating from 1 (not soft) to 5 (extremely soft).

The weight loss and shrinkage extent were monitored during 3 consecutive household wash cycles, using a model WFL 1300 washing machine (Bosch, Germany); the program specific for wool (i.e. 35 min of washing at 30 ºC) was used, after addition of 3 g/L of standard IEC detergent without bleaching ingredients – under a total wash load of 2 kg (dry mass). After each wash cycle, the fabrics were air-dried and conditioned at 30 ºC for at least 24 h prior to evaluation (unless otherwise stated).

The weight of the fabrics was measured with an electronic scale after cooling on a Petri dish inside an excicator. The weight loss of the fabric was recorded as dried sample weight loss – with drying conditions set at 105 ºC and 1 h (Yu et al. 2005); the percent weight loss (% WL) was calculated as $\%WL=[(W_1-W_2)/W_1]\ x100$ where W_1 and W_2 denote the weights of the fabric before and after treatment, respectively.

The shrinkage was calculated based on the original size (length and width) of the fabric prior to enzyme treatment (Schroeder et al. 2004). Measurements in each direction of the specimens were made, before and after each of the 3 consecutive household washing cycles; and the average dimensional change (% DC) was calculated according to $\%DC=[(A-B)/B]\ x100$ where A and B denote the area of fabric after and before each cycle, respectively (Anonimous 2006).

The bursting strength was assessed on 7.3 cm^2-pieces of wool fabrics, after performance of 3 washing cycles – according to ISO 13938-2, using a *Tru-Burst 610*.

4.1.3.8 Tests of protease in laundry detergents

Sources of detergents

The compatibility and stability of the enzyme extract towards selected solid laundry detergents available in the market – viz. Persil Progress (Henkel, Portugal), Skip Effervescent Active Clean Hygiene Protection (Unilever, Portugal), Skip Active Clean (Unilever), Xau 101 (Reckitt Benckiser, Portugal), Tide (Procter & Gamble, Portugal) and Skip Baby (Unilever), were examined as described elsewhere (Mukherjee et al. 2009).

Enzymatic compatibility

Aqueous solutions of each detergent (7.0 g L^{-1}, to accurately simulate typical washing conditions) were heated at 100 ºC for 90 min, so as to denature the indigenous enzyme activity (if any); this was confirmed by the protease standard assay procedure. The enzyme extract was then added to a final concentration of 20 U mL^{-1}, via mixing at a ratio of 1:1 (v/v) – and incubated at 35 ºC for 1 and 2 h. The remaining protease activity was measured by the standard assay procedure described above, and compared with a control – i.e. enzyme diluted to 1:1 (v/v), in tap water without detergent.

Performance in stain removal

The effectiveness of the enzyme extract in removal of three types of common stains (chocolate, egg and tomato) was assessed. A piece of the test fabric was thoroughly wet out in a bath containing the staining source, over 24 h. The starting temperature of the chocolate (1:1, chocolate:water) and tomato

pulp (1:1, tomato pulp:water) was 95 ºC; the starting temperature of the egg yolk solution (5% v/v) was 20 ºC. The fabric was then padded to remove excess stain, before drying for 1 h at 105 ºC. To evaluate the performance of the enzyme in stain removal during 30 min or 1 h washing at 35 ºC, four wash liquors were used: enzyme extract, diluted to 20 U mL^{-1} in tap water; commercial detergent (at 7 g L^{-1}); detergent at 7 g L^{-1} and enzyme extract (at 20 U mL^{-1}); and tap water (used as control). After each treatment, the fabrics were hand-rinsed 3 times with tap water. Each fabric stained was then padded to remove excess water, and oven dried at 105 ºC for 1 h. The fabric samples were finally conditioned and assessed for color change.

4.1.4. Results

4.1.4.1 Effects of surfactants and oxidants upon protease activity

The protease extract was rather stable in the presence of non-ionic surfactants, e.g. Triton X-100 and Tween 20 – as shown in Table 4.1.1. It was also stable in the presence of SDS, a strong anionic surfactant – with ca. 96 %, 85 % and 34 % of its initial activity remaining after incubation, for 1 h at room temperature, with 0.1 %, 0.5 % and 1.0 % (w/v) of that compound, respectively.

The stability of the enzyme extract was enhanced in the presence of H_2O_2 and sodium perborate – as apparent from the increase in activity observed (Table 4.1.1). The residual activity after incubation with 1 %, 5 % and 10 % (v/v) of that oxidant was ca. 134 %, 112 % and 100 %, respectively; whereas

incubation with sodium perborate conveyed residual activities of ca. 142 %, 121 % and 104 %, respectively.

4.1.4.2 Effects of enzyme inhibitors and metal ions upon protease activity

The AEBSF inhibitor strongly constrained the action of the enzyme extract: essentially all its original activity was lost upon pre-incubation, for 30 min at room temperature, with 2 mM AEBSF. On the other hand, the chelator EDTA could just mildly affect the protease residual activity: ca. 80% of its original activity was still kept after incubation with 2 mM EDTA (Table 4.1.1).

The metal ions Cu^{2+} and Mg^{2} were particularly successful in promoting enzyme activity – whereas Ca^{2+} and Ba^{2+} proved essentially inert (Table 4.1.1). Interestingly, Mn^{2+} and Zn^{2+} inhibited the enzyme significantly, with quantitative inhibition by 30 min of incubation.

4.1.4.3 Effects of pH and temperature upon protease activity and stability

The relative activity of the enzyme extract at various pH values is depicted in Figure 4.1.1a. The enzyme was chiefly active between pH 7.0 and 9.0, with an optimum at pH 8.0. The pH stability profile indicates that said protease is fairly stable in the pH range 6.0–9.0 (Figure 4.1.1a). At pH 10 and 11, the enzyme was able to retain ca. 70% and 50% of its original activity, respectively.

Table 4.1.1 Effects of surfactants, oxidizing agents, inhibitors and metal ions upon activity of protease extract

Substance	Concentration	% residual actvity	
		Mean	SD
None	--	100,0	0,0
Surfactants			
SDS	0.1 % (w/v)	95,8	0,8
	0.5	84,7	0,6
	1.0	33,9	0,9
Triton X-100	1.0 % (v/v)	100,0	0,4
	5.0	97,9	0,8
Tween 20	1.0 % (v/v)	102,9	0,8
	5.0	100,0	0,2
Oxidants			
H_2O_2	1 % (v/v)	133,9	0,4
	5	112,4	0,1
	10	100,0	0,4
Sodium perborate	0.1 % (v/v)	141,5	0,8
	0.5	120,9	0,8
	1.0	103,8	1,2
Inhibitors			
AEBSF	1 mM	7,9	0,5
	2 mM	0,0	0,0
EDTA	1 mM	100,0	0,4
	2 mM	80,4	1,5
Metal ions			
Ca^{2+}	5 mM	99,6	0,4
Cu^{2+}	5 mM	120,9	0,6
Ba^{2+}	5 mM	91,9	0,6
Mg^{2+}	5 mM	124,8	1,3
Mn^{2+}	5 mM	0,0	0,0
Zn^{2+}	5 mM	0,0	0,0

The effects of temperature upon enzyme activity and stability are depicted in Figures 4.1.1b and 4.1.1c. The optimum temperature for protease activity was 35 ºC. The temperature stability unfolds a highly stable protease at temperatures below 40 ºC – and able to retain ca. 80 % and 90 % of its original activity upon incubation for 60 min, at 30 and 35 ºC, respectively. After 45 min at 55 ºC or 1 h at 50 ºC, the enzyme became inactivated in full.

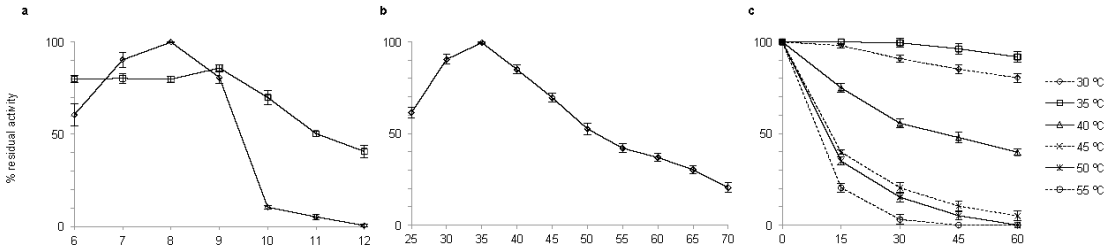


Figure 4.1.1 Effects of pH and temperature (mean±SD) upon activity and stability of protease extract: a) pH stability (□) and activity (◇) profiles; b) temperature activity profile; c) temperature stability profile

4.1.4.4 Tests of protease in wool processing

The results of the water penetration time and the perceived softness degree of wool, with or without proteolytic treatment, are depicted in Figure 4.1.2. It is apparent that the enzymatic treatment, either with commercial protease or with protease extract, decreased significantly the wetting time of wool samples (Figure 4.1.2a) relative to the untreated samples.

The results plotted in Figure 4.1.2b indicate that both enzymatic treatments produced an increase in perceived softness – from a score of ca. 2 in control samples, up to a score of ca. 4 following enzymatic treatment.

The enzymatic treatment of woven wool fabrics with protease extract was able to reduce fabric shrinkage to ca. 27% of that observed in the control; however, it also caused a decrease of ca. 15% in the bursting strength of wool fibers (Figure 4.1.3). Treatment with the commercial protease unfolded a worse performance in terms of area shrinkage and weight loss, when compared with samples treated with our protease extract.

a

Treatment	Wetting Time (s)
Untreated	>1200
Treated without protease	1097 ± 64
Treated with E1	263 ± 33
Treated with E2	232 ± 23

b

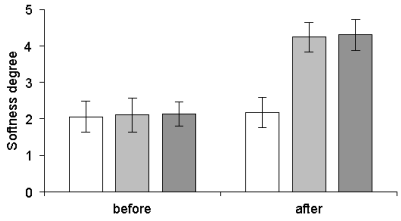


Figure 4.1.2 (a) Wettability and (b) perceived softness degree of untreated and protease-treated wool fabrics (mean±SD): control (white bars), after treatment with commercial protease (light grey bars) and after treatment with enzyme extract (dark grey bars). E1 – commercial enzyme; E2 – crude protease from *Bacillus* sp. HTS 102

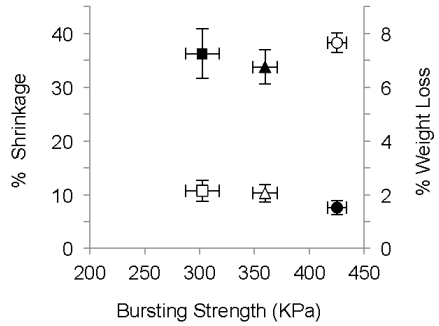


Figure 4.1.3 Area shrinkage (unfilled markers) and weight loss (filled markers) of untreated and protease-treated wool fabrics (mean±SD), as a function of bursting strength: control (○), after treatment with commercial protease (□), and after treatment with protease extract (△)

4.1.4.5 Tests of protease in laundry detergents

The data presented in Figure 4.1.4 illustrate the effect of several commercial laundry detergents upon enzyme stability, over a 2 h-incubation period; one concludes that the protease extract was extremely stable in the presence of all solid detergents tested – except Skip AC. In the presence of the latter, the enzyme could only retain ca. 34 % and 22 % of its original activity, after incubation for 1 h and 2 h, respectively. Approximately 70% of activity

remained following incubation for 2 h with Skip Effervescent ACHP – and the remainder four commercial detergents performed even better.

The wool fabric pieces that had been deliberately stained were washed for 30 min or 1 h, at 35 ºC, with protease extract – supplemented, or not, with solid laundry detergent. As apparent from inspection of Figure 4.1.5, a limited washing performance resulted when only detergent was used. Incubation of the protease extract with tomato-, egg yolk- and chocolate-stained wool fabrics produced a considerable degree of stain removal – even without the need for any detergent; however, a faster stain removal was obviously noticed upon supplementation with commercial detergent.

4.1.5. Discussion

This work has focused on the biochemical characterization of a crude protease from *Bacillus* sp. HTS 102 – in attempts to assess its feasibility to impart shrink-resistance to wool fabrics, together with its compatibility with laundry detergents currently available in the market.

The reasonable stability found in the presence of SDS is worth mentioning, since SDS-stable enzymes are indeed not widely available. The stability of our extract was similar to that observed by other authors for proteases synthesized by species of *Bacillus* and other bacterial genera (Oberoi et al. 2001; Doddapaneni et al. 2007; Sellami-Kamoun et al. 2008; Haddar et al. 2009; Hmidet et al. 2009; Haddar et al. 2010; Manni et al. 2010). Furthermore, its stability upon exposure to oxidizing agents and other surfactants is of great importance for eventual detergent formulation; the particularly high stability to

both hydrogen peroxide and sodium perborate, relative to the control, is to be emphasized. The oxidizing agents tested did not only stabilize the enzyme, but could even improve its activity.

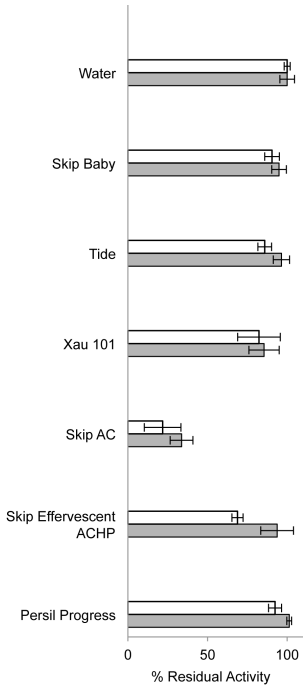


Figure 4.1.4 Stability of protease extract (mean±SD) following incubation for 1 h (white bars) and 2 h (grey bars), in the presence of commercial laundry detergents

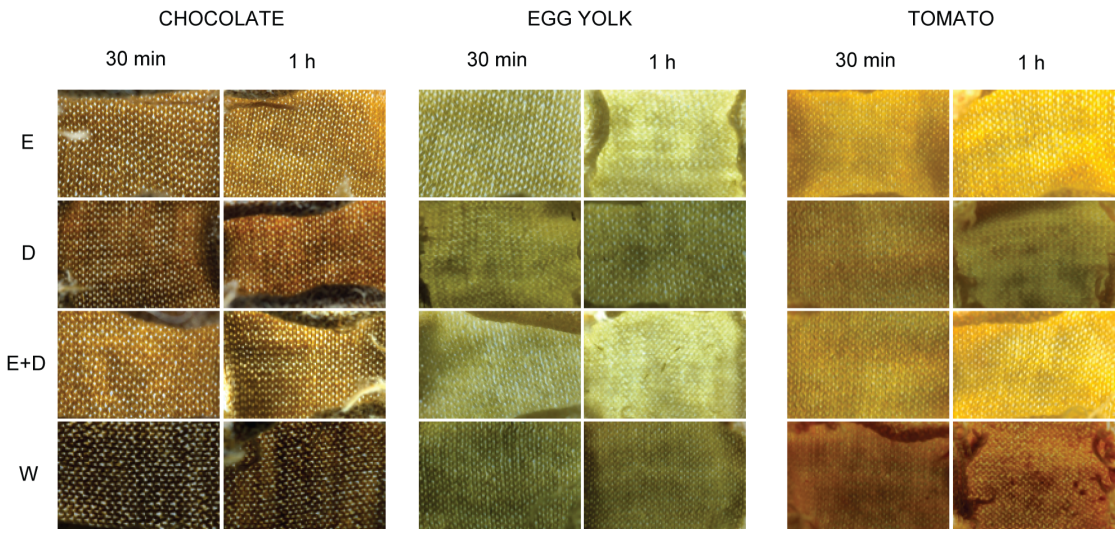


Figure 4.1.5 Washing performance upon chocolate, egg yolk and tomato stains, following incubation for 30 min and 1 h: control (W), after treatment with detergent (D), after treatment with protease extract (E), and after treatment with both detergent and protease extract (E+D).

The enzyme extract was fully inhibited by AEBSF, and a slight inhibition was observed upon pre-incubation with EDTA. Note that such chelating agents as EDTA are included as water softeners in detergent formulation, to overcome water hardness while assisting in removal of stains (Gessesse et al. 2003; Haddar et al. 2010). Hence, the behavior of our enzyme extract in the presence of EDTA unfolds a potential application as detergent additive.

Among the metal ions tested, Ca^{2+} and Ba^{2+} did not affect the activity of the enzyme extract. The former has a great relevance for application of this enzyme in detergents, because such chelators as EDTA in detergents will easily strip Ca^{2+} off its weak binding to the enzyme – and might otherwise greatly affect its thermal stability (Gessesse et al. 2003).

The protease extract was reasonably stable at pH values between 7.0 and 9.0, and even kept ca. 80% of its original activity after incubation at room temperature for 1 h. Said pH stability profile was similar to those found with proteases synthesized by several *Bacillus* spp. (Hmidet et al. 2009; Fakhfakh-Zouari et al. 2010; Manni et al. 2010).

The crude enzyme showed an optimum activity at 35 ºC – thus denoting a mesophilic character. This represents a competitive advantage when one seeks detergents for home laundering that are efficient at mild temperatures; reduction of energy consumption, as well as in wear and tear of fabrics are obvious advantages derived from cold washing (Gerday et al. 2000). Nevertheless, the stability of mesophilic and psychrophilic enzymes is usually lower than that of their thermophilic counterparts. It is possible to improve the stability of such enzymes, though – while maintaining their high catalytic

efficiency at low temperatures, using biocatalyst engineering (should the need arise).

The hydrophobic nature of wool is more directly related with the properties of the outmost layer of its fibers (Wang et al. 2010); the exocuticle is indeed hydrophobic owing to its high degree of disulphide crosslinking – and the epicuticle surrounding each cuticle cell of the wool fiber consists of fatty acids, with 18-methyleicosanoic acid being its chief component (Walawska et al. 2006). The way the chains of this acid are covalently bound to cysteine on the wool fiber, and their orientation away from the fiber bring hydrophobic features to the epicuticle (Wang et al. 2009). It has been reported (Walawska et al. 2006; Wang et al. 2010) that chemical or enzymatic treatments may decrease the degrees of lipids bound to, and increase the wettability of the wool surface; hence, pretreatments may facilitate access of protease to the surface of wool fibers, where proteolytic reactions will be promoted – thus allowing hydrophilic proteins located deeper in the fiber to become exposed on the surface (Wang et al. 2009). For individual protease treatments, the wetting time of wool did distinctly decrease with regard to that of untreated samples. On the other hand, wool treated without protease also underwent an increase in wettability – even though distinctively different from that brought about by enzymatic treatment. The alkaline conditions prevailing in the wool treatment without proteases could account for their decreased wetting time (Pascual & Juliá 2001; Montazer & Ramin 2010). Furthermore, the increase in perceived softness of protease-treated samples agrees with the putative modification of wool fiber surface thereby.

The results pertaining to dimensional area changes and weight losses of wool treated with commercial and crude proteases, as a function of bursting strength, show that the enzyme extract imparts a significant shrink-resistance to woven wool fabrics (ca. 10%), without significantly damaging the fibers – as revealed by the reasonably high bursting strength, coupled with acceptable levels of weight loss. Other authors (Hossain et al. 2008; Ge et al. 2009) have produced similar results regarding area shrinkage, yet the existing reports on tests of bursting strength refer to treatment of fabrics with a combination of proteases and other enzymes, e.g. transglutaminases. Hence, our crude enzyme will be of great relevance in large-scale attempts to impart shrink-resistance to wool fabrics, without resorting to supplementary enzymes.

It was interesting to realize that the protease extract was highly stable to most solid detergents tested; however, the diversity of stability profiles obtained indicates clearly that the enzyme performance depends on a number of factors, including detergent composition.

Finally, detergent proteases are supposed to hydrolyze large insoluble proteins, viz. those attached to solid surfaces (Haddar et al. 2010). Several reports have indeed pointed at the usefulness of proteases to promote removal of proteinaceous stains, e.g. blood, chocolate, egg and spaghetti sauce (Oberoi et al. 2001; Schroeder et al. 2006; Vasconcelos et al. 2006; Mukherjee 2007). The wash performance analysis conducted here revealed that the protease extract allows removal of protein stains – so, once again, the potential application in the formulation of detergents efficient at low washing temperatures may be claimed.

4.1.6. Conclusions

Bacillus sp. HTS 102 – a wild strain isolated from Portuguese Merino sheep wool, is very promising for biotechnological applications. The crude protease secreted is stable and active over a wide pH range, with an optimum temperature of ca. 35 ºC. When allowed to act on woven wool fabrics, noticeable improvements to fabric properties result – e.g. decreased area shrinkage, as well as increased wettability and softness degree, without substantial strength loss.

Said protease also exhibits excellent performance in the presence of several commercial laundry detergents. Considering its good activity and stability in the pH range 7–9, and its relatively good stability towards surfactants and oxidizing agents (which, in addition, is independent of Ca^{2+}), said protease appears as an interesting candidate toward detergent formulation.

Acknowledgments

A.C. Queiroga received a Ph.D. fellowship (ref. SFRH/BD/19212/2004), granted by Fundação para a Ciência e a Tecnologia (Portugal) and supervised by F.X. Malcata. Partial funding to cover research expenses was via projects "GoBlue" (POCTI 13-02-03-SDR-01254), granted by Agência de Inovação (Portugal), and "BioTex" (POCI/CTM/58312/2004), granted by Fundação para a Ciência e a Tecnologia (Portugal).

4.1.7. References

Anonimous. 2006. AATCC Testing Method 135-2004, Dimensional changes of fabrics after home laundering. In. American Association of Textile Chemists and Colourists. pp. 231–234.

Araújo R, Casal M, Cavaco-Paulo A. 2008. Application of enzymes for textile fibres processing. Biocatal Biotransfor 26:332-349.

Cardamone JM, Yao J, Nunez A. 2004. DCCA shrinkproofing of wool: Part I: Importance of antichlorination. Textile Res J 74:555-560.

Cardamone JM, Yao J, Phillips JG. 2005. Combined bleaching, shrinkage prevention, and biopolishing of wool fabrics. Textile Res J 75:169-174.

Chauhan B, Gupta R. 2004. Application of statistical experimental design for optimization of alkaline protease production from *Bacillus* sp. RGR-14. Process Biochem 39:2115-2122.

Chen QH, Au KF, Yuen CWM, Yeung KW. 2004. An analysis of the felting shrinkage of plain knitted wool fabrics. Textile Res J 74:399-404.

Doddapaneni KK, Tatineni R, Vellanki RN, Gandu B, Panyala NR, Chakali B, Mangamoori LN. 2007. Purification and characterization of two novel extra cellular proteases from *Serratia rubidaea*. Process Biochem 42:1229-1236.

Fakhfakh-Zouari N, Haddar A, Hmidet N, Frikha F, Nasri M. 2010. Application of statistical experimental design for optimization of keratinases production by *Bacillus pumilus* A1 grown on chicken feather and some biochemical properties. Process Biochem 45:617-626.

Fonollosa J, Campos L, Marti M, Maza A, Parra JL, Coderch L. 2004. X-Ray diffraction analysis of internal wool lipids. Chem Phys Lip 130:159-166.

Galland-Irmouli AV, Fleurence J, Lamghari R, Lucon M, Rouxel C, Barbaroux O, Bronowicki JP, Villaume C, Gueant JL. 1999. Nutritional value of proteins from edible seaweed *Palmaria palmata* (dulse). J Nutrit Biochem 10:353-359.

Ge FY, Cai ZS, Zhang HY, Zhang RP. 2009. Transglutaminase treatment for improving wool fabric properties. Fiber Polym 10:787-790.

Gerday C, Aittaleb M, Bentahir M, Chessa J-P, Claverie P, Collins T, d'Amico S, Dumont J, Garsoux G, Georlette D, Hoyoux A, Lonhienne T, Meuwis M-A, Feller G. 2000. Cold-adapted enzymes: from fundamentals to biotechnology. TIBTECH 18:103-107.

Gessesse A, Hatti-Kaul R, Gashe BA, Mattiasson B. 2003. Novel alkaline proteases from alkaliphilic bacteria grown on chicken feather. Enzyme Microb Technol 32:519-524.

Haddar A, Bougatef A, Agrebi R, Sellami-Kamoun A, Nasri M. 2009. A novel surfactant-stable alkaline serine-protease from a newly isolated *Bacillus mojavensis* A21. Purification and characterization. Process Biochem 44:29-35.

Haddar A, Sellami-Kamoun A, Fakhfakh-Zouari N, Hmidet N, Nasri M. 2010. Characterization of detergent stable and feather degrading serine proteases from *Bacillus mojavensis* A21. Biochem Eng J 51:53-63.

Hmidet N, el-Hadj Ali N, Haddar A, Kanoun S, Alya S-K, Nasri M. 2009. Alkaline proteases and thermostable α-amylase co-produced by *Bacillus licheniformis* NH1: characterization and potential application as detergent additive. Biochem Eng J 47:71-79.

Hossain KMG, Juan AR, Tzanov T. 2008. Simultaneous protease and transglutaminase treatment for shrink resistance of wool. Biocatal Biotransfor 26:405-411.

Kumar D, Savitri N, Thakur R, Verma TCB. 2008. Microbial proteases and application as laundry detergent additive. Res J Microbiol 3 661-672.

Manni L, Jellouli K, Ghorbel-Bellaaj O, Agrebi R, Haddar A, Sellami-Kamoun A, Nasri M. 2010. An oxidant- and solvent-stable protease produced by *Bacillus cereus* SV1: application in the deproteinization of shrimp wastes and as a laundry detergent additive. Appl Biochem Biotech 160:2308-2321.

Molina R, Jovančić P, Comelles F, Bertran E, Erra P. 2002. Shrink-resistance and wetting properties of keratin fibres treated by glow discharge. J Adhesion Sci Technol 16:1469-1485.

Montazer M, Ramin A. 2010. Influences of proteases and trans-glutaminases on wool. Fibres Text East Eur 18:98-102.

Mukherjee A. 2007. Potential application of cyclic lipopeptide biosurfactants produced by *Bacillus subtilis* strains in laundry detergent formulations. Lett Appl Microbiol 45:330-335.

Mukherjee AK, Borah M, Rai SK. 2009. To study the influence of different components of fermentable substrates on induction of extracellular α-amylase synthesis by *Bacillus subtilis* DM-03 in solid-state fermentation and exploration of feasibility for inclusion of α-amylase in laundry detergent formulations. Biochem Eng J 43:149-156.

Oberoi R, Beg QK, Puri S, Saxena RK, Gupta R. 2001. Characterization and wash performance analysis of an SDS-stable alkaline protease from a *Bacillus* sp. World J Microbiol Biotechnol 17:493-497.

Pascual E, Juliá MR. 2001. The role of chitosan in wool finishing. J Biotechnol 89:289-296.

Plowman JE. 2003. Proteomic database of wool components. J Chromat B 787:63-76.

Queiroga AC, Pintado MM, Malcata FX. 2007. Novel microbial-mediated modifications of wool. Enzyme Microb Technol 40:1491-1495.

Schroeder M, Lenting H, Kandelbauer A, Silva C, Cavaco-Paulo A, Guebitz G. 2006. Restricting detergent protease action to surface of protein fibres by chemical modification. Appl Microbiol Biotechnol 72:738-744.

Schroeder M, Schweitzer M, Lenting HBM, Guebitz GM. 2004. Chemical modification of proteases for wool cuticle scale removal. Biocatal Biotransfor 22:299-305.

Sellami-Kamoun A, Haddar A, Ali NE-H, Ghorbel-Frikha B, Kanoun S, Nasri M. 2008. Stability of thermostable alkaline protease from *Bacillus*

licheniformis RP1 in commercial solid laundry detergent formulations. Microbiol Res 163:299-306.

Vasconcelos A, Silva C, Schroeder M, Guebitz G, Cavaco-Paulo A. 2006. Detergent formulations for wool domestic washings containing immobilized enzymes. Biotechnol Lett 28:725-731.

Walawska A, Rybicki E, Filipowska B. 2006. Physicochemical changes on wool surface after an enzymatic treatment. Progr Colloid Polym Sci 132:131-137.

Wang P, Wang QA, Cui L, Fan XR, Yuan JG, Gao MR. 2010. A comparative evaluation of the action of savinase and papain to the cutinase-pretreated wool. Fiber Polym 11:586-592.

Wang Q, Wang P, Fan XR, Cui L, Zhao XF, Gao XX. 2009. A comparative study on wool bio-antifelting based on different chemical pretreatments. Fiber Polym 10:724-730.

Yu X-W, Guan W-J, Li Y-Q, Guo T-J, Zhou J-D. 2005. A biological treatment technique for wool textile. Braz Arch Biol Techn 48:675-680.

Section 4.2

Potential use of wool-associated *Bacillus* species for biodegradation of keratinous materials

4.2.1. Abstract

Five wool-degrading bacterial strains were selected from a set of 115 isolates from a less conventional source, i.e. the wool of Portuguese Merino sheep, based on their promising keratinolytic abilities. All selected strains belong to the genus *Bacillus*, likely *B. subtilis/ licheniformis*, according to 16S rRNA sequencing. They were able to grow and hydrolyse feathers and wool (in both native and milled forms), and human hair and nails to a lesser extent. The maximum keratinase activity was recorded on milled chicken feathers. Biodegradation of such keratin-rich matrices increased the amount of soluble proteins in the fermentation broth, and implied the action of extracellular enzymes from those adventitious microorganisms. Therefore, such novel strains have a potential for effective use in solid waste management strategies,

encompassing keratin-rich materials and based on submerged fermentation. Concomitantly, the nutritional value of the broth may be improved for eventual formulation of animal feed.

4.2.2. Introduction

Keratin-rich materials – e.g. hair, wool, feather, horn, hoof, claw, nail and beak, are quite abundant in nature. In view of the intrinsic stability and insolubility of these proteins, said materials are resistant to degradation carried out by most proteolytic enzymes (Lateef et al. 2010). The complex structure of α- and β-keratin – including the presence of a high degree of disulfide crosslinking further to numerous hydrogen bonds and strong hydrophobic interactions, has been implicated in their recalcitrant characteristics (Riessen & Antranikian 2001; Riffel & Brandelli 2006).

Despite the great resistance of keratins to enzyme attack, keratin-rich materials do not excessively accumulate in Nature to excessive levels. This suggests the existence of natural decomposers (or users) of such matrices (Lucas et al. 2003). Keratinolytic activity has indeed been reported in several fungi, and especially gram-positive bacteria (Gradisar et al. 2005): those best studied to date are keratinases from dermatophytic fungi, as well as from bacteria belonging to the *Bacillus* and *Streptomyces* genera (Daroit et al. 2009).

Textile and agro-industrial processes produce large amounts of keratin-rich wastes that pose serious environmental and sanitary problems. Hence, efficient ways to degrade/recycle them are urged. Classical processing of keratinous wastes, produced by poultry and leather industries, encompass alkali

hydrolysis and steam pressure-cooking – which release digestible dietary proteins that are safe for animal feeding (Mazotto et al. 2011). However, this physicochemical treatment is expensive and energy-consuming; and leads as well to loss of integrity of certain types of amino acids (Haddar et al. 2010). Hence, establishment of low environmental footprint conditions is urged in search for cleaner processes.

Keratinases have received an increased interest after realising that microbial degradation of keratin-rich matrices occurs spontaneously in nature – so an alternative, eco-friendly technology may be hypothesized in attempts to upgrade agro-industrial byproducts, especially in view of the mild reaction conditions required (Adıgüzel et al. 2009; Haddar et al. 2010). Furthermore, microbial keratinases may hold applications in the textile industry (e.g. modification of wool fibers) and in the pharmaceutical industry (e.g. production of bioactive peptides, and personal care hair removal and peeling agents). Finally, keratinases have been reported in association with the production of bio-hydrogen, biodegradable films and keratin composites; and in medicine, keratinases may find applications in breakdown of prions and improvement of ungual drug delivery (Gupta & Ramnani 2006; Mazotto et al. 2011).

Despite bacterial keratinases showing a great potential for biotechnological and industrial applications, the underlying mechanisms of enzymatic hydrolysis of keratin remain essentially unknown; hence, identification and test of new keratin-degrading isolates will be of interest to expand the pool of enzymes available, coupled with efforts to enhance enzyme activities and improve yields – so that keratinases may eventually be available in

sufficient amounts for large-scale applications and in-depth integrated studies (Kim et al. 2001; Lucas et al. 2003).

In this chapter, reports will be presented on a few novel keratinolytic bacteria isolated from the wool of Portuguese Merino sheep – including results on their feasibility to degrade common keratin-rich wastes.

4.2.3. Materials and Methods

4.2.3.1 Selection of microorganisms

A total of 156 wool-associated bacteria, previously isolated from healthy Portuguese Merino sheep, were screened for proteolytic activity on calcium caseinate agar plates, supplemented with 1 % skim milk (CCA) – as detailed in previous chapters. The 115 isolates showing caseinolytic activity on CCA were cultured on saline solution (0.85 % NaCl), supplemented with 5 wool fibres (with an average diameter of 19.5 μm and an average length of 5 cm), for 4 d at 37 ºC – in order to select keratinolytic microorganisms, based on their ability to grow and hydrolyse said fibres.

4.2.3.2 Identification of microorganisms

The wool degrading strains were identified based on partial sequencing of 16S rRNA. The 5 sequences have been deposited in GenBank, under accession numbers JF795474 – JF795476, HQ698269 and HQ698270.

4.2.3.3 Performance in submerged fermentation

The ability of wool-associated keratinolytic microorganisms to degrade distinct keratinous substrates was assessed using the following medium formulation: 0.5 g l^{-1} NaCl, 0.3 g l^{-1} K_2HPO_4 and 0.4 g l^{-1} KH_2PO_4, supplemented with 10 g l^{-1} of either feather (FB), human hair (HB), human nail (NB), or wool (WB) – both intact (W) and milled (M) (Daroit et al. 2009).

Keratinous substrates were thoroughly washed with warm tap water and then with distilled water, and dried at 45 ºC for 48 h (Syed et al. 2009). In medium formulations containing whole feathers (WFB), hair (WHB) or nails (WNB), keratinous substrates were immediately incorporated after drying and prior to medium sterilization. On the other hand, raw woven wool fabric was cut into small pieces of ca. 5x5 cm before incorporation into the broth medium (WWB). A blade grinder was used to chop all keratinous substrates selected, so as to help incorporate them in the milled media (MFB, MHB, MNB and MWB). The initial pH of all 8 medium formulations was adjusted to 7.0, before sterilization by autoclaving at 121 ºC for 20 min. Erlenmeyer flasks (500 ml), containing 100 ml of broth medium were inoculated with 1 % bacterial suspension (with an optical density of 0.6 at 660 nm), and incubated at 37 ºC for up to 7 d.

By the end of the experiment, the cultures were centrifuged (at 4 000 rpm and 4 ºC, for 10 min) and filter-sterilized (0.45 μm), and the supernatant was used as (crude) enzyme extract for further determination of keratinase activity and amount of dissolved protein.

4.2.3.4 Assay for extent of degradation

To assay for the degradation of keratinous substrates in milled form, the residual hydrolysates were considered – which encompassed cells and undigested keratinous wastes. After cultivation, cultures in those substrates were centrifuged (at 4000 rpm and 4 ºC, for 10 min), and filtered through 0.45 µm filter paper. The filter paper and debris retained thereon were then dried at 105 ºC for at least 1 h, until constant weight. The weight of the residual hydrolysates (WRH) was obtained as $WRH = [W(RH+FP)] - WFP$ where $W(RH + FP)$ denotes the sum of the weight of filter-retained particles and the filter paper itself, and *WFP* refers to the weight of only the filter paper (dried to constant weigh) (Cai et al. 2008).

When using whole keratinous substrates, degradation of keratin was determined by visual inspection and calculation of the weigh loss ratio. The weight was measured with an electronic scale: the sample was weighed after cooling on a Petri dish, in an excicator. The whole keratin substrate weight loss was recorded as dried sample weight loss (after thoroughly washing with warm tap water, and then with distilled water) – with drying conditions set at 105 ºC and 1 h; the ratio of weight loss (% WL) was calculated according to $\%WL=[(W_1-W_2)/W_1]x100$ where W_1 and W_2 are the weights of the fibers before and after treatment, respectively (Yu et al. 2005).

4.2.3.5 Assay for enzyme activity

The keratinolytic activity was determined as described elsewhere (Suntornsuk & Suntornsuk 2003), using Keratin Azure K-8500 (Sigma Aldrich,

USA) as substrate. Briefly, the reaction mixture containing 1 ml of enzyme solution and 1 ml of keratin azure suspension was incubated at 37 °C for 1 h; then, it was boiled for 5 min, and centrifuged at 4 000 rpm for 15 min to remove unutilized substrate. The supernatant was spectrophotometrically assayed for release of the azo-dye, at 595 nm. A mixture of enzyme and substrate was boiled before the assay and taken as control. All assays were done in duplicate. One unit (U) of keratinase was defined as the amount of enzyme causing an absorbance increment of 0.1 between sample and control, at 595 nm, within 1 h under the indicated assay conditions.

For total protein determination, the BCA™ Protein Assay Kit (Pierce, USA) was used, according to supplier's instructions.

4.2.3.6 Effects of processing conditions

The experiments pertaining to the effect of pH upon enzyme stability were carried out by incubating the enzyme solution at pH 5, 7 and 9, in 100 mM of various buffers (acetate buffer, pH 5; phosphate buffer, pH 7; and Tris-HCl buffer, pH 9), at 30 ºC, for 10, 30 and 60 min.

The effect of temperature on enzyme stability was ascertained by incubating the enzyme solution at 4, 30 and 50 ºC, in 100 mM phophate buffer (pH 8), for 10, 30 and 60 min.

4.2.4. Results

A total of 115 caseinase-producing wild strains of bacteria, previously isolated from healthy Portuguese Merino sheep, were screened for their ability

to grow and hydrolyze wool fibers as sole source of carbon and nitrogen. After an incubation period of 4 d, the fragmentation of wool fibers assessed by visual inspection, differed significantly among the 115 isolates. The outstanding activities of 5 of those isolates eventually justified their selection for complementary work of identification, coupled with assessment of feasibility to degrade distinct keratinous substrates.

Analysis of the 16s rRNA partial gene sequences revealed that all 5 isolates belong to the genus *Bacillus*. Complementary phylogenetic analysis indicated that they belong to *B. subtilis/licheniformis* with a high degree of homology.

The wool-degrading strains *Bacillus* sp. HTS 85, *Bacillus* sp. HTS 102, *Bacillus* sp. HTS 119A, *Bacillus* sp. HTS 120 and *Bacillus* sp. HTS 126 were then tested for their capacity to degrade several keratin-containing wastes. Cultivation thereon permitted detection of keratinase production. Maximum levels were obtained on (milled or whole) feathers, followed by wool, as unfolded by the high percent of whole keratin substrate weight losses, and by the decrease in weight of the residual hydrolysates (see Table 4.2.1).

The extents of hydrolysis of the whole keratinous substrate were variable, probably due to structural differences of the (heterogeneous) surface of such substrates. Nevertheless, there was always a strong positive correlation between keratinase activity and percent weight loss, characterized by high correlation coefficients ($R>0.90$). *Bacillus* sp. HTS 85 and *Bacillus* sp. HTS 126 produced maximum hydrolysis levels when milled or whole feathers were used. By 96 h of incubation, only ca. 30% of the initial feather substrate remained intact in the fermentation broth. On the other hand, wool was degraded by those two isolates

only to ca. 20% of its initial weight, whereas hair and nails did not go beyond 5% (Table 4.2.1). Conversely, *Bacillus* sp. HTS 120 conveyed the lowest rates of hydrolysis of all substrates in all medium formulations tested with.

Table 4.2.1 Hydrolysis degree of various keratinous substrates, expressed as % weight loss (% WL) and weight of residual hydrolysates (%WRH), keratinase activity, expressed as U ml^{-1}, and soluble protein content, expressed as μg ml^{-1}, in terms of (W) whole keratin and (M) milled keratin, under submerged cultivation of HTS 85, HTS 102, HTS 119A, HTS 120 and HTS 126 strains. All data presented are the means of two independent replicates.

Strain	Incubation period (h)	Substrate Treatment	HAIR			WOOL			FEATHER			NAIL		
			hydolysis*	Keratinase (U ml^{-1})	Protein (μg ml^{-1})	hydolysis*	Keratinase (U ml^{-1})	Protein (μg ml^{-1})	hydolysis*	Keratinase (U ml^{-1})	Protein (μg ml^{-1})	hydolysis*	Keratinase (U ml^{-1})	Protein (μg ml^{-1})
HTS 85	24	W	2,13	0,55	13,90	5,21	1,45	15,77	21,55	2,57	17,50	3,20	0,89	13,94
		M	0,67	0,76	15,27	0,44	1,95	16,96	0,89	4,26	19,64	0,63	0,70	15,21
	48	W	3,53	0,86	17,41	9,81	1,76	22,65	54,55	3,17	25,92	4,40	1,17	17,05
		M	0,63	1,26	20,75	0,21	2,80	25,39	0,57	5,90	27,14	0,53	1,58	20,97
	96	W	4,45	1,10	20,81	20,40	2,67	25,75	64,15	4,83	27,31	5,50	1,88	20,45
		M	0,51	1,89	22,31	0,09	2,72	27,61	0,11	6,65	29,00	0,41	2,21	22,69
HTS 102	24	W	1,10	0,36	12,38	3,09	0,80	15,38	10,99	1,53	16,07	0,96	0,41	12,44
		M	0,70	0,35	10,98	0,45	1,21	14,44	0,91	2,68	18,21	0,66	0,26	11,21
	48	W	1,62	0,87	15,56	5,37	1,17	18,49	21,57	1,83	20,52	1,58	0,72	15,52
		M	0,67	0,97	15,28	0,42	2,00	16,77	0,83	3,21	20,19	0,62	0,47	16,10
	96	W	2,49	1,18	18,01	6,90	2,02	20,23	35,84	2,44	22,11	1,87	0,86	17,90
		M	0,60	1,48	18,76	0,39	2,38	21,91	0,76	5,28	22,80	0,58	0,94	19,14
HTS 119A	24	W	1,60	0,18	10,72	3,37	1,02	14,27	11,26	1,85	16,64	1,35	0,26	12,40
		M	0,68	0,57	11,96	0,46	1,31	18,38	0,88	2,26	18,68	0,68	1,03	11,98
	48	W	1,30	1,04	16,33	4,96	1,46	18,69	24,19	2,17	20,65	2,04	0,90	17,15
		M	0,67	1,27	15,83	0,42	1,68	20,00	0,82	3,88	20,15	0,62	1,53	16,91
	96	W	2,25	1,34	18,44	7,59	1,68	19,91	31,78	2,41	22,20	2,44	0,91	18,26
		M	0,64	1,58	18,66	0,37	2,04	22,51	0,76	5,42	23,67	0,60	1,86	18,87
HTS 120	24	W	0,50	0,12	7,84	1,42	0,26	10,03	7,41	0,77	10,85	0,80	0,12	11,34
		M	0,68	0,29	10,18	0,49	0,31	11,97	0,88	0,73	14,27	0,62	0,16	11,10
	48	W	1,10	0,36	12,18	2,42	0,42	14,57	9,11	1,28	15,46	1,31	0,23	13,60
		M	0,67	0,62	13,34	0,47	0,84	15,73	0,83	1,16	19,48	0,61	0,36	13,53
	96	W	1,35	0,89	15,71	5,15	1,26	16,68	12,15	1,38	16,96	1,64	0,57	15,36
		M	0,66	0,84	16,76	0,43	1,00	19,98	0,81	2,42	21,65	0,59	0,67	16,71
HTS 126	24	W	3,30	0,27	14,30	7,15	1,71	18,78	23,76	2,04	18,83	2,71	0,75	16,81
		M	0,69	1,00	16,18	0,47	1,31	19,70	0,90	2,90	19,14	0,64	0,72	17,09
	48	W	3,95	0,69	18,35	9,45	2,33	22,09	54,10	2,67	24,58	4,69	0,94	18,34
		M	0,62	1,52	20,93	0,32	2,63	23,19	0,64	4,64	22,40	0,53	1,42	20,91
	96	W	5,20	1,17	20,55	20,85	3,22	26,00	67,67	3,74	25,90	5,55	1,66	20,63
		M	0,56	2,17	22,83	0,10	2,89	25,05	0,17	5,88	27,05	0,34	2,25	22,89

* Measured as % of weigh loss for whole (W) keratin substrates and weigh of residual hydrolysates (g) for milled (M) keratin substrates

Bacillus sp. HTS 102 and *Bacillus* sp. HTS 119A strains exhibited identical patterns of keratin degradation. Their enzyme production was consistent with this observation, since the keratinase activity curves (presented in Table 4.2.1) showed similar increases. Even though keratinase production does not differ much among isolates acting on whole keratin substrates, the resulting percent WL was quite dissimilar. The keratinase activity values presented in Table 4.2.1

further confirm that *Bacillus* sp. HTS 120 performed the worst in terms of keratinase synthesis. It should be emphasized that the keratinase activity measured was always higher for the milled keratin medium formulations than for their whole keratin counterparts (see Figure 4.2.1).

The production of soluble protein by the 5 isolates, during cultivation on 8 different medium formulations, was also monitored (see Table 4.2.1). One easily realises that the production of soluble protein underwent a trend similar to the evolution of keratinase activity. The high protein content recorded in fermented broths suggests their feasibility for further concentration, towards eventual use as a protein ingredient of animal feeds.

To infer on thermal stability, the enzyme solution from each of the 5 *Bacillus* strains was incubated in phosphate buffer (100 mM, pH 8) for 10, 30 and 60 min, at various temperatures in the absence of substrate; the enzyme activity prior to heating was taken as reference (i.e. 100%). The results depicted in Figure 4.2.2A indicate that the extent of loss of activity was chiefly dependent on temperature (as expected): enzymes were relatively stable at 30 ºC for 30 min, but decreases in stability were in general observed at higher temperatures and longer times (Figure 4.2.2A). At 50 ºC, the enzymatic activities of all 5 isolates dropped to 10-20% of their initial values (Figure 4.2.2A).

A remarkable decrease in stability was observed at pH 5 (Figure 4.2.2B), for all isolates. On the other hand, greater enzyme stabilities (ca. 80%) were recorded at pH 7 and 9 for all *Bacillus* sp. isolates – except for the keratinase synthesized by *Bacillus* sp. HTS 126, which lost ca. half of its activity by 60 min at pH 7.

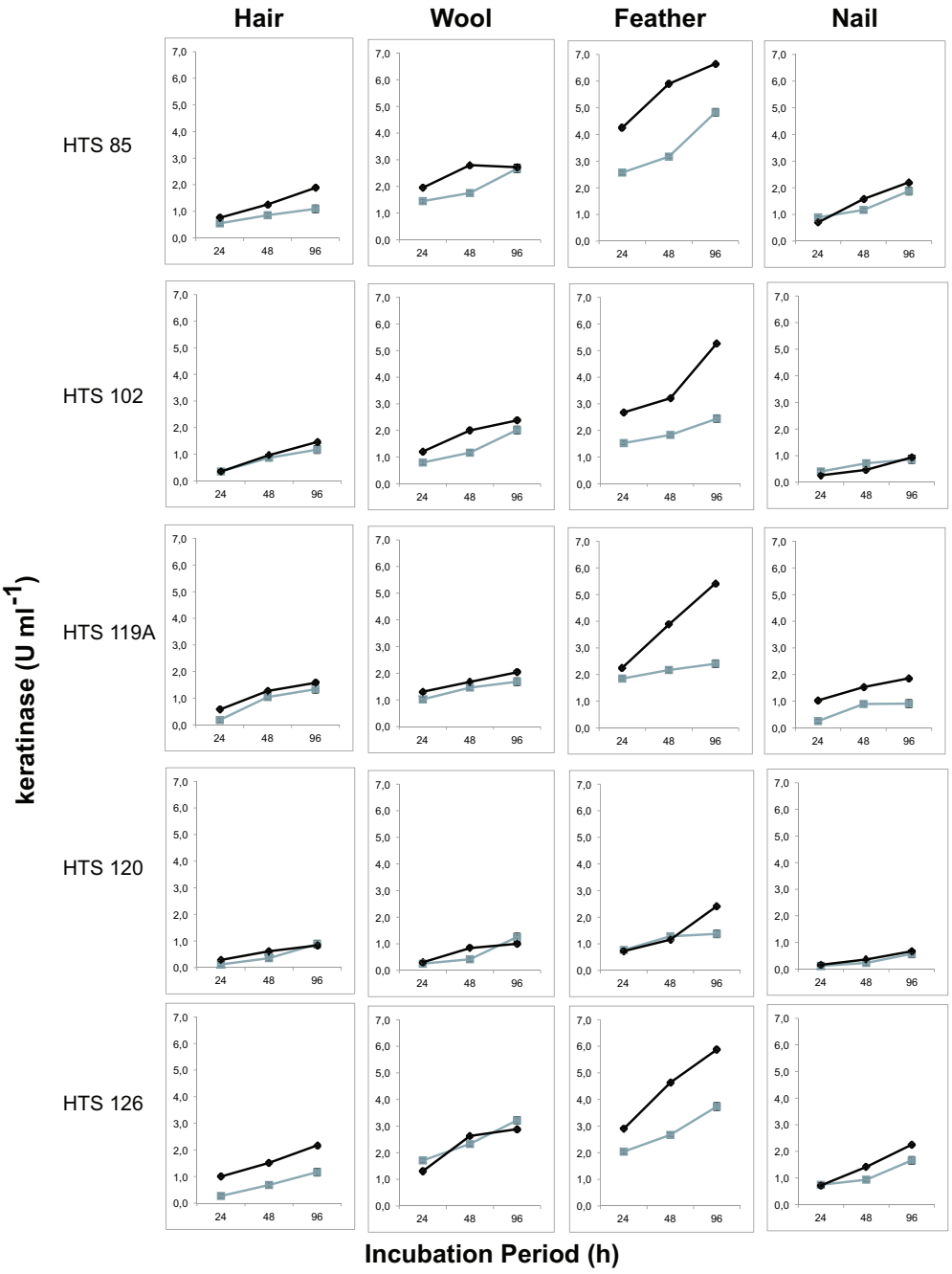


Figure 4.2.1 Keratinase activity, expressed as U ml^{-1}, in terms of whole keratin (grey lines) and milled keratin (black lines), under submerged cultivation of HTS 85, HTS 102, HTS 119A, HTS 120 and HTS 126 strains. All data presented are the means of two independent replicates.

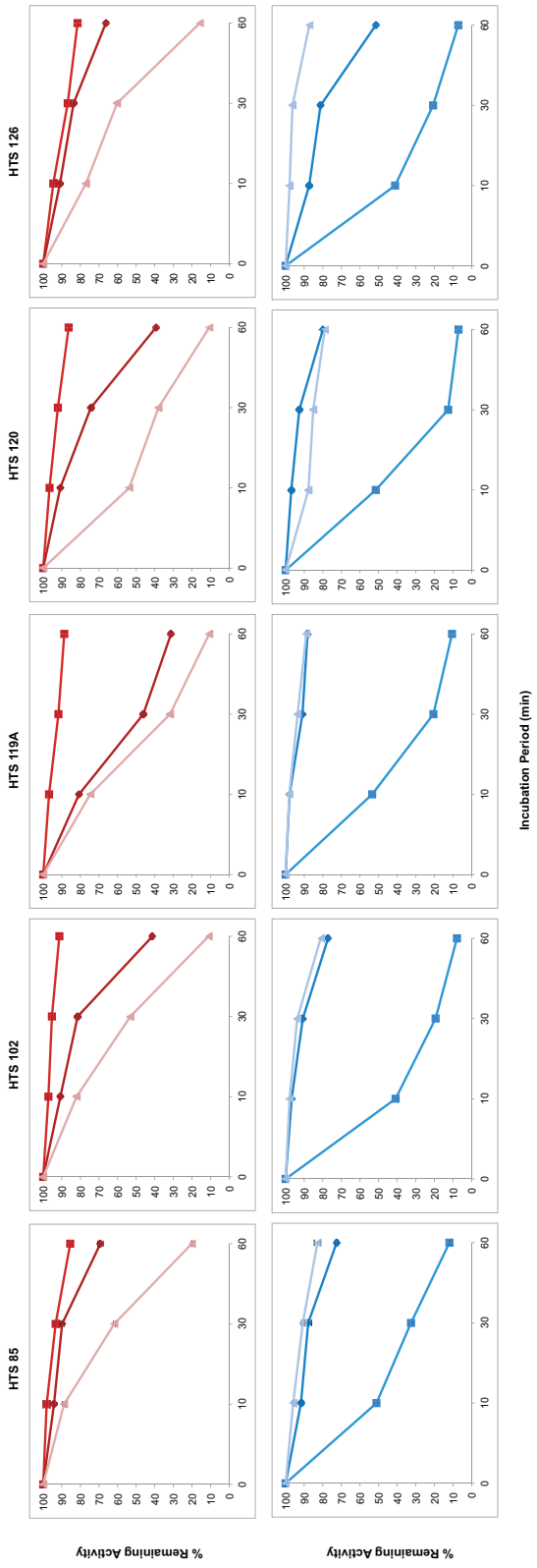


Figure 4.2.2 Decay of keratinolytic activity produced by various strains, under several (A) temperatures and (B) pH values: T= 4 ºC, pH= 5 (■), T= 37 ºC, pH= 7 (◆) and T= 50 ºC, pH= 9 (light and dark ▲, respectively). Light lines and symbols refer to temperature data, whereas dark lines and symbols refer to pH data. All data presented are the means of two independent replicates.

4.2.5. Discussion

Previous studies have indicated that *Bacillus* spp. are ubiquitous sources of keratinases (Brandelli et al. 2010). On the other hand, levels of keratinase produced by our 5 *Bacillus* isolates varied over time thus suggesting their inducible character. Even though a few authors have reported constitutive or partially inducible keratinase production by *Bacillus* strains, most keratinolytic microorganisms produce truly inducible keratinase when keratinous materials are used as sole substrates (Kim et al. 2001; el-Refai et al. 2005; Cai et al. 2008).

An interesting feature associated with our data is that both α-keratin (from hair, wool and nails) and β-keratin (from feathers) are appropriate substrates for the wool-associated *Bacillus* strains isolated. The nature of the keratinous substrate and its form influenced the capacity of the microorganism to degrade it; e.g. visual disruption of hair and nails (on whole broth media) occurred only in old cultures (5–7 d). The distinct structural conformations that are characteristic of α- and β-keratins may explain the differences recorded between degradation of feathers and of the other substrates tested (Daroit et al. 2009).

The correlation unfolded between the levels of keratinase produced and the degree of hydrolysis observed suggests that biodegradation of keratinous substrates is indeed associated with keratinase activity. However, further experiments are recommended, to conclude on whether only one or a set of peptidases account for keratin hydrolysis.

Milled keratinous substrates were, in general, degraded more readily and to a greater extent than their intact counterparts, probably because milling and

heating brought about physical modifications that make keratin molecules more susceptible to enzymatic digestion. In particular, the ability of *Bacillus* sp. HTS 85 and *Bacillus* sp. HTS 126 to efficiently and rapidly hydrolyse whole wool and feather makes them particularly promising, because no requirement for milling (or any other prior treatment) reduces overall processing costs.

An increase in pH was observed for all isolates and media – with a maximum of 9.5 in the case of *Bacillus* sp. HTS 85 grown in whole feather broth for up to 3 d. This trend is similar to that observed with other microorganisms bearing keratinolytic features (Riffel & Brandelli 2006; Daroit et al. 2009). Such a tendency for alkalinization of the medium has been associated with release of ammonia via deamination of peptides and amino acids from the first stage of keratin degradation. The exact mechanism underlying keratinolysis has not yet to been fully elucidated, yet deamination leading to alkaline conditions appears to aid in substrate swelling, sulphitolysis and subsequent proteolytic attack (Cai et al. 2008).

Overall, our findings on keratinolytic activity revealed the potential of the 5 wild strains to act as vectors for eco-friendly bioconversion of keratin-rich wastes to animal feed, or even added-value products.

4.2.6. Conclusions

Owing to the inducible character of most microbial keratinases, their potential application in keratin-rich waste upgrade justifies large-scale production of their source microorgisms. Keratinases may indeed prove useful in handling textile and agro-industrial residual matrices.

The 5 wool-associated *Bacillus* sp. tested successfully add to a number of other strains of the same genus with activity alike, and possess a high degree of homology with *B. subtilis/licheniformis*. These isolates appear promising toward degradation of feathers and other keratinous materials, to eventually convert crude keratin mixtures into ingredients for feed formulation. However, further tests are still required to fully validate this hypothesis.

Acknowledgments

A.C. Queiroga held a Ph.D. fellowship (ref. SFRH/BD/19212/2004), granted by Fundação para a Ciência e a Tecnologia (Portugal) and supervised by F.X. Malcata. Partial funding to cover research expenses was via projects "GoBlue" (POCTI 13-02-03-SDR-01254), granted by Agência de Inovação (Portugal); and "BioTex" (POCI/CTM/58312/2004), granted by Fundação para a Ciência e a Tecnologia (Portugal).

4.2.7. References

Adıgüzel A, Bitlisli B, Yaşa İ, Eriksen N. 2009. Sequential secretion of collagenolytic, elastolytic, and keratinolytic proteases in peptide-limited cultures of two *Bacillus cereus* strains isolated from wool. J Appl Microbiol 107:226-234.

Brandelli A, Daroit DJ, Riffel A. 2010. Biochemical features of microbial keratinases and their production and applications. Appl Microbiol Biotechnol 85:1735-1750.

Cai C-G, Lou B-G, Zheng X-D. 2008. Keratinase production and keratin degradation by a mutant strain of *Bacillus subtilis*. J Zhejiang Univ Sci B 9:60-67.

Daroit DJ, Corría AP, Brandelli A. 2009. Keratinolytic potential of a novel *Bacillus* sp. P45 isolated from the Amazon basin fish *Piaractus mesopotamicus*. Int Biodeter Biodegrad 63:358-363.

el-Refai HA, AbdelNaby MA, Gaballa A, el-Araby MH, Abdel Fattah AF. 2005. Improvement of the newly isolated *Bacillus pumilus* FH9 keratinolytic activity. Process Biochem 40:2325-2332.

Gradisar H, Friedrich J, Krizaj I, Jerala R. 2005. Similarities and specificities of fungal keratinolytic proteases: comparison of keratinases of *Paecilomyces marquandii* and *Doratomyces microsporus* to some known proteases. Appl Environ Microbiol 71:3420-3426.

Gupta R, Ramnani P. 2006. Microbial keratinases and their prospective applications: an overview. Appl Microbiol Biotechnol 70:21-33.

Haddar A, Sellami-Kamoun A, Fakhfakh-Zouari N, Hmidet N, Nasri M. 2010. Characterization of detergent stable and feather degrading serine proteases from *Bacillus mojavensis* A21. Biochem Eng J 51:53-63.

Kim JM, Lim WJ, Suh HJ. 2001. Feather-degrading *Bacillus* species from poultry waste. Process Biochem 37:287-291.

Lateef A, Oloke JK, Kana EBG, Sobowale BO, Ajao SO, Bello BY. 2010. Keratinolytic activities of a new feather-degrading isolate of *Bacillus cereus* LAU 08 isolated from Nigerian soil. Int Biodeter Biodegrad 64:162-165.

Lucas FS, Broennimann O, Febbraro I, Heeb P. 2003. High diversity among feather-degrading bacteria from a dry meadow soil. Microb Ecol 45:282-290.

Mazotto A, de Melo A, Macrae A, Rosado A, Peixoto R, Cedrola S, Couri S, Zingali R, Villa A, Rabinovitch L, Chaves J, Vermelho A. 2011. Biodegradation of feather waste by extracellular keratinases and gelatinases from *Bacillus* spp. World J Microbiol Biotechnol 27:1355-1365.

Riessen S, Antranikian G. 2001. Isolation of *Thermoanaerobacter keratinophilus* sp. nov., a novel thermophilic, anaerobic bacterium with keratinolytic activity. Extremophiles 5:399-408.

Riffel A, Brandelli A. 2006. Keratinolytic bacteria isolated from feather waste. Braz J Microbiol 37:395-399.

Suntornsuk W, Suntornsuk L. 2003. Feather degradation by *Bacillus* sp. FK 46 in submerged cultivation. Bioresource Technol 86:239-243.

Syed DG, Lee JC, Li WJ, Kim CJ, Agasar D. 2009. Production, characterization and application of keratinase from *Streptomyces gulbargensis.* Bioresource Technol 100:1868-1871.

Yu X-W, Guan W-J, Li Y-Q, Guo T-J, Zhou J-D. 2005. A biological treatment technique for wool textile. Braz Arch Biol Techn 48:675-680.

Chapter 5

Final Remarks

Section 5.1

Concluding remarks

An integrated, overall view of the conclusions deriving from the work developed and presented in this dissertation is provided below.

Despite the ubiquitous nature of proteolytic enzymes, a few habitats constitute obviously important microbial sources thereof; hence, ecological issues are in order for better (and further) understanding. The isolation of bacterial strains from healthy raw wool from Portuguese Merino ewe's breed in this work shed further light onto this still poorly known habitat. A total of 156 morphotypes were initially isolated in an effort focused on screening for mesophilic and thermophilic bacteria exhibiting relevant proteolytic activities. Data revealed that bacteria are present throughout the sheep whole body surface, but follow a non-uniform distribution; there was also a strong indication that a distinction ought to be made between abundance and diversity, as the various body parts presented total bacterial numbers correlating negatively to bacterial diversity. Several bacterial isolates exhibited remarkably strong

proteolytic activities at temperatures below 60 ºC – particularly *Bacillus* sp. HTS 102 and *Bacillus* sp. HTS 119A, which were able to grow and release protease at 37 ºC, while operating well under alkaline conditions; altogether, they appeared as noteworthy candidates for alternative applications in the wool industry.

The classical low productivity of fermentation processes remains a major constraint for application of many microbial metabolites (including enzymes) at industrial level – so a comprehensive effort was implemented to enhance the rate of protease synthesis by *Bacillus* sp. HTS 102. Its protease productivity was increased from 24.6 U/mL at basal fermentation conditions, up to 56.8 U/mL when optimized fermentation conditions were at stake. At this point, cell-free (crude) extracts were used to assess protease applicability in wool industry – via laboratory-scale testing. Two sets of experiments were thus made: one regarding the potential applications of its crude protease in wool processing and laundry; and on other regarding potential use thereof for biodegradation of keratinous materials.

Decreased area shrinkage, combined with increasing wettability and softness degree – and without substantial strength loss, suggested the suitability of said protease to impart improvements to woolen fabrics. Additionally, said enzyme exhibited an excellent performance in the presence of commercial laundry detergents, and a good washing performance of stains of proteinaceous nature (e.g. chocolate, egg yolk and tomato). Therefore, a promising role for eventual biotechnological applications of protease from *Bacillus* sp. HTS 102 was unfolded. Furthermore, the 5 wool-associated *Bacillus* sp. used in the second dataset may be employed in processes aimed at bioconverting keratin-rich

wastes (e.g. feather or other keratinous material) into feed ingredients or added-value products.

Overall, a high potential of our wool-associated bacteria exists for practical application in several steps of wool processing and laundering, as well as of upgrading of keratin-rich wastes. This deserves complementary studies, which will certainly help further understand the mechanism of action of said proteases in keratinous substrates of interest – besides allowing a more rational development and optimization of their industrial uses.

Section 5.2

Future Prospects

The genetic diversity present in nature is still a major asset in terms of proteolytic enzymes for use in textile and agro-industries; the great many habitats on Earth justify comprehensive efforts of bioprospection. Nevertheless, it is clear from the data presented in this thesis that most research efforts in this field appear to be "application-driven" – with anticipated target applications being the trigger for efforts to isolate novel microorganisms. Therefore, it is important to shift a considerable amount of research effort from novel microorganism prospection (via classical or modern techniques) to screening for alternative applications of already existing (and somehow characterized) microbial isolates. A comprehensive research effort in this field can open new research avenues, while helping understand the ecology of the microflora from several unexploited habitats.

Since crude extracts of proteases and other metabolites are frequently used to commercial levels in the textile and agro-industries, purification of such

extracts is desirable before attempts to extend the knowledge on the operational features of any metabolite. A purification protocol for the protease produced by *Bacillus* sp. HTS 102 should accordingly be tested and implemented, and further kinetic and biophysical characterization of said enzyme(s) could be accomplished. Finally, the potential use of the aforementioned enzyme(s) for several biotechnological applications in the textile and agro-industries may justify its large-scale production, so the predesign of a process for continuous, efficient and economically viable enzyme production at larger-scale should take place.

Chapter 6

References

2006. AATCC Testing Method 135-2004, Dimensional changes of fabrics after home laundering. In American Association of Textile Chemists and Colourists. pp. 231–234.

Abusham RA, Rahman R, Salleh AB, Basri M. 2009. Optimization of physical factors affecting the production of thermo-stable organic solvent-tolerant protease from a newly isolated halo-tolerant *Bacillus subtilis* strain Rand. Microb Cell Fact 8:20-28.

Adıgüzel A, Bitlisli B, Yaşa İ, Eriksen N. 2009. Sequential secretion of collagenolytic, elastolytic, and keratinolytic proteases in peptide-limited cultures of two *Bacillus cereus* strains isolated from wool. J Appl Microbiol 107:226-234.

Akhurst RJ, Lyness EW, Zhang QY, Cooper DJ, Pinnock DE. 1997. A 16S rRNA gene oligonucleotide probe for identification of *Bacillus thuringiensis* isolates from sheep fleece. J Invertebr Pathol 69:24-30.

Akolkar A, Bharambe N, Trivedi S, Desai A. 2009. Statistical optimization of medium components for extracellular protease production by an extreme haloarchaeon, *Halobacterium* sp. SP1(1). Lett Appl Microbiol 48:77-83.

Alcaraz LD, Moreno-Hagelsieb G, Eguiarte LE, Souza V, Herrera-Estrella L, Olmedo G. 2010. Understanding the evolutionary relationships and major traits of *Bacillus* through comparative genomics. BMC Genomics 11:332-348.

Anandan D, Marmer W, Dudley R. 2007. Isolation, characterization and optimization of culture parameters for production of an alkaline protease isolated from *Aspergillus tamarii*. J Ind Microbiol Biotechnol 34:339-347.

Anbu P, Annadurai G, Lee J-F, Hur B-K. 2009. Optimization of alkaline protease production from *Shewanella oneidensis* MR-1 by response surface methodology. J Chem Technol Biotechnol 84:54-62.

Anbu P, Gopinath SCB, Hilda A, Lakshmipriya T, Annadurai G. 2007. Optimization of extracellular keratinase production by poultry farm isolate *Scopulariopsis brevicaulis*. Bioresource Technol 98:1298-1303.

Anbu P, Gopinath SCB, Hilda A, Priya TL, Annadurai G. 2005. Purification of keratinase from poultry farm isolate – *Scopulariopsis brevicaulis* and statistical optimization of enzyme activity. Enzyme Microb Technol 36:639-647.

Antão CM, Malcata FX. 2005. Plant serine proteases: biochemical, physiological and molecular features. Plant Physiol Biochem 43:637-650.

Anwar A, Saleemuddin M. 1998. Alkaline proteases: A review. Bioresource Technol 64:175-183.

Araújo R, Casal M, Cavaco-Paulo A. 2008. Application of enzymes for textile fibres processing. Biocatal Biotransf 26:332-349.

Arnold FH, Volkov AA. 1999. Directed evolution of biocatalysts. Curr Opin Chem Biol 3:54-59.

Banik RM, Prakash M. 2004. Laundry detergent compatibility of the alkaline protease from *Bacillus cereus*. Microbiol Res 159:135-140.

Beilen JB, Li Z. 2002. Enzyme technology: an overview. Curr Opin Biotechnol 13:338-344.

Benucci I, Liburdi K, Garzillo AMV, Esti M. 2011. Bromelain from pineapple stem in alcoholic-acidic buffers for wine application. Food Chem 124:1349-1353.

Beynon RJ, Bond JS. 2001. Proteolytic Enzymes: A Practical Approach. Oxford: Oxford University Press.

Bhaskar N, Benila T, Radha C, Lalitha RG. 2008. Optimization of enzymatic hydrolysis of visceral waste proteins of Catla (*Catla catla*) for

preparing protein hydrolysate using a commercial protease. Bioresource Technol 99:335-343.

Bhaskar N, Mahendrakar NS. 2008. Protein hydrolysate from visceral waste proteins of Catla (*Catla catla*): Optimization of hydrolysis conditions for a commercial neutral protease. Bioresource Technol 99:4105-4111.

Bhaskar V, Raj JTR, Kandasamy SKJ, Vijaykumar P, Achary A. 2008. Optimization of production of subtilisin in solid substrate fermentation using response surface methodology. Afr J Biotechnol 7:2286-2291.

Brandelli A, Daroit DJ, Riffel A. 2010. Biochemical features of microbial keratinases and their production and applications. Appl Microbiol Biotechnol 85:1735-1750.

Brandelli A. 2008. Bacterial keratinases: useful enzymes for bioprocessing agroindustrial wastes and beyond. Food Bioprocess Technol 1:105-116.

Breithaupt H. 2001. The hunt for living gold. EMBO Rep 2:968-071.

Burg BVd. 2003. Extremophiles as a source for novel enzymes. Curr Opin Microbiol 6:213-218.

Burton SG, Cowan DA, Woodley JM. 2002. The search for the ideal biocatalyst. Nat Biotechnol 20:37-45.

Cai C-G, Lou B-G, Zheng X-D. 2008. Keratinase production and keratin degradation by a mutant strain of *Bacillus subtilis*. J Zhejiang Univ Sci B 9:60-67.

Cai CG, Chen JS, Qi JJ, Yin Y, Zheng XD. 2008. Purification and characterization of keratinase from a new *Bacillus subtilis* strain. J Zhejiang Univ-SCI B 9:713-720.

Cai CG, Zheng XD. 2009. Medium optimization for keratinase production in hair substrate by a new *Bacillus subtilis* KD-N2 using response surface methodology. J Ind Microbiol Biotechnol 36:875-883.

Cai S-B, Huang Z-H, Zhang X-Q, Cao Z-J, Zhou M-H, Hong F. 2011. Identification of a keratinase-producing bacterial strain and enzymatic study for its improvement on shrink resistance and tensile strength of wool- and polyester-blended fabric. Appl Biochem Biotechnol 163:112-126.

Cardamone JM, Yao J, Nunez A. 2004. DCCA shrinkproofing of wool: Part I: Importance of antichlorination. Textile Res J 74:555-560.

Cardamone JM, Yao J, Phillips JG. 2005. Combined bleaching, shrinkage prevention, and biopolishing of wool fabrics. Textile Res J 75:169-174.

Castro G, Santopietro L, Siñeriz F. 1992. Acid pullulanase from *Bacillus polymyxa* MIR-23. Appl Biochem Biotechnol 37:227-233.

Chauhan B, Gupta R. 2004. Application of statistical experimental design for optimization of alkaline protease production from *Bacillus* sp. RGR-14. Process Biochem 39:2115-2122.

Chen BY, Wang HT. 2008. Utility of enzymes from *Fibrobacter succinogenes* and *Prevotella ruminicola* as detergent additives. J Ind Microbiol Biotechnol 35:923-930.

Chen K-N, Huang J-C, Chung C-I, Kuo W-Y, Chen M-J. 2011. Identification and characterization of H10 enzymes isolated from *Bacillus cereus* H10 with keratinolytic and proteolytic activities. World J Microbiol Biotechnol 27:349-358.

Chen QH, Au KF, Yuen CWM, Yeung KW. 2004. An analysis of the felting shrinkage of plain knitted wool fabrics. Textile Res J 74:399-404.

Cheng K, Lu FP, Li M, Liu LL, Liang XM. 2010. Purification and biochemical characterization of a serine alkaline protease TC4 from a new isolated *Bacillus alcalophilus* TCCC11004 in detergent formulations. Afr J Biotechnol 9:4942-4953.

Chi Z, Ma C, Wang P, Li HF. 2007. Optimization of medium and cultivation conditions for alkaline protease production by the marine yeast *Aureobasidium pullulans*. Bioresource Technol 98:534-538.

Chu W-H. 2007. Optimization of extracellular alkaline protease production from species of *Bacillus*. J Ind Microbiol Biotechnol 34:241-245.

Cortez J, Bonner PLR, Griffin M. 2004. Application of transglutaminases in the modification of wool textiles. Enzyme Microb Technol 34 64-72.

Daroit DJ, Corría AP, Brandelli A. 2009. Keratinolytic potential of a novel *Bacillus* sp. P45 isolated from the Amazon basin fish *Piaractus mesopotamicus*. Int Biodeter Biodegrad 63:358-363.

Demirjian DC, Morís-Varas F, Cassidy CS. 2001. Enzymes from extremophiles. Curr Opin Chem Biol 5:144-151.

Devaraj KB, Kumar PR, Prakash V. 2008. Purification, characterization, and solvent-induced thermal stabilization of ficin from *Ficus carica*. J Agric Food Chem 56:11417-11423.

Devi NKA, Balakrishnan K, Gopal R, Padmavathy S. 2008. *Bacillus clausii* MB9 from the east coast regions of India: isolation, biochemical characterization and antimicrobial potentials. Curr Sci 95:627-636.

Dienes D, Borjesson J, Hagglund P, Tjerneld F, Liden G, Reczey K, Stalbrand H. 2007. Identification of a trypsin-like serine protease from *Trichoderma reesei* QM9414. Enzyme Microb Technol 40:1087-1094.

Doddapaneni KK, Tatineni R, Vellanki RN, Gandu B, Panyala NR, Chakali B, Mangamoori LN. 2007. Purification and characterization of two novel extracellular proteases from *Serratia rubidaea*. Process Biochem 42:1229-1236.

Dubey VK, Pande M, Singh BK, Jagannadham MV. 2007. Papain-like proteases: applications of their inhibitors. Afr J Biotechnol 6:1077-1086.

Elíades L, Cabello M, Voget C, Galarza B, Saparrat M. 2010. Screening for alkaline keratinolytic activity in fungi isolated from soils of the biosphere reserve "Parque Costero del Sur" (Argentina). World J Microbiol Biotechnol 26:2105-2111.

Esposito TS, Amaral IPG, Buarque DS, Oliveira GB, Carvalho LB, Bezerra RS. 2009. Fish processing waste as a source of alkaline proteases for laundry detergent. Food Chem 112:125-130.

Eyal E. 1963. Shorn and unshorn Awassi sheep. 4. Skin temperature and changes in temperature and humidity in fleece and its surface. J Agric Sci 60:183-193.

Fakhfakh-Zouari N, Haddar A, Hmidet N, Frikha F, Nasri M. 2010. Application of statistical experimental design for optimization of keratinases production by *Bacillus pumilus* A1 grown on chicken feather and some biochemical properties. Process Biochem 45:617-626.

Fang Y, Liu S, Wang S, Lv M. 2009. Isolation and screening of a novel extracellular organic solvent-stable protease producer. Biochem Eng J 43:212-215.

Fonollosa J, Campos L, Marti M, Maza A, Parra JL, Coderch L. 2004. X-Ray diffraction analysis of internal wool lipids. Chem Phys Lipids 130:159-166.

Frazier WC, Rupp P. 1928. Studies on the proteolytic bacteria of milk I. A medium for the direct isolation of caseolytic milk bacteria. J Bacteriol 16:57-63.

Frazier WC, Rupp P. 1928. Studies on the proteolytic bacteria of milk I. A medium for the direct isolation of caseolytic milk bacteria. J Bacteriol 16:57-63.

Fujinami S, Fujisawa M. 2010. Industrial applications of alkaliphiles and their enzymes – past, present and future. Environ Technol 31:845 - 856.

Galland-Irmouli AV, Fleurence J, Lamghari R, Lucon M, Rouxel C, Barbaroux O, Bronowicki JP, Villaume C, Gueant JL. 1999. Nutritional value of proteins from edible seaweed *Palmaria palmata* (dulse). J Nutrit Biochem 10:353-359.

Garcia-Kirchner O, Bautista-Ramirez M, Segura-Granados M. 1998. Submerged culture screening of two strains of *Streptomyces* sp. with high keratinolytic activity. Appl Biochem Biotechnol 70-72:277-284.

Ge FY, Cai ZS, Zhang HY, Zhang RP. 2009. Transglutaminase treatment for improving wool fabric properties. Fiber Polym 10:787-790.

Genckal H, Tari C. 2006. Alkaline protease production from alkalophilic *Bacillus* sp. isolated from natural habitats. Enzyme Microb Technol 39:703-710.

Geok LP, Razak CNA, Rahman RNZ. 2003. Isolation and screening of an extracellular organic solvent-tolerant protease producer. Biochem Eng J 13:73-77.

Gerday C, Aittaleb M, Bentahir M, Chessa J-P, Claverie P, Collins T, d'Amico S, Dumont J, Garsoux G, Georlette D, Hoyoux A, Lonhienne T, Meuwis M-A, Feller G. 2000. Cold-adapted enzymes: from fundamentals to biotechnology. TIBTECH 18:103-107.

Gessesse A, Hatti-Kaul R, Gashe BA, Mattiasson B. 2003. Novel alkaline proteases from alkaliphilic bacteria grown on chicken feather. Enzyme Microb Technol 32:519-524.

Giongo J, Lucas F, Casarin F, Heeb P, Brandelli A. 2007. Keratinolytic proteases of *Bacillus* species isolated from the Amazon basin showing remarkable de-hairing activity. World J Microbiol Biotechnol 23:375-382.

Giongo J, Lucas F, Casarin F, Heeb P, Brandelli A. 2007. Keratinolytic proteases of *Bacillus* species isolated from the Amazon basin showing remarkable de-hairing activity. World J Microbiol Biotechnol 23:375-382.

Gradisar H, Friedrich J, Krizaj I, Jerala R. 2005. Similarities and specificities of fungal keratinolytic proteases: comparison of keratinases of *Paecilomyces marquandii* and *Doratomyces microsporus* to some known proteases. Appl Environ Microbiol 71:3420-3426.

Gu XB, Zheng ZM, Yu HQ, Wang J, Liang FL, Liu RL. 2005. Optimization of medium constituents for a novel lipopeptide production by *Bacillus subtilis* MO-01 by a response surface method. Process Biochem 40:3196-3201.

Gubitz GM, Cavaco-Paulo A. 2001. Biotechnology in the textile industry – perspectives for the new millennium. J Biotechnol 89 89-90.

Gupta A, Roy I, Patel RK, Singh SP, Khare SK, Gupta MN. 2005. One-step purification and characterization of an alkaline protease from haloalkaliphilic *Bacillus* sp. J Chromat A 1075:103-108.

Gupta R, Beg Q, Khan S, Chauhan B. 2002a. An overview on fermentation, downstream processing and properties of microbial alkaline proteases. Appl Microbiol Biotechnol 60:381-395.

Gupta R, Beg Q, Lorenz P. 2002b. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 59:15-32.

Gupta R, Ramnani P. 2006. Microbial keratinases and their prospective applications: an overview. Appl Microbiol Biotechnol 70:21-33.

Haddar A, Agrebi R, Bougatef A, Hmidet N, Sellami-Kamoun A, Nasri M. 2009. Two detergent stable alkaline serine-proteases from *Bacillus mojavensis* A21: purification, characterization and potential application as a laundry detergent additive. Bioresource Technol 100:3366-3373.

Haddar A, Bougatef A, Agrebi R, Sellami-Kamoun A, Nasri M. 2009. A novel surfactant-stable alkaline serine-protease from a newly isolated *Bacillus mojavensis* A21. Purification and characterization. Process Biochem 44:29-35.

Haddar A, Sellami-Kamoun A, Fakhfakh-Zouari N, Hmidet N, Nasri M. 2010. Characterization of detergent stable and feather degrading serine proteases from *Bacillus mojavensis* A21. Biochem Eng J 51:53-63.

Hajji M, Rebai A, Gharsallah N, Nasri M. 2008. Optimization of alkaline protease production by *Aspergillus clavatus* ES1 in *Mirabilis jalapa* tuber powder using statistical experimental design. Appl Microbiol Biotechnol 79:915-923.

Haki GD, Rakshit SK. 2003. Developments in industrially important thermostable enzymes: a review. Bioresource Technol 89:17-34.

Halliday LA. 2002. Woolscouring, carbonising and effluent treatment. In: Simpson WS, Crawshaw GH, Editors. Woodhead Textile Series. Cambridge, U.K.: Woodhead Publishing. pp. 21-22.

Hmidet N, el-Hadj AN, Haddar A, Kanoun S, Alya S-K, Nasri M. 2009. Alkaline proteases and thermostable α-amylase co-produced by

Bacillus licheniformis NH1: characterization and potential application as detergent additive. Biochem Eng J 47:71-79.

Horikoshi K. 1999. Alkaliphiles: some applications of their products for biotechnology. Microbiol Mol Biol Res 63:735-750.

Hossain KMG, Juan AR, Tzanov T. 2008. Simultaneous protease and transglutaminase treatment for shrink resistance of wool. Biocatal Biotransfor 26:405-411.

Huang GR, Dai DH, Hu WL, Jiang JX. 2008. Optimization of medium composition for thermostable protease production by *Bacillus* sp. HS08 with a statistical method. Afr J Biotechnol 7:1115-1122.

Ibrahim ASS, al-Salamah AA. 2009. Optimization of media and cultivation conditions for alkaline protease production by alkaliphilic *Bacillus halodurans*. Res J Microbiol 4:251-259.

Infante I, Morel M, Ubalde M, Martínez-Rosales C, Belvisi S, Castro-Sowinski S. 2010. Wool-degrading *Bacillus* isolates: extracellular protease production for microbial processing of fabrics. World J Microbiol Biotechnol 26:1047-1052.

Jackson TA, Pearson JF, Young SD, Armstrong J, O'Callaghan M. 2002. Abundance and distribution of microbial populations in sheep fleece. New Zeal J Agr Res 45:49-55.

Jaouadi B, Abdelmalek B, Fodil D, Ferradji FZ, Rekik H, Zarai N, Bejar S. 2010. Purification and characterization of a thermostable keratinolytic serine alkaline proteinase from *Streptomyces* sp. strain AB1 with high stability in organic solvents. Bioresource Technol 101:8361-8369.

Jaouadi B, Ellouz-Chaabouni S, Ali M, Messaoud E, Naili B, Dhouib A, Bejar S. 2009. Excellent laundry detergent compatibility and high dehairing ability of the *Bacillus pumilus* CBS alkaline proteinase (SAPB). Biotechnol Bioproc Eng 14:503-512.

Joo H-S, Chang C-S. 2005. Production of protease from a new alkalophilic *Bacillus* sp. I-312 grown on soybean meal: optimization and some properties. Process Biochem 40:1263-1270.

Joo H-S, Chang C-S. 2006. Production of an oxidant and SDS-stable alkaline protease from an alkalophilic *Bacillus clausii* I-52 by submerged fermentation: feasibility as a laundry detergent additive. Enzyme Microb Technol 38:176-183.

Joshi R, Dodia M, Singh S. 2008. Production and optimization of a commercially viable alkaline protease from a haloalkaliphilic bacterium. Biotechnol Bioproc Eng 13:552-559.

Kainoor PS, Naik GR. 2010. Production and characterization of feather degrading keratinase from *Bacillus* sp. JB 99. Indian J Biotechnol 9:384-390.

Kalaiarasi K, Sunitha PU. 2009. Optimization of alkaline protease production from *Pseudomonas fluorescens* isolated from meat waste contaminated soil. Afr J Biotechnol 8:7035-7041.

Kamal M, Hoog JO, Kaiser R, Shafqat J, Razzaki T, Zaidi ZH, Jornvall H. 1995. Isolation, characterization and structure of subtilisin from a thermostable *Bacillus subtilis* isolate. FEBS Letters 374:363-366.

Kamath P, Subrahmanyam VM, Rao JV, Raj PV. 2010. Optimization of cultural conditions for protease production by a fungal species. Indian J Pharm Sci 72:161-166.

Kanekar PP, Nilegaonkar SS, Sarnaik SS, Kelkar AS. 2002. Optimization of protease activity of alkaliphilic bacteria isolated from an alkaline lake in India. Bioresource Technol 85:87-93.

Karbalaei-Heidari H, Amoozegar M, Hajighasemi M, Ziaee A-A, Ventosa A. 2009. Production, optimization and purification of a novel extracellular protease from the moderately halophilic bacterium *Halobacillus karajensis*. J Ind Microbiol Biotechnol 36:21-27.

Kim CM, Kang SM, Jeon HJ, Shin SH. 2007. Production of *Vibrio vulnificus* metalloprotease VvpE begins during the early growth phase: usefulness of gelatin-zymography. J Microbiol Meth 70:96-102.

Kim JM, Lim WJ, Suh HJ. 2001. Feather-degrading *Bacillus* species from poultry waste. Process Biochem 37:287-291.

Kirk O, Borchet TV, Fuglsang CC. 2002. Industrial enzyme applications. Curr Opin Biotechnol 13:345-351.

Klomklao S. 2008. Digestive proteinases from marine organisms and their applications. Songklanakarin J Sci Technol 30:37-46.

Kumar CG, Takagi H. 1999. Microbial alkaline proteases: from a bioindustrial viewpoint. Biotechnol Adv 17:561-594.

Kumar D, Savitri N, Thakur R, Verma TCB. 2008. Microbial proteases and application as laundry detergent additive. Res J Microbiol 3:661-672.

Kumar R, Balaji S, Uma T, Mandal A, Sehgal P. 2010. Optimization of influential parameters for extracellular keratinase production by *Bacillus subtilis* (MTCC9102) in solid state fermentation using horn meal — a biowaste management. Appl Biochem Biotechnol 160:30-39.

Ladeira SA, Andrade MVV, Delatorre AB, Perez VH, Martins MLL. 2010. Utilização de resíduos agroindustriais para a produção de proteases pelo termofílico *Bacillus* sp. em fermentação submersa: optimização do meio de cultura usando a técnica de planejamento experimental. Quím Nova 33:5.

Lateef A, Oloke JK, Kana EBG, Sobowale BO, Ajao SO, Bello BY. 2010. Keratinolytic activities of a new feather-degrading isolate of *Bacillus cereus* LAU 08 isolated from Nigerian soil. Int Biodeter Biodegr 64:162-165.

Lazim H, Mankai H, Slama N, Barkallah I, Limam F. 2009. Production and optimization of thermophilic alkaline protease in solid-state fermentation by *Streptomyces* sp. CN902. J Ind Microbiol Biotechnol 36:531-537.

Leber TM, Balkwill FR. 1997. Zymography: a single-step staining method for quantitation of proteolytic activity on substrate gels. Anal Biochem 249:24-28.

Li C, Yu H, Liu S, Xing R, Guo Z, Li P. 2005. Factors affecting the protease activity of venom from jellyfish *Rhopilema esculentum* Kishinouye. Bioorg Med Chem Lett 15:5370-5374.

Li W, Zhou X, Lu P. 2004. Bottlenecks in the expression and secretion of heterologous proteins in *Bacillus subtilis*. Res Microbiol 155:605-610.

Liu J, Xing J, Chang T, Ma Z, Liu H. 2005. Optimization of nutritional conditions for nattokinase production by *Bacillus natto* NLSSE using statistical experimental methods. Process Biochem 40:2757-2762.

Liu S, Fang Y, Lv M, Wang S, Chen L. 2010. Optimization of the production of organic solvent-stable protease by *Bacillus sphaericus* DS11 with response surface methodology. Bioresource Technol 101:7924-7929.

London CJ, Griffith IP, Kortt AA. 1984. Proteinases produced by Pseudomonads isolated from sheep fleece. Appl Environ Microbiol 47:75-79.

London CJ, Griffith IP. 1984. Characterization of Pseudomonads isolated from diseased fleece. Appl Environ Microbiol 47 993-997.

Lucas FS, Broennimann O, Febbraro I, Heeb P. 2003. High diversity among feather-degrading bacteria from a dry meadow soil. Microb Ecol 45:282-290.

Lyness EW, Pinnock DE, Cooper DJ, Milner R. 1994. Microbial ecology of sheep fleece. Agric Ecosyst Environ 49:103-112.

Mabrouk MEM. 2008. Feather degradation by a new keratinolytic *Streptomyces* sp. MS-2. World J Microbiol Biotechnol 24:2331-2338.

Macedo AJ, da Silva WOB, Gava R, Driemeier D, Henriques JAP, Termignoni C. 2005. Novel keratinase from *Bacillus subtilis* S14 exhibiting remarkable dehairing capabilities. Appl Environ Microbiol 71:594-596.

Mala M, Srividya S. 2010. Partial purification and properties of a laundry detergent-compatible alkaline protease from a newly isolated *Bacillus* species Y. Indian J Microbiol 50:309-317.

Manczinger L, Rozs M, Vágvölgyi C, Kevei F. 2003. Isolation and characterization of a new keratinolytic *Bacillus licheniformis* strain. World J Microbiol Biotechnol 19:35-39.

Manni L, Jellouli K, Ghorbel-Bellaaj O, Agrebi R, Haddar A, Sellami-Kamoun A, Nasri M. 2010. An oxidant- and solvent-stable protease produced by *Bacillus cereus* SV1: application in the deproteinization of shrimp wastes and as a laundry detergent additive. Appl Biochem Biotechnol 160:2308-2321.

Mansfeld J, Ulbrich-Hofmann R. 2007. The stability of engineered thermostable neutral proteases from *Bacillus stearothermophilus* in organic solvents and detergents. Biotechnol Bioeng 97:672-679.

Marshall WA. 1998. Aerial transport of keratinaceous substrate and distribution of the fungus *Geomyces pannorum* in Antarctic soils. Microb Ecol 36:212-219.

Martley FG, Jayashankar SR, Lawrence RC. 1970. An improved agar medium for the detection of proteolytic organisms in total bacterial counts. J Appl Microbiol 33:363-370.

Matta H, Punj V. 1998. Isolation and partial characterization of a thermostable extracellular protease of *Bacillus polymyxa* B-17. Int J Food Microbiol 42:139-145.

Maurer K-H. 2004. Detergent proteases. Curr Opinion Biotechnol 15:1-5.

Mazotto A, de Melo A, Macrae A, Rosado A, Peixoto R, Cedrola S, Couri S, Zingali R, Villa A, Rabinovitch L, Chaves J, Vermelho A. 2011. Biodegradation of feather waste by extracellular keratinases and gelatinases from *Bacillus* spp. World J Microbiol Biotechnol Online 27:1355:1365.

Mehrotra S, Pandey PK, Gaur R, Darmwal NS. 1999. The production of alkaline protease by a *Bacillus* species isolate. Bioresource Technol 67:201-203.

Mendes CM, Brito MA, Porto TS, Porto ALF, Bezerra RS, Carvalho LB, Caneiro-Leao AMA, Carneiro-da-Cunha MG. 2009. Aquaculture by-product: a source of proteolytic enzymes for detergent additives. Chem Papers 63:662-669.

Meza JC, Auria R, Lomascolo A, Sigoillot JC, Casalot L. 2007. Role of ethanol on growth, laccase production and protease activity in *Pycnoporus cinnabarinus* ss3. Enzyme Microb Technol 41:162-168.

Molina R, Jovančić P, Comelles F, Bertran E, Erra P. 2002. Shrink-resistance and wetting properties of keratin fibres treated by glow discharge. J Adhesion Sci Technol 16:1469-1485.

Montazer M, Ramin A. 2010. Influences of proteases and trans-glutaminases on wool. Fibres Text East Eur 18:98-102.

Montazer M, Ramin A. 2010. Influences of proteases and trans-glutaminases on wool. Fibres Text East Eur 18:98-102.

Moradian F, Khajeh K, Naderi-Manesh H, Sadeghizadeh M. 2009. Isolation, purification and characterization of a surfactants-, laundry

detergents- and organic solvents-resistant alkaline protease from *Bacillus* sp. HR-08. Appl Biochem Biotechnol 159:33-45.

Moreira KA, Porto TS, Teixeira MFS, Porto ALF, Lima-Filho JL. 2003. New alkaline protease from *Nocardiopsis* sp.: partial purification and characterization. Process Biochem 39:67-72.

Mukherjee A. 2007. Potential application of cyclic lipopeptide biosurfactants produced by *Bacillus subtilis* strains in laundry detergent formulations. Lett Appl Microbiol 45:330-335.

Mukherjee AK, Adhikari H, Rai SK. 2008. Production of alkaline protease by a thermophilic *Bacillus subtilis* under solid-state fermentation (SSF) condition using *Imperata cylindrica* grass and potato peel as low-cost medium: characterization and application of enzyme in detergent formulation. Biochem Eng J 39:353-361.

Mukherjee AK, Borah M, Rai SK. 2009. To study the influence of different components of fermentable substrates on induction of extracellular α-amylase synthesis by *Bacillus subtilis* DM-03 in solid-state fermentation and exploration of feasibility for inclusion of α-amylase in laundry detergent formulations. Biochem Eng J 43:149-156.

Mukhtar H, Ikram Ul H. 2007. Optimization of volume of fermentation medium for the production of alkaline protease by an EMS mutant strain of *Bacillus subtillis* IH-72. Pakist J Bot 39:2705-2715.

Myers RH, Montgomery DC. 2002. Response Surface Methodology: Process and Product Optimization Using Designed Experiments. New York, NY: Wiley Interscience.

Nadeem M, Qazi JI, Baig S, Syed QUA. 2007. Studies on commercially important alkaline protease from *Bacillus licheniformis* N-2 isolated from decaying organic soil. Turk J Biochem 32:171-177.

Nadeem M, Qazi JI, Syed QUA, Baig S. 2008. Optimization of process parameters for alkaline protease production by *Bacillus licheniformis* N-2 and kinetics studies in batch fermentation. Turk J Biol 32:243-251.

Najafi MF, Deobagkar DN, Mehrvarz M, Deobagkar DD. 2006. Enzymatic properties of a novel highly active and chelator resistant protease from a *Pseudomonas aeruginosa* PD100. Enzyme Microb Technol 39:1433-1440.

Nascimento WCA, Rocha da Silva RVC, Martins MLL. 2007. Otimização de um meio de cultura para a produção de proteases por um *Bacillus* sp. termofílico. Ciênc Tecnol Aliment 27:5.

Navaneeth S, Bhuvanesh S, Bhaskar V, Kumar PV, Kandaswamy SKJ, Achary A. 2009. Optimization of medium for the production of subtilisin from *Bacillus subtilis* MTCC 441. Afr J Biotechnol 8:6327-6331.

Niehaus F, Bertoldo C, Kähler M, Antranikian G. 1999. Extremophiles as a source of novel enzymes for industrial application. Appl Microbiol Biotechnol 51:711-729.

NIST/SEMTECH e-Handbook of Statistical Methods.

Norris BJ, Colditz IG, Dixon TJ. 2008. Fleece rot and dermatophilosis in sheep. Vet Microbiol 128:217-230.

O'Fagain C. 2003. Enzyme stabilization – recent experimental progress. Enzyme Microb Technol 33:137-149.

Oberoi R, Beg QK, Puri S, Saxena RK, Gupta R. 2001. Characterization and wash performance analysis of an SDS-stable alkaline protease from a *Bacillus* sp. World J Microbiol Biotechnol 17:493-497.

Olivera N, Sequeiros C, Sineriz F, Breccia JD. 2006. Characterization of alkaline proteases from a novel alkali-tolerant bacterium *Bacillus patagoniensis*. World J Microbiol Biotechnol 22:737-743.

Onifade AA, al-Sane NA, al-Musallam AA, al-Zarban S. 1998. A review: potentials for biotechnological applications of keratin-degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources. Bioresource Technol 66:1-11.

Oskouie SFG, Tabandeh F, Yakhchali B, Eftekhar F. 2008. Response surface optimization of medium composition for alkaline protease production by *Bacillus clausii*. Biochem Eng J 39:37-42.

Oskouie SFG, Tabandeh F, Yakhchali B, Eftekhar F. 2008. Response surface optimization of medium composition for alkaline protease production by *Bacillus clausii*. Biochem Eng J 39:37-42.

Pascual E, Juliá MR. 2001. The role of chitosan in wool finishing. J Biotechnol 89:289-296.

Peričin D, Mađarev-Popović S, Radulović-Popović L. 2009. Optimization of conditions for acid protease partitioning and purification in aqueous two-phase systems using response surface methodology. Biotechnol Lett 31:43-47.

Perrière G, M. G. 1996. WWW-Query: An on-line retrieval system for biological sequence banks. Biochimie 78:364-369.

Pillai P, Archana G. 2008. Hide depilation and feather disintegration studies with keratinolytic serine protease from a novel *Bacillus subtilis* isolate. Appl Microbiol Biotechnol 78:643-650.

Plowman JE. 2003. Proteomic database of wool components. J Chromat B 787:63-76.

Prakash M, Banik R, Koch-Brandt C. 2005. Purification and characterization of *Bacillus cereus* protease suitable for detergent industry. Appl Biochem Biotech 127:143-155.

Puri S, Beg QK, Gupta R. 2002. Optimization of alkaline protease production from *Bacillus* sp. by response surface methodology. Curr Microbiol 44:286-290.

ul-Qadar SA, Shireen E, Iqbal S, Anwar A. 2009. Optimization of protease production from newly isolated strain of *Bacillus* sp. PCSIR EA-3. Indian J Biotechnol 8:286-290.

Quesada AR, Fajardo I, Rodríguez-Agudo D, Pachón JM, Medina MÂ. 1996. Zymography of extracellular matrix proteases. Biochem Educ 24:170-171.

Rachadech W, Navacharoen A, Ruangsit W, Pongtharangkul T, Vangnai A. 2010. An organic solvent-, detergent-, and thermostable alkaline protease from the mesophilic, organic solvent-tolerant *Bacillus licheniformis* 3C5. Microbiology 79:620-629.

Radha S, Gunasekaran P. 2007. Cloning and expression of keratinase gene in *Bacillus megaterium* and optimization of fermentation conditions for the production of keratinase by recombinant strain. J Appl Microbiol 103:1301-1310.

Rahman RNZRA, Geok LP, Basri M, Salleh AB. 2005. An organic solvent-tolerant protease from *Pseudomonas aeruginosa* strain K: nutritional factors affecting protease production. Enzyme Microb Technol 36:749-757.

Rai SK, Konwarh R, Mukherjee AK. 2009. Purification, characterization and biotechnological application of an alkaline β-keratinase produced by *Bacillus subtilis* RM-01 in solid-state fermentation using chicken-feather as substrate. Biochem Eng J 45:218-225.

Rai SK, Mukherjee AK. 2009. Ecological significance and some biotechnological application of an organic solvent stable alkaline serine protease from *Bacillus subtilis* strain DM-04. Bioresource Technol 100:2642-2645.

Rai SK, Mukherjee AK. 2010. Statistical optimization of production, purification and industrial application of a laundry detergent and organic solvent-stable subtilisin-like serine protease (Alzwiprase) from *Bacillus subtilis* DM-04. Biochem Eng J 48:173-180.

Raja ASM, Thilagavathi G. 2010. Comparative study on the effect of acid and alkaline protease enzyme treatments on wool for improving handle and shrink resistance. J Text Inst 101:823-834.

Rao MB, Tanksale MSG, Deshpande VV. 1998. Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Res 62:597-635.

Rao YK, Tsay KJ, Wu WS, Tzeng YM. 2007. Medium optimization of carbon and nitrogen sources for the production of spores from *Bacillus amyloliquefaciens* B128 using response surface methodology. Process Biochem 42:535-541.

Rawlings ND, Barrett AJ, Bateman A. 2010. MEROPS: the peptidase database. Nucleic Acids Research 38:D227-D233.

Reddy LVA, Wee YJ, Ryu HW. 2008a. Purification and characterization of an organic solvent and detergent-tolerant novel protease produced by *Bacillus* sp. RKY3. J Chem Technol Biotechnol 83:1526-1533.

Reddy LVA, Wee YJ, Yun JS, Ryu HW. 2008b. Optimization of alkaline protease production by batch culture of *Bacillus* sp. RKY3 through Plackett-Burman and response surface methodological approaches. Bioresource Technol 99:2242-2249.

el-Refai HA, AbdelNaby MA, Gaballa A, el-Araby MH, Abdel Fattah AF. 2005. Improvement of the newly isolated *Bacillus pumilus* FH9 keratinolytic activity. Process Biochem 40:2325-2332.

Riessen S, Antranikian G. 2001. Isolation of *Thermoanaerobacter keratinophilus* sp. nov., a novel thermophilic, anaerobic bacterium with keratinolytic activity. Extremophiles 5:399-408.

Riffel A, Brandelli A. 2006. Keratinolytic bacteria isolated from feather waste. Braz J Microbiol 37:395-399.

Riffel A, Lucas Fo, Heeb P, Brandelli A. 2003a. Characterization of a new keratinolytic bacterium that completely degrades native feather keratin. Arch Microbiol 179:258-265.

Riffel A, Ortolan S, Brandelli A. 2003b. De-hairing activity of extracellular proteases produced by keratinolytic bacteria. J Chem Technol Biotechnol 78:855-859.

Romsomsa N, Chim-Anagae P, Jangchud A. 2010. Optimization of silk degumming protease production from *Bacillus subtilis* C4 using Plackett-Burman design and response surface methodology. Sci Asia 36:118-124.

Saeki K, Ozaki K, Kobayashi T, Ito S. 2007. Detergent alkaline proteases: enzymatic properties, genes, and crystal structures. J Biosci Bioeng 103:501-508.

Šafařík I, Šafaříková M. 1994. A modified procedure for the detection of microbial producers of extracellular proteolytic enzymes. Biotechnol Tech 8:627-628.

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

Sambrook J, Russell DW. 2001. Molecular Cloning: A Laboratory Manual. New York, NY, USA: Cold Spring Harbor Laboratory Press.

al-Sane NA, al-Musallam AA, Onifade AA. 2002. The isolation of keratin degrading microorganisms from Kuwaiti soil: production and characterization of their keratinases. Kuwait J Sci Eng 29:125-138.

Saran S, Isar J, Saxena R. 2007a. Statistical optimization of conditions for protease production from *Bacillus* sp. and its scale-up in a bioreactor. Appl Biochem Biotechnol 141:229-239.

Saran S, Isar J, Saxena RK. 2007. A modified method for the detection of microbial proteases on agar plates using tannic acid. J Biochem Biophys Meth 70:697-699.

Schiraldi C, de Rosa M. 2002. The production of biocatalysts and biomolecules from extremophiles. Trends Biotechnol 20:515-521.

Schmid A, Hollmann F, Park JB, Buhler B. 2002. The use of enzymes in the chemical industry in Europe. Curr Opin Biotechnol 13 359-366.

Schroeder M, Lenting H, Kandelbauer A, Silva C, Cavaco-Paulo A, Guebitz G. 2006. Restricting detergent protease action to surface of protein fibres by chemical modification. Appl Microbiol Biotechnol 72:738-744.

Schroeder M, Schweitzer M, Lenting HBM, Guebitz GM. 2004. Chemical modification of proteases for wool cuticle scale removal. Biocatal Biotransf 22:299-305.

Schumacher K, Heine E, Hicker H. 2001. Extremozymes for improving wool properties. J Biotechnol 89 281-288.

Sellami-Kamoun A, Haddar A, Ali NE-H, Ghorbel-Frikha B, Kanoun S, Nasri M. 2008. Stability of thermostable alkaline protease from *Bacillus licheniformis* RP1 in commercial solid laundry detergent formulations. Microbiol Res 163:299-306.

Seong CN, Jo JS, Choi SK, Kim SW, Kim SJ, Lee OH, Han JM, Yoo JC. 2004. Production, purification, and characterization of a novel thermostable serine protease from soil isolate, *Streptomyces tendae*. Biotechnol Lett 26:907-909.

Shafee N, Aris SN, Rahman RNZA, Basri M, Salleh AB. 2005. Optimization of environmental and nutritional conditions for the production of alkaline protease by a newly isolated bacterium *Bacillus cereus* strain 146. J Appl Sci Res 1:1-8.

Shanmughapriya S, Krishnaveni J, Selvin J, Gandhimathi R, Arunkumar M, Thangavelu T, Kiran G, Natarajaseenivasan K. 2008. Optimization of extracellular thermotolerant alkaline protease produced by marine *Roseobacter* sp. (MMD040). Bioproc Biosyst Eng 31:427-433.

Shrinivas D, Naik GR. 2011. Characterization of alkaline thermostable keratinolytic protease from thermoalkalophilic *Bacillus halodurans* JB 99 exhibiting dehairing activity. Int Biodeter Biodegr 65:29-35.

Silva CJSM, Sousa F, Guebitz G, Cavaco-Paulo A. 2004. Chemical modifications on proteins using glutaraldehyde. Food Technol Biotechnol 42:51-56.

Sindhu R, Suprabha GN, Shashidhar S. 2009. Optimization of process parameters for the production of alkaline protease from *Penicillium godlewskii* SBSS 25 and its application in detergent industry. Afr J Microbiol Res 3:515-522.

Singh AK, Chhatpar HS. 2010. Optimization of protease production by *Streptomyces* sp. A6 using statistical approach for reclamation of shellfish waste. World J Microbiol Biotechnol 26:1631-1639.

Singh J, Vohra RM, Sahoo DK. 1999. Alkaline protease from a new obligate alkalophilic isolate of *Bacillus sphaericus*. Biotechnol Lett 21:921-924.

Skolpap W, Nuchprayoon S, Scharer JM, Grisdanurak N, Douglas PL, Moo-Young M. 2008. Fed-batch optimization of α-amylase and protease-producing *Bacillus subtilis* using genetic algorithm and particle swarm optimization. Chem Eng Sci 63:4090-4099.

Son H-J, Park H-C, Kim H-S, Lee C-Y. 2008. Nutritional regulation of keratinolytic activity in *Bacillus pumilis*. Biotechnol Lett 30:461-465.

Subba Rao C, Sathish T, Mahalaxmi M, Suvarna Laxmi G, Sreenivas Rao R, Prakasham RS. 2008. Modelling and optimization of fermentation factors for enhancement of alkaline protease production by isolated *Bacillus circulans* using feed-forward neural network and genetic algorithm. J Appl Microbiol 104:889-898.

el-Sukhon SN. 2002. Isolation and characterization of *Pseudomonas aeruginosa* from sheep with fleece rot in northern and middle Jordan. Vet Dermatol 13:247-251.

Sundin GW, Jacobs JL. 1999. Ultraviolet radiation (UVR) sensitivity analysis and UVR survival strategies of a bacterial community from the phyllosphere of field-grown peanut (*Arachis hypogeae* L.). Microb Ecol 38:27-38.

Suntornsuk W, Suntornsuk L. 2003. Feather degradation by *Bacillus* sp. FK 46 in submerged cultivation. Bioresource Technol 86:239-243.

Syed DG, Lee JC, Li WJ, Kim CJ, Agasar D. 2009. Production, characterization and application of keratinase from *Streptomyces gulbargensis*. Bioresource Technol 100:1868-1871.

Tamhane AC. 2009. Statistical Analysis of Designed Experiments: Theory and Applications. New Jersey, NJ: Wiley.

Tang XY, Pan Y, Li S, He BF. 2008. Screening and isolation of an organic solvent-tolerant bacterium for high-yield production of organic solvent-stable protease. Bioresource Technol 99:7388-7392.

Tari C, Genckal H, Tokatli F. 2006. Optimization of a growth medium using a statistical approach for the production of an alkaline protease from a newly isolated *Bacillus* sp. L21. Process Biochem 41:659-665.

Tatineni R, Doddapaneni K, Potumarthi R, Mangamoori L. 2007. Optimization of keratinase production and enzyme activity using response surface methodology with *Streptomyces* sp7. Appl Biochem Biotechnol 141:187-201.

Tatineni R, Doddapaneni KK, Potumarthi RC, Vellanki RN, Kandathil MT, Kolli N, Mangamoori LN. 2008. Purification and characterization of an alkaline keratinase from *Streptomyces* sp. Bioresource Technol 99:1596-1602.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acid Res 24:4876-4882.

Thys RCS, Guzzon SO, Cladera-Olivera F, Brandelli A. 2006. Optimization of protease production by *Microbacterium* sp. in feather meal using response surface methodology. Process Biochem 41:67-73.

Tiwary E, Gupta R. 2010. Medium optimization for a novel 58 kDa dimeric keratinase from *Bacillus licheniformis* ER-15: biochemical characterization and application in feather degradation and dehairing of hides. Bioresource Technol 101:6103-6110.

Vasconcelos A, Silva C, Schroeder M, Guebitz G, Cavaco-Paulo A. 2006. Detergent formulations for wool domestic washings containing immobilized enzymes. Biotechnol Lett 28:725-731.

Vavrová L, Muchová K, Barák I. 2010. Comparison of different *Bacillus subtilis* expression systems. Res Microbiol 161:791-797.

Venil CK, Lakshmanaperumalsamy P. 2009. Application of response surface methodology in medium optimization for protease

production by the new strain of *Serratia marcescens* SB08. Pol J Microbiol 58:117-124.

Vermelho AB, Meirelles MNL, Lopes A, Petinate SDG, Chaia AA, Branquinha MH. 1996. Detection of extracellular proteases from microorganisms on agar plates. Mem Inst Oswaldo Cruz 91:755-760.

Vidyasagar M, Prakash S, Jayalakshmi S, Sreeramulu K. 2007. Optimization of culture conditions for the production of halothermophilic protease from halophilic bacterium *Chromohalobacter* sp. TVSP101. World J Microbiol Biotechnol 23:655-662.

Vishwanatha K, Rao A, Singh S. 2010. Acid protease production by solid-state fermentation using *Aspergillus oryzae* MTCC 5341: optimization of process parameters. J Ind Microbiol Biotechnol 37:129-138.

Vonothini G, Murugan M, Sivakumar K, Sudha S. 2008. Optimization of protease production by an *Actinomycete* strain, PS-18A isolated from an estuarine shrimp pond. Afr J Biotechnol 7:3225-3230.

Wahler D, Reymond J-L. 2001. High-throughput screening for biocatalysts. Curr Opin Biotechnol 12:535-544.

Walawska A, Rybicki E, Filipowska B. 2006. Physicochemical changes on wool surface after an enzymatic treatment. Progr Colloid Polym Sci 132:131-137.

Wang P, Wang QA, Cui L, Fan XR, Yuan JG, Gao MR. 2010. A comparative evaluation of the action of savinase and papain to the cutinase-pretreated wool. Fiber Polym 11:586-592.

Wang Q, Hou Y, Xu Z, Miao J, Li G. 2008a. Optimization of cold-active protease production by the psychrophilic bacterium *Colwellia* sp. NJ341 with response surface methodology. Bioresource Technol 99:1926-1931.

Wang Q, Wang P, Fan XR, Cui L, Zhao XF, Gao XX. 2009. A comparative study on wool bio-antifelting based on different chemical pretreatments. Fiber Polym 10:724-730.

Wang S-L, Yang C-H, Liang T-W, Yen Y-H. 2008b. Optimization of conditions for protease production by *Chryseobacterium taeanense* TKU001. Bioresource Technol 99:3700-3707.

Wang SL, Chio YH, Yen YH, Wang CL. 2007. Two novel surfactant-stable alkaline proteases from *Vibrio fluvialis* TKU005 and their applications. Enzyme Microb Technol 40:1213-1220.

Westers L, Westers H, Quax WJ. 2004. *Bacillus subtilis* as cell factory for pharmaceutical proteins: a biotechnological approach to optimize the host organism. BBA Mol Cell Res 1694:299-310.

Wilson ED. 1930. Studies in bacterial proteases I. The relation of protease production to the culture medium. J Bacteriol 20:41-59.

Xu B, Zhong QF, Tang XH, Yang YJ, Huang ZX. 2009. Isolation and characterization of a new keratinolytic bacterium that exhibits significant feather-degrading capability. Afr J Biotechnol 8:4590-4596.

Yallop CA, Edwards C, Williams ST. 1997. Isolation and growth physiology of novel thermoactinomycetes. J Appl Microbiol 83:685-692.

Yang CY, Wang F, Hao JH, Zhang K, Yuan N, Sun M. 2010. Identification of a proteolytic bacterium, HW08, and characterization of its extracellular cold-active alkaline metalloprotease Ps5. Biosci Biochem Biotechnol 74:1220-1225.

Yu X-W, Guan W-J, Li Y-Q, Guo T-J, Zhou J-D. 2005. A biological treatment technique for wool textile. Braz Arch Biol Technol 48:675-680.

Zhang B, Jiang DD, Zhou WW, Hao HK, Niu TG. 2009. Isolation and characterization of a new *Bacillus* sp. 50-3 with highly alkaline

keratinase activity from *Calotes versicolor* faeces. World J Microbiol Biotechnol 25:583-590.

Zhang Q, Smith E, Shen J, Bishop D. 2006. An ethoxylated alkyl phosphate (anionic surfactant) for the promotion of activities of proteases and its potential use in the enzymatic processing of wool. Biotechnol Lett 28:717-723.

Zhou LH, Zhang YQ, Wang RJ, Shen XL, Li YQ, Guan WJ. 2009. Optimization of mycelial biomass and protease production by *Laccocephalum mylittae* in submerged fermentation. Afr J Biotechnol 8:1591-1601.

Zweers JC, Barak I, Becher D, Driessen AJM, Hecker M, Kontinen VP, Saller MJ, Vavrova L, van Dijl JM. 2008. Towards the development of *Bacillus subtilis* as a cell factory for membrane proteins and protein complexes. Microb Cell Fact 7:10-29.