FABIANO JARES CONTESINI

"PRODUÇÃO, CARACTERIZAÇÃO E APLICAÇÃO DE PROTEASES DE *Bacillus* sp."

"PRODUCTION, CHARACTERIZATION AND APPLICATION OF PROTEASES FROM Bacillus sp."

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"PRODUCTION, CHARACTERIZATION AND APPLICATION OF PROTEASE FROM Bacillus sp."

Tese apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Ciência de Alimentos

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Orientadora: Profa. Dra. Hélia Harumi Sato

Este exemplar corresponde à versão final da tese defendida pelo aluno Fabiano Jares Contesini e orientada pela Profa. Dra. Hélia Harumi Sato

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"Àquele que é capaz de fazer infinitamente mais do que tudo o que pedimos ou pensamos, de acordo com o seu poder que atua em nós, a ele seja a glória na igreja e em Cristo Jesus, por todas as gerações, para todo o sempre! Amém!"

(Efésios 3:20, 21)

“Get up off the floor and believe in life...”

(Noel Gallagher)

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RESUMO GERAL

Proteases bacterianas são enzimas de elevada importância comercial, amplamente aplicadas em diversas áreas como nas indústrias de detergentes, de alimentos, farmacêutica e têxtil. Este trabalho teve como principais objetivos selecionar entre 59 linhagens de *Bacillus* sp., da coleção de culturas do Laboratório de Bioquímica de Alimentos da Faculdade de Engenharia de Alimentos, aquelas que apresentam potencial de maior produção de proteases com características tais como estabilidade em diferentes condições de temperatura, pH, detergentes e solventes orgânicos, atividade em ampla faixa de pH e capacidade de lisar células de *Xanthomonas campestris.* Em seguida, visou-se otimizar a produção de proteases pela linhagem selecionada, determinar as características bioquímicas da protease parcialmente purificada e estudar a aplicação do extrato enzimático bruto e preparação parcialmente purificada.

Entre as cinquenta e nove linhagens de *Bacillus* sp. testadas foram selecionadas nove linhagens que produziram maior atividade de proteases. A produção de protease pelas nove linhagens foi testada em frascos agitados contendo o meio de cultura nº 1 (10g/L de caseína, 1g/L de extrato de levedura e sais), meio nº 2 (35 g/L de melaço de cana de açúcar, 20g/L de água de maceração de milho, 3g/L de extrato de levedura Prodex-Lac SD® e 20g/L de soro de queijo), e por fermentação em meio sólido nº 3 (farelo de trigo e água, na proporção 1:1, m:m). As linhagens de *Bacillus* sp. LBA 07, LBA 46 e LBA 08 fermentadas nos meios de cultura nº 1, nº 2 e nº 3 produziram 222 U/mL, 548 U/mL e 13480 U/grama de substrato seco (gss) respectivamente. As proteases dos extratos enzimáticos brutos obtidos das nove linhagens fermentadas nos três meios de cultura apresentaram atividade ótima na faixa de pH 7 a 9 e 60°C, estabilidade na faixa de pH 5 a 9 por 24h a 4º C , e em pH 7,0 a 50°C por 1h.

Entre os extratos enzimáticos brutos de proteases testados, aqueles obtidos da fermentação de *Bacillus* sp. LBA 46 nos três meios de cultura foram as mais estáveis em detergente Ariel®. Quando incubadas em solventes orgânicos alguns extratos enzimáticos brutos de proteases mantiveram mais de 60% de atividade residual após 24h em acetona (*Bacillus* sp. LBA 8 e 44), hexano (*Bacillus* sp. LBA 19, 29, 44, 46 e 60), clorofórmio (*Bacillus* sp. LBA 44 and 60) e etanol (*Bacillus* sp. LBA 60). Os extratos enzimáticos brutos de proteases obtidos do cultivo da linhagem de *Bacillus* sp. LBA 46 nos meios n° 2 e n° 3 foram as mais eficientes na lise de células de *Xanthomonas campestris*, aumentando cerca de 30% a transmitância a 620 nm (Trans $_{620nm}$) do meio fermentado de goma xantana.

A linhagem de *Bacillus* sp. LBA 46 foi selecionada como melhor produtora de protease e estudos preliminares de identificação biomolecular indicam que se trata de uma linhagem de *Bacillus licheniformis*. Utilizando-se a linhagem de *Bacillus* sp LBA 46 e o meio de cultura otimizado (meio n° 4) por metodologia de superfície de resposta (MSR), composto de 40g/L de melaço de cana de açúcar, 6g/L de água de maceração de milho, 2g/L de extrato de levedura Prodex-Lac SD® e 20g/L de soro de queijo, foi obtido 3000 U/mL de protease após 96h de fermentação a 30°C e 200 rpm. No estudo da aplicação da enzima para a remoção de manchas de tecidos de algodão foram obtidos melhores resultados de remoção de manchas de sangue e molho de tomate com carne moída, utilizando-se a combinação de extrato bruto de protease (100 ou 1000U) com o detergente Omo®.

O extrato enzimático bruto da linhagem de *Bacillus* sp. LBA 46 foi parcialmente purificado por fracionamento com sulfato de amônio (80% de saturação), diálise e cromatografia de filtração em gel (Sephadex G100), resultando em fator de purificação de 3,69. Após caracterização com MSR

observou-se que a protease da preparação parcialmente purificada apresentou atividade ótima a 55°C e pH 7,5 e considerável estabilidade (95% de atividade residual) na faixa de pH 5,7 – 9,3 após 1h de incubação a 30 – 36°C, e acima de 78,9% quando incubadas por 1h em pH 7,5 e 50°C. A condição ótima de lise das células de *X. campestris* do meio fermentado de goma xantana utilizando-se o extrato enzimático bruto de protease e a preparação parcialmente purificada de proteases, foi observada utilizando 42 U de protease /mL de suspensão celular de *X. campestris* a 60°C, resultando em aumento de mais de 20% da Trans $_{620nm}$ do meio fermentado de goma xantana. Um aumento de quase 40% de Trans $_{620nm}$ foi observado após 2h de reação utilizando extrato enzimático bruto de protease (42 U de protease/mL de suspensão celular de *X. campestris*) a 65°C. A produção de proteases de *Bacillus* sp. LBA 46 por fermentação em estado sólido foi otimizada utilizando MSR, sendo obtido 5000 U/grama de substrato seco utilizando-se meio de cultura composto de farelo de trigo e água (60%:40%) após 96h de fermentação a 30°C.

Palavras-chave: proteases, *Bacillus* sp., fermentação, clarificação de goma xantana, detergentes, remoção de manchas de tecidos.

SUMMARY

Bacterial proteases are commercially relevant enzymes widely applied in several industrial areas, such as in detergent, food, pharmaceutical and textile industries. The aims of this work were selecting among fifty nine *Bacillus* sp. strains from culture collection of Foood Biochemistry Laboratory of College of Food Engineering those ones that have higher potential of production of proteases with biochemical characteristics, such as stability in different conditions of temperature, pH, detergents and organic solvents, activity in a wide range of pH and capability of lysing cells of *Xanthomonas campestris*. Afterwards, it was aimed the optimization of the production of proteases by the selected *Bacillus* sp. strain and the determination of the biochemical characteristics of the partially purified protease and the application of the crude and partially purified protease.

Nine *Bacillus* sp. strains were selected as the best protease producers among fifty nine *Bacillus* sp. strains tested. The protease production by the nine strains was carried out in Erlenmeyer flasks containing medium no. 1 (10g /L of casein, 1g/L of yeast extract and salts), medium no. 2 (35 g/L of sugar cane molasses, 20g/L corn steep liquor, 3g/L of yeast extract Prodex-Lac SD® and 20g/L of dried whey), and by fermentation using solid substrate medium no. 3 (wheat bran and water, 1:1, m:m). The strains *Bacillus* sp. LBA 07, LBA 46 and LBA 08 when fermented in medium no. 1, no. 2 e no. 3 produced 222 U/mL, 548 U/mL and 13480 U/gram of dried substrate (gds) respectively. Proteases from the crude enzymatic extracts obtained from the fermentation of the nine *Bacillus* sp. strains in the three media showed optimal activity in pH range 7-9 and 60°C, stability in pH range 5-9 for 24 hours at 4°C and pH 7.0 for 1h at 50°C. The protease preparations from the fermentation of *Bacillus* sp. LBA 46 in the three media were the most stable when incubated in detergent Ariel®, among the

proteases tested from the *Bacillus* sp. strains. In addition, some proteases presented more than 60% residual activity after 24h in the organic solvents acetone (*Bacillus* sp. LBA 8 and 44), hexane (*Bacillus* sp. LBA 19, 29, 44, 46 and 60), chloroform (*Bacillus* sp. LBA 44 and 60) and ethanol (*Bacillus* sp. LBA 60). The protease preparations obtained from the cultivation of *Bacillus* sp. LBA 46 in medium no. 2 and no. 3 presented the best results on the lysis of *Xanthomonas campestris* cells, resulting in an increase of approximately 30% in transmittance at 620 nm (Trans $_{620nm}$) of the fermented broth of xanthan.

Bacillus sp. LBA 46 strain was selected as the best protease producer and after preliminary biomolecular analysis of identification, the results indicate that this microorganism correspond to a *Bacillus licheniformis* strain. Protease preparation containing 3000 U/mL was obtained from *Bacillus* sp. LBA 46 cultivated in Erlenmeyer flasks containing medium no. 4 composed of 40g/L of sugar cane molasses, 6g/L of corn steep liquor, 2g/L of yeast extract Prodex-Lac SD® and 20 g/L of dried whey after 96h of fermentation at 30°C and 200 rpm, optimized with response surface methodology (RSM). In the washing tests, the best results of the removal of blood and tomato sauce with ground beef stains from cotton fabrics were observed using the combination of crude extract of protease (100 or 1000U) with detergent Omo®.

Crude protease extract of the *Bacillus* sp. LBA 46 was partially purified by ammonium sulfate fractionation (80% saturation), dialysis and gel filtration chromatography (Sephadex G100), resulting in the purification fold of 3.69. After characterization with RSM it was observed that the crude protease extract and partially purified proteases presented optimal activity at 55°C and pH 7.5 and considerable stability (95% of residual activity) in pH range 5.7 – 9.3 after 1h incubation at 30-36°C and more than 78.9% when incubated at pH 7.5 and 50°C for 1h. The optimal conditions of the lysis of *X. campestris* cells contained in the

fermentation broth using crude and partially purified protease preparations were observed using 42 U of protease/mL of cell suspension of *X. campestris* at 60°C, resulting in a increase of more than 20% in Trans $_{620\ nm}$ of the fermented broth of xanthan. It was observed an increase of almost 40% in Trans $_{620\ nm}$ after 2h reaction using crude protease (42 U de protease/mL of cell suspension of *X. campestris*) at 65° C. The production of proteases by *Bacillus* sp. LBA 46 under solid state fermentation was optimized using RSM, resulting in 5000 U/gram of dry substrate utilizing wheat bran and water (60%:40%) after 96h of fermentation at 30°C.

Keywords: proteases, *Bacillus* sp., fermentation, xanthan gum clarification, detergents, stain removal from fabrics.

INTRODUÇÃO GERAL

Proteases são enzimas com diversas aplicações industriais como na indústria de alimentos, sendo aplicadas na obtenção de aromas, como também na obtenção de peptídeos com atividade biológica. Na indústria de detergentes as proteases são utilizadas como parte da formulação destes produtos para o auxílio na remoção de manchas de proteínas que são difíceis de remover apenas com os componentes dos detergentes. Outra área importante de aplicação das proteases é na indústria têxtil onde estas enzimas são utilizadas no tratamento do couro e outros tecidos para a retirada de resíduos de pelos e pele de animais. Estas enzimas também são utilizadas em meios orgânicos para síntese de diversos compostos com alto valor agregado devido à sua alta seletividade pelo substrato. Uma destacada aplicação de proteases se encontra na clarificação da goma xantana, que corresponde a uma importante etapa de purificação, que é necessária para aplicação deste produto em indústrias farmacêuticas e de alimentos. A clarificação da goma é possível uma vez que as proteases são capazes de lisar os peptídeoglucanos presentes na parede celular das células de *Xanthomonas campestris*. Esta aplicação de proteases tem se mostrado atrativa uma vez que as demais técnicas apresentam diversas desvantagens, como a degradação da goma ou diluição excessiva da mesma, devido à alta viscosidade do meio fermentado de *X. campestris* contendo goma.

As proteases podem ser obtidas de diversas fontes como fungos filamentosos, leveduras e bactérias e as características das proteases dependem totalmente do micro-organismo utilizado. Dentre as fontes de proteases as de *Bacillus* sp. são muito interessantes para a produção da enzima devido à elevada produtividade, bem como propriedades bioquímicas que estas podem apresentar como alta estabilidade em soluções alcalinas e compostos oxidantes,

termoestabilidade, além de estabilidade em solventes orgânicos. As proteases de *Bacillus* sp. podem ser produzidas por fermentação submersa e em estado sólido, sendo que cada tipo de fermentação apresenta vantagens e desvantagens. O uso de resíduos agroindustriais como substratos tais como melaço de cana de açúcar e água de maceração de milho é uma alternativa, uma vez que são consideravelmente de menor custo.

Este trabalho teve como principais objetivos selecionar linhagens de *Bacillus* sp. para a produção de proteases por fermentação submersa e em estado sólido, baseado nas propriedades bioquímicas das enzimas, estabilidade em detergentes e capacidade de lise de células de *X. campestris* do meio fermentado contendo goma xantana. A linhagem de *Bacillus* sp. LBA 46 foi selecionada para a produção de proteases e foi estudada a otimização do meio de cultura para a fermentação submersa da bactéria utilizando resíduos agroindustriais. O extrato enzimático bruto de proteases obtido foi aplicado na remoção de manchas de sangue e molho de tomate com carne, de tecidos. O extrato enzimático de protease foi parcialmente purificado, caracterizado e aplicado na clarificação de goma xantana. Este trabalho está apresentado na forma de capítulos e a estrutura da tese foi organizada da seguinte forma:

O capítulo 1 trata de uma revisão bibliográfica sobre proteases de *Bacillus* sp. abordando desde a produção até as aplicações desta enzima.

O capítulo 2 descreve a seleção de nove linhagens (LBA 07, 08, 19, 39, 44, 46, 48, 50 e 60) maiores produtoras de proteases entre 59 linhagenss de *Bacillus* sp. Estas nove linhagens foram fermentadas em meio n° 1, composto de caseína, extrato de levedura e sais, e em meio n° 2, composto de melaço de cana de açúcar, água de maceração de milho, extrato de levedura Prodex-Lac SD® e soro de queijo e meio n° 3, composto de farelo de trigo e água por fermentação

em estado sólido. Estes três diferentes meios foram testados, uma vez que proteases podem se apresentar na forma de isoenzimas e com isso a atividade, seletividade e estabilidade podem variar em função do meio de cultivo utilizado para a sua produção. Os extratos brutos de protease das nove linhagens foram caracterizados com relação à atividade ótima e estabilidade em diferentes valores de pH e temperatura, estabilidade em solventes orgânicos e detergentes. Os extratos também foram aplicados ao meio fermentado contendo goma xantana para avaliar a lise de células de *X. campestris*. A linhagem de *Bacillus* sp. LBA 46 foi selecionada para estudos de produção de protease.

O capítulo 3 trata da produção de proteases pela linhagem *Bacillus* sp. LBA 46 em meio de cultivo composto de resíduos e subprodutos agroindustriais. Inicialmente foi estudada a cinética de produção de protease, crescimento bacteriano e variação de pH do meio de cultura. As concentrações dos substratos do meio de cultura, melaço de cana de açúcar, água de maceração de milho e extrato de levedura Prodex-Lac SD® foram otimizadas, utilizando metodologia de superfície de resposta (MSR). Posteriormente foram realizados experimentos univariáveis para determinar concentração a do soro de queijo adequada no meio de cultura para a produção de proteases. O soro de queijo não foi considerado no delineamento composto central rotacional (DCCR) uma vez que diversos experimentos foram realizados, porém não foi possível obter resultados satisfatórios nas análises estatísticas, provavelmente devido à interferência dos efeitos da alta concentração dos carboidratos presentes tanto no melaço como do soro de queijo. O extrato enzimático bruto foi aplicado na remoção de manchas de sangue e de molho de tomate com carne moída de tecidos de algodão em conjunto com detergentes de lavar roupas.

O capítulo 4 trata da purificação do extrato bruto de protease obtido da fermentação da linhagem de *Bacillus* sp. LBA 46 no meio otimizado (meio n° 4)

como descrito no capítulo anterior composto de melaço de cana de açúcar (40g/L), água de maceração de milho (6g/L), extrato de levedura Prodex-Lac SD® (2 g/L) e soro de queijo (20g/L). As proteases do extrato enzimático bruto foram inicialmente concentradas por precipitação com sulfato de amônio a 80% de saturação seguida de diálise. Posteriormente foi utilizada coluna de filtração em gel Sephadex G-100 e a fração com maior atividade foi selecionada. As proteases do extrato enzimático bruto e da fração parcialmente purificada foram caracterizadas quanto ao efeito do pH e temperatura na atividade e estabilidade das proteases utilizando MSR. O extrato enzimático bruto e a fração de protease parcialmente purificada foram aplicados na lise de *X. campestris* no meio fermentado contendo goma xantana, utilizando MSR para otimizar as condições de lise e a temperatura e a quantidade de protease por mL de suspensão celular de *X. campestris* foram avaliadas. Os resultados de caracterização e aplicação das proteases do extrato enzimático bruto e fração de protease parcialmente purificada foram comparados entre si.

No anexo está relatada a otimização da produção de proteases da linhagem de *Bacillus* sp. LBA 46 por fermentação em estado sólido sendo avaliadas os efeitos da proporção de farelo de trigo e água (m:m) do meio de cultura, quantidade de inóculo e temperatura.

I. CAPITULO 1 - AN OVERVIEW ON *Bacillus* sp. PROTEASES: FROM THE PRODUCTION TO THE APPLICATION

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RESUMO

As proteases apresentam diversas aplicações industriais e estão entre a maioria das enzimas mundialmente comercializadas. Dentre as fontes de proteases microbianas, as do gênero *Bacillus* são as fontes bacterianas mais importantes, uma vez que produzem enzimas proteolíticas alcalinas extracelulares com alto rendimento e interessantes propriedades, tais como alta estabilidade em condições extremas de temperatura, pH, em contato com solventes orgânicos, detergentes e agentes oxidantes. Desta forma, diversas estratégias têm sido estudadas para o desenvolvimento de processos de produção de proteases de *Bacillus* sp. de modo economicamente viável. Estas estratégias incluem parâmetros de fermentação, como nutrientes, temperatura e tempo de fermentação. Desta forma, existem diversos trabalhos relatando o uso de substratos de baixo custo para fermentação submersa e em estado sólido, como melaço de cana de açúcar e farelo de trigo. Antes da aplicação a protease deve ser caracterizada e purificada, preferencialmente utilizando técnicas de baixo custo. As proteases de *Bacillus* sp. têm sido estudadas para aplicação na indústria de detergentes para remoção de manchas de sangue em tecidos, clarificação da goma xantana, obtenção de peptídeos bioativos, processamento de alimentos, em reações enantioseletivas e na remoção de pelos de peles de animais. Este trabalho de revisão descreve a produção, purificação, caracterização e aplicações de proteases de espécies de *Bacillus* sp.

Palavras-chave: *Bacillus*, proteases, goma xantana, peptídeos bioativos, detergentes

ABSTRACT

Proteases find a broad range of industrial applications and correspond to the majority of worldwide enzyme sales. Among the microbial proteases sources, the genus *Bacillus* are probably the most important bacterial source, producing extracellular alkaline proteolytic enzymes with high yield and remarkable properties, such as high stability towards extreme temperatures, pH, organic solvents, detergents and oxidizing compounds. Based on that, several strategies have been developed for a cost effective production of *Bacillus* sp. proteases, which include fermentation parameters, for instance, nutrients, temperature and fermentation time. In addition, there are many works on the use of low cost substrates for submerged and solid state fermentation, i.e. molasses and wheat bran. Before application, proteases should be characterized and purified, preferentially using inexpensive methods. *Bacillus* sp. proteases have been studied for use in detergent formulation for the removal of blood stains from fabrics, the clarification of xanthan gum, obtaining of bioactive peptides, food processing, in enantioselective reactions, and in dehairing of animal skins. Therefore, this review highlights the production, purification, characterization, applications of proteases from *Bacillus* sp. species.

Keywords: *Bacillus*, proteases, xanthan gum, bioactive peptides, detergents

I.1.Introduction

It is well reported and established that in many different industrial steps a selective and efficient catalyst is necessary. Conventional chemical catalysts have frequently low cost and result in high yield of production. On the other side, the chemical catalysts present disadvantages when used in food and pharmaceutical industries. In this context, enzymes can be applied in several reactions with high selectivity and specificity allowing the obtaining of high value added products, at the same time, resulting in none or few byproducts. In addition, this biocatalysts act in mild reactions conditions regarding pH and temperature.

Hydrolases are an important group of enzymes widely applied in different industrial fields. Among these enzymes, proteases (E.C. 3.4.21.14) are highlighted, corresponding to approximately 60% of the total worldwide enzyme sales, and they represent one of the most important enzymes from the industrial point of view. Proteases, proteolytic enzymes or proteinases hydrolyse peptide bonds of proteins and their specificity is based on the bonds they break. The importance of the proteases in nature is in the decomposition of plant, animal and microbial residues. They represent one of the largest groups of industrial enzymes which have increasing market demands due to their applications in the detergent, food, pharmaceutical, chemical, leather, paper, pulp and silk industries (Kuddus and Ramteke, 2012).

Proteases occur in animals (Ahmed et al., 2013), plants (Van der Hoorn et al., 2004) and microorganisms (Cheng et al., 2012). Microorganisms produce enzymes easily and faster compared to mammalian and plant cells, and the enzyme production is neither influenced by climatic conditions or seasonal changes, nor by regulatory or ethical issues related to animal slaughter or tree or plant felling. Additionally, extracellular enzymes produced by microorganisms are preferred, considering this simplifies downstream processing, hence further

lowering costs (Tufvesson et al., 2010). The immense biodiversity of microorganisms improves their biotechnological importance and justifies the search for new proteases. Literature reports several microorganisms capable of producing extracellular proteases, such as *Aspergillus oryzae* (Belmessikh et al., 2013), *Aureobasidium pullulans* (Chi et al., 2007), *Serratia marcescens* (Bach et al., 2012) and *Bacillus subtilis* (Helal et al., 2012). Within this context, bacteria are one of the most important groups of protease producers with the genus *Bacillus* being the most prominent source. A myriad of *Bacillus* species from different exotic environments have been explored and studied for extracellular alkaline protease production and the strains of *Bacillus licheniformis, B. subtilis, Bacillus amyloliquefaciens* are the most reported (Gupta et al. 2002a; Kalisz 1988).

Taking into account the relevance of the proteases from *Bacillus* sp., the cost-effective production of this enzyme is extremely necessary, and the use of agro-industrial residues is an interesting alternative, including the use of sugar cane molasses and corn steep liquor for submerged fermentation (SmF) (Shikha et al., 2007; Helal et al., 2012) and different types of brans and cakes for solid state fermentation (SSF) (Sangeetha et al., 2011; Shivasharana and Naik, 2012). The immobilization of different species of *Bacillus* sp. cells for protease production under SmF has also been reported (Adinarayana et al., 2005). With this technique the protease can be produced in repeated fermentations batches which can make the process more cost effective, in some cases. Furthermore, another important field is the genetic improvement of *Bacillus* sp. strains to increase protease production or result in proteases with differentiated properties.

The proteases from *Bacillus* strains are widely studied and the one of the main reasons are the interesting properties that they present, including high activity, stability and in some cases selectivity for the substrate. Therefore, there

are several studies focused on the screening, purification and characterization of these enzymes (Jain et al., 2012; Rajkumar et al., 2011). In this context, *Bacillus* sp. proteases present several remarkable characteristics for many industrial applications. Taking into consideration the broad pH and temperature range of activity and stability, *Bacillus* sp. proteases are applied in detergent industries for removal of stains in fabrics (Annamalai et al., 2013). For this application the enzyme has to be highly tolerant to alkaline environment and not be inactivated by several toxic compounds including oxidants and surfactants. In spite of this application, the *Bacillus* sp. proteases can be applied in the food industry for clarification of xanthan gum (Armentrout et al., 1999), obtaining of bioactive peptides and processing of different foods (Bougatef et al., 2012; Ozcan and Kurdal, 2012). Another feature of these proteases is the stability in organic solvents and therefore their application in organic synthesis (Caille et al., 2002). For this last application the immobilization of the enzyme can be very attractive and the reuse of the enzyme can be performed (Corîci et al. 2011).

Based on all this interesting aspects of proteases from the genus *Bacillus,* these enzymes are marketed and several studies using these commercial proteases are frequently reported, including strains from *B. licheniformis* and *B. subtilis* (Ferreira et al., 2003; Ozcan and Kurdal, 2012). The present manuscript provides an overview of developments on different aspects of extracellular proteases from *Bacillus* sp. strains from the production to the application as summarized in the Figure I.1.

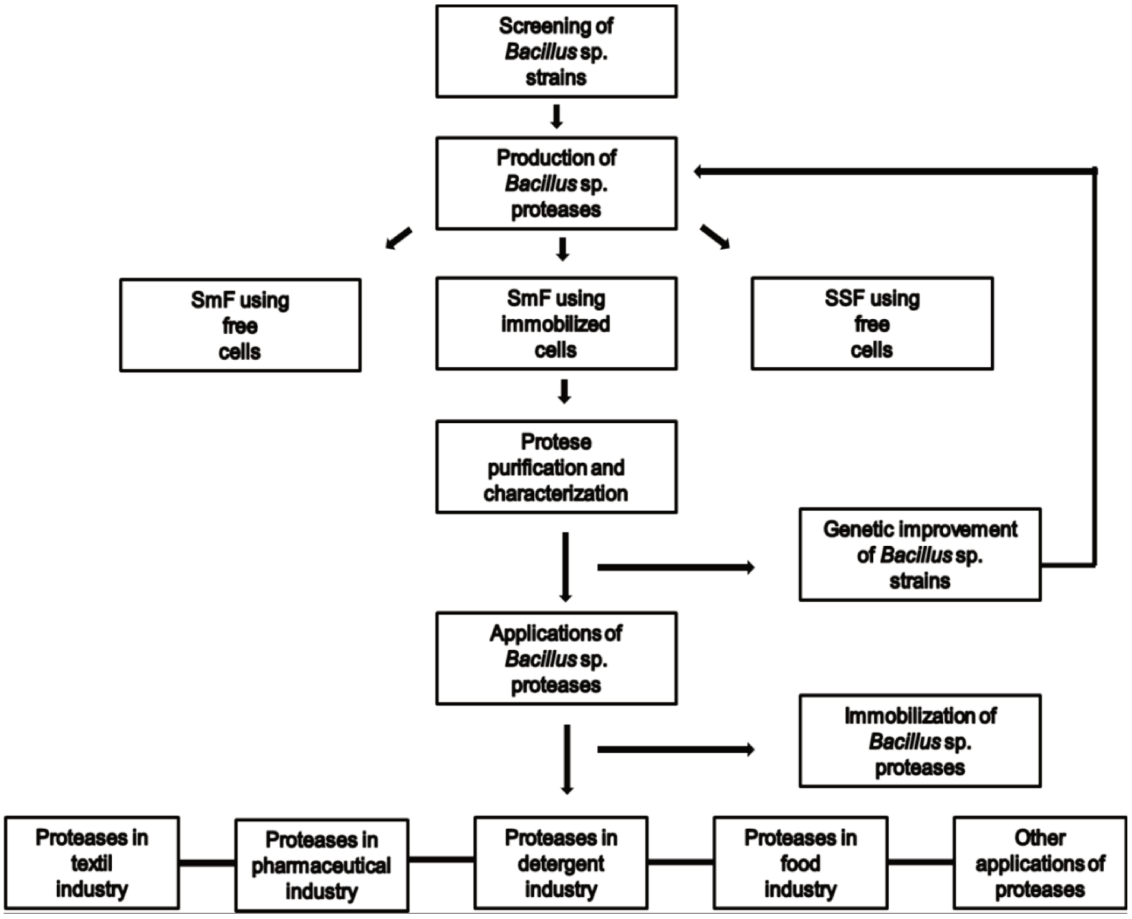

Figure I.1. Schematic diagram outlining the general steps of proteases from *Bacillus* sp. strains from the production to the application.

SmF – Submerged Fermentation; SSF – Solid State Fermentation

I.2. Production of proteases from *Bacillus* sp.

The use of microorganisms for protease production presents advantages compared to other sources, but it is not necessarily cost effective, simply for the fact that the media components and the large scale fermentation processing is highly expensive and frequently does not allow the feasibility of the enzyme production. Thus, the first important step is the selection of a *Bacillus* sp. strain capable of producing high quantities of proteases with desirable properties.

With the increasing emphasis on cost effectiveness for enzyme production, the use of low-cost fermentation medium should be of immense importance for commercial gain, considering the cost of the fermentation media components correspond to approximately 30 a 40% of the whole cost (Kirk et al., 2002). In this case, the use of agricultural residues and byproducts are the focus of several studies to make them alternatives to the expensive components including, monosaccharides and oligosaccharides, and proteins sources such as peptone and casein. Table I.1 shows the optimized conditions for protease production by different *Bacillus* sp. strains under SmF and SSF using agro-industrial residues or byproducts as component of culture media.

Another important aspect is the optimal production of the protease and for this purpose the concentration of substrates, temperature, pH, agitation and aeration must be optimized. The use of statistic techniques, including Placket Burman design for the selection of the variables and central composite design and response surface methodology for the optimization of the variables is also very important (Cheng et al., 2012).

In this section the most important aspects of protease production is reported and discussed, such as the production of the enzyme under SmF, SSF and immobilization of the *Bacillus* sp. cells for protease production under SmF.

Table I.1. Optimized conditions for cultivation of protease-producing *Bacillus* sp. strains using agro-industrial residues or by products under submerged fermentation (SmF) and solid state fermentation (SSF).

***Bacillus* sp. strain (SmF)**	**Initial pH of culture medium**	**Temperature (°C)**	**Agitation (rpm)**	**Incubation time (h)**	**Substrate (SmF)**	**Protease Activity (U/mL)**	**Reference**
Bacillus sp. BGS	11	37	150	48	Molasses	2,992.75	Moorthy and Bascar, 2013
B. subtilis PCSIR-5	7.5	37	120	48	Soybean meal	107	Nadeem et al., 2006
B. subtilis	10	37	150	48	Molasses and corn steep liquor	401	Helal et al., 2012
Bacillus pantotheneticus	10	30	120	48	Molasses and wheat bran	285	Shikha et al., 2007
***Bacillus* sp. strain (SSF)**	**Moisture (%)**	**Temperature (°C)**	**Incubation time (h)**		**Substrate (SSF)**	**Protease Activity (U/g)**	**Reference**
Bacillus sp. JB-99	90-95	50	96		Wheat bran and sugarcane bagasse	7000	Shivasharana and Naik, 2012
B. subtilis KHS-1	60	37	72		Green gram husk	8848	Ramakrishna et al., 2012
Bacillus pumilus SG2	60	60	72		*Pongamia pinnata* seed cake	9840	Sangeetha et al., 2011
Bacillus cereus	50	40	48		Red gram husk	258	Rathakrishnan and Nagarajan, 2011

I.2.1. Production of *Bacillus* sp. protease under submerged fermentation

Approximately 90% of all industrial biocatalysts are produced by SmF, using specifically optimized media, resulting in enzyme titers in the range of g/L. SmF provides several advantages including reduction of contamination due to relatively short growth period, higher conversion levels of the raw medium substrates due to the controlled growth period, less production monitoring labor and well developed scale-up methods (Gupta et al., 2002b).

The effect of nutritional and environmental conditions on protease synthesis plays an important role in the repression or expression of the enzyme (Gupta et al., 2002b). Furthermore each microorganism has its own specific conditions for maximum production of protease. The most difficult part in optimization of culture conditions is the presence of interactive effects of medium components and culture condition factors.

Proteases are massively produced during stationary phase and, therefore, are generally regulated by carbon and nitrogen stress. They are associated with the beginning of stationary phase and it marks by the transition from vegetative growth to sporulation stage in spore-formers. Hence, production of protease is often related to the sporulation stage in many *Bacilli*, including *B. subtilis* (O'Hara and Hageman 1990), and *B. licheniformis* (Hanlon and Hodges 1981). On the other hand, based on some reports, it can be concluded that sporulation and protease production are not related, taking into consideration that the spore-deficient strains of *B. licheniformis* produced proteases (Fleming et al. 1995).

Besides carbon and nitrogen sources, pH is a very important parameter for *Bacillus* sp. protease production under SmF, considering generally pH of culture media affects both the morphological and physiological characteristics of an organism and moreover strongly affects many enzymatic processes and transport of compounds across the cell membrane. In addition, the production

of *Bacillus* sp. proteases can be very influenced by the agitation parameter rate, due to the difficulty to maintain sufficient dissolved oxygen (DO) for the cell growth as described in the work of Joo and Chang (2006).

Taking into account that several parameters interfere in the protease production, some works have been used to illustrate that. Moorthy and Baskar, (2013) studied the production of proteases by the bacterial strain *Bacillus* sp. BGS. The strain was isolated from effluent of a milk processing industry and some variables were evaluated for the enzyme production. The extracellular protease secretion is influenced not only by carbon and nitrogen sources, but also by culture conditions, for instance pH, temperature, DO, and inoculum density. Molasses, peptone, pH, and inoculum size were selected as the most important for protease production, using Plackett–Burman design. The bacterial strain chosen for this study showed a gradual increase in the growth and production of protease with an increase in pH (7-11), indicating the alkalophilic nature of the bacterial strain. Inoculum size was also important, considering high inoculum size can attenuate enzyme production due to competition for available nutrients, whereas low inoculum size results in a mitigation of enzyme secretion, owing to a decrease in cell numbers. After optimization using a hybrid system of response surface methodology followed by genetic algorithm, the optimal concentration of media components and culture conditions was found to be 16.82 g/L of peptone, 11.28 g/L of molasses, pH value of 11, and 2% (v/v) of inoculum size. The protease activity increased significantly with an optimized medium (2,992.75 U/mL) as opposed to basal medium prior to the optimization (470.35 U/mL).

The protease from the *B. amyloliquefaciens* B7 was studied by Cheng et al. (2012). The nutritional medium requirement for alkaline protease production was optimized initially screening the nutrients through one-factor-at-a-time experiments and then Plackett–Burman design, by which fructose and yeast extract were identified as the most significant variables. The

protease production was increased approximately 3.92-fold comparing the protease production using the original medium (51.49 U/mL) to the optimized medium (242.45 U/mL) composed of 54.14 g/L of fructose and 16 g/L of yeast extract. After the optimization the authors studied the production of protease in batch cultivations in a 10 L bioreactor, resulting in 3120.55 U/mL of the maximum enzyme activity.

Bacillus sp. SW-2 was studied for protease production in shake-flask fermentation using low-cost residues. The maximum protease yield of 2.69±0.19 U/mL was obtained at 72 h, initial pH 9.0, 45°C and using 5% of inoculum and soy meal (10 g/L) and sugar cane bagasse (10 g/L) at 180 rpm. The protease production using the low cost substrates was significantly higher than that obtained from expensive substrates (sucrose and peptone) (George-Okafor and Mike-Anosike 2012).

The production and characteristics of a protease from *Bacillus clausii* I-52 was studied by Joo and Chang (2006). The enzyme was produced under SmF and the authors observed that the initial pH of the medium plays a critical role for the cell growth and protease production taking into account the protease yield and cell growth was reduced substantially at around neutral pH indicating that *B. clausii* I-52 was an obligate alkalophile. The optimal environmental parameters for protease production were observed at an agitation rate of 700 rpm and aeration rate of 1.5 vvm at 37°C. Thus, the maximum enzyme activity (137,020 U/mL) was obtained when cells were grown under the conditions at 37°C for 48 h with an aeration rate of 1.5 vvm and agitation rate of 700 rpm in a medium (pH 10.6) containing soybean meal, wheat flour, liquid maltose, K_2HPO_4, Na_2HPO_4, $MgSO_4 \cdot 7H_2O$, NaCl, $FeSO_4 \cdot 7H_2O$, and Na_2CO_3.

I.2.2. Production of *Bacillus* sp. protease under solid state fermentation

The solid state fermentation (SSF) involves the growth of microorganisms in solid substrates, like wheat bran, and other agro-industrial products under reduced moisture conditions. The substrate must possess enough moisture to support growth and metabolism of microorganisms (Pandey, 1992). In spite of the great industrial importance of SmF, SSF presents some advantages when compared to the former, including simplicity of the media, economy of space for fermentation, no requirement for complex machinery, compactness of the fermentation vessel due to the smaller volume of water, equipment or control systems, superior yields, less capital investment and recurring expenditure and less energy demand (Lonsane et al., 1985). Additionally, such systems can be applied in solid waste management, biomass energy conservation and in the production of secondary metabolites. However, SSF present some limitations, including the controlling and monitoring of parameters such as temperature, pH, humidity and air flow and a limited choice of microorganisms capable of growing under reduced moisture conditions (Nahara et al., 1982; Lonsane et al., 1985). SSF offers numerous opportunities in processing of agro-industrial residues and byproducts. Part of this is because solid-state processes require less energy, and produce lesser wastewater. In addition, they are environmentally-friendly, taking into account they resolve the problem of solid wastes disposal.

Different from SmF, SFF has some other parameters that must be studied cautiously, such as the particle size of the solid substrate. The availability of surface area is vitally important for microbial attachment, mass transfer of various nutrients, substrates, and hence microbial growth and enzyme formation. The availability of surface area truly depends on particle size of the substrate/support matrix. Moisture content/water content is also one of the most critical factors, considering in SSF microbial growth and

product formation occur at or near the surface of the solid substrate particle having low moisture contents (Pandey et al., 2000).

Several agro-residues were tested for α-amylase and protease production by *B. licheniformis* ZB-05. For both enzymes rice husk was selected as the best substrate resulting in protease activity of 469,000 U/g. The best conditions for protease production was observed to be 30% initial moisture, 30% inoculum volume at 36h of fermentation. The addition of 20 g/L of ammonium sulfate and 10 g/L of Bacto casaminoacid served as the best inorganic and organic nitrogen sources for protease production, respectively. Regarding the carbon source it was possible to observe that 10g/L of maltose enhanced protease production (Karatas et al., 2013).

In the work of Madhuri et al. (2012) castor husk waste was used as substrate for alkaline protease production by *Bacillus altitudinis* GVC11 in SSF. Protease production increased with incubation period up to 96h and decreased after this time. Maximum enzyme production was obtained with particle size smaller than 1 mm and substrate particle size higher than this resulted in reduction of alkaline protease production. At 80% of moisture content, maximum protease activity was observed resulting in 128,357 U/gram of dried substrate (gds). Linearity between moisture content and enzyme production was observed up to 80% and after that further increase in moisture level in the fermentation medium resulted in separation of liquid medium from solid substrate. Based on that, the authors commented that the reduction in protease production at reduced moisture level is probably associated with reduced availability of water for microbial growth. Thus, the highest enzyme production of 419,293 units per gram husk was obtained after 96h at moisture content of 80% with particle size smaller than 1 mm.

The *Bacillus* sp. RRM1 was isolated from the red seaweed *Kappaphycus alvarezii* (Doty) Doty ex Silva by the authors Renganathan et al., (2011). The production of proteases by this strain under SSF was studied

using inexpensive agricultural residues like pigeon pea husk, black gram husk, rice bran, green gram hull, and orange peel. The wheat bran was found to be the best solid substrate for protease production. It was observed that the best moistening agent was sea water, when compared to tap water and salt solution and the best ratio of wheat bran and moisture was 60%. The optimum temperature for protease production was 37°C, which was the same for bacterium growth. At temperatures below 37°C and above 45°C the enzyme production greatly decreased. Regarding the inoculum, it was noticed that 15% was the best concentration and higher quantities of inoculum decreased the protease yield. The authors also tested the addition of some nitrogen sources and observed that maltose at 15 g/L and yeast extract at 10 g/L supported the maximum protease production (2081U/g).

In the work of Ramakrishna et al. (2012) the best conditions of protease by *B. subtilis* KS1 (MTCC No-10110) were green gram husk (12.5g), glucose (10 g/L) and casein (10 g/L) pH 9.0, 37°C and incubation period 72h. In another work, a metalloprotease was produced in rice bran after 72h with 20% inoculum and 1:3 moisture content (Saxena and Singh, 2011).

I.2.3. Immobilization of *Bacillus* sp. cells for protease production

Bacillus sp. proteases are usually obtained from free or immobilized cells. In the latter case the cells are usually entrapped in a natural or synthetic polymer. The immobilization technique has to be mild enough to not inactivate the cells and maintain the metabolism or even improve it. Different aspects of immobilized cells must be evaluated, such as gel concentration, solidification period and $CaCl_2$ for ionic gelation, cell concentration, bead size and porosity. Whole cell immobilization presents several advantages when compared to free cell systems such as higher yield of enzyme activity, higher operational stability, greater resistance to environmental perturbations, lower

enzyme cost and repeated use of biocatalyst (Mamo and Gessesse 2000; Nampoothiri et al. 2005). After immobilization of the cells the fermentation can be carried out in SmF. The most common immobilization matrix used nowadays are polysaccharides as alginate, k-carrageenan, agar, chitosan, taking into account the formation of these gels occurs under very mild conditions and is characterized by the low cost (Bladino et al. 2001). The immobilization of microbial cells in alginate by ionic gelation using divalent cations has been extensively applied for entrapment of viable cells for production of a broad number of enzymes (Mrudula and Shyam, 2012; Jamuna and Ramakrishna, 1992; Kar et al., 2008).

Mrudula and Shyam, 2012 reported the optimization of immobilization parameters for protease production from the *Bacillus megaterium* MTCC2444 strain. In the study, calcium alginate was used and they observed that immobilization was most effective with 4% gel concentration, bead size of 3 mm, 24h aged immobilized cells for a solidification period of 12 h at 1.5 % initial biomass concentration. Smaller bead diameter resulted in higher protease production, probably due to an increase in the surface volume ratio. As the biomass concentration in the gel beads increased, the cell leakage into the fermentation medium increased as well. The authors attributed that to the fact that when the cell loading in the beads increased, the nutrient/cell ratio decreased, which might become limiting. Prolongation of solidification time improved the stability of the beads. The optimum solidification time for the maximum protease production was 12 h followed by decrease in cell leakage. The authors observed that there was 1.5-fold increase in the enzyme yield by the immobilized cells when compared to the free cells. When the immobilized cells were applied in repeated batches, it was observed that the immobilized cells maintained approximately 80% of the operational stability up to the sixth batch of 72h.

In the work of Shrinivas et al. (2012) the *Bacillus halodurans* JB 99 cells were immobilized in calcium alginate for protease production in SmF. It was observed that the alkaline protease production increases with increasing alginate concentration from 1 to 2.5% and reached a maximal value at 2.5%. The protease production also increased as a function of the number of beads and maximal production was observed when 350 beads per flask was used. When the free and immobilized cells were compared regarding protease production, it was observed that in the immobilized system the protease production was enhanced by 23.2% at the same time interval of 24h, resulting in 5,275 ± 39.4 U/mL. When the immobilized cells were applied in the semicontinuous mode of cultivation, a considerable level of protease was observed in up to nine cycles, reaching maximal value of 5,975 U/mL after the seventh cycle. The protease was applied in degradation of chicken feathers in the management of keratin-rich waste and obtaining value-added products.

In the work of Mashhadi-Karim et al. (2011) *B. licheniformis* cells were immobilized in calcium alginate beads and applied for production of protease. In the optimal conditions of immobilization the beads were stable at pH 8, 8.5, and 9 for 8, 5, and 4 days, respectively. When the free cells were studied, the highest level of protease production, (623 U/mL) was reached after 35h. On the other hand, in the immobilized cell process, the highest amount of enzyme, 1,083 U/mL, has been produced after 48 h, which was 74% higher than the former. After optimization of immobilization of cells and culture medium a 7.3-fold higher productivity was observed compared to the free cells in the non-optimized medium. The immobilized cells were applied for the production of the protease for 13 batches in 19 days.

Cells of *B. subtilis* PE-11 were immobilized in various matrices, including calcium alginate, k-carrageenan, polyacrylamide, agar-agar, and gelatin, and then applied for the production of alkaline protease. The authors observed that calcium alginate was the most effective and suitable matrix for

higher protease productivity when compared to other matrices studied. The average of specific volumetric productivity using calcium alginate was 15.11 U/mL/hour, corresponding to a production 79.03% higher over the conventional free-cell fermentation. The specific volumetric productivity by repeated batch fermentation was 13.68 U/mL/hour with k-carrageenan, 12.44 U/mL/hour with agar-agar, 11.71 U/mL/hour with polyacrylamide, and 10.32 U/mL/hour with gelatin. An optimum level of enzyme production was maintained for 9 days using calcium alginate immobilized cells, in repeated batch fermentations of the shake flasks (Adinarayana et al., 2005).

Okita and Kirwan (1987) reported the production of protease by immobilized cells of a strain of *B. licheniformis* compared to the production using free cells. The cells were immobilized by ionic adsorption to Cellex E, an anionic exchange cellulose. Under conditions of low glutamate and high dilution rates in the reactor, the immobilized cells presented specific protease production rates greatly exceeding those of free cells.

I.3. Purification and characterization of proteases from *Bacillus* sp.

There are different types of proteases and their classification depends on different criteria, such as pH that the enzyme acts and the active site of the enzyme. Most of the *Bacillus* sp. proteases are alkaline acting in pH values above 7.0. The characterization of proteases is very important taking into account the application of the enzyme, considering the biochemical, catalytic and kinetics parameter. If optimal conditions of the proteases are known, this enzyme can be industrially applied. Activity parameters include optimal pH, temperature, substrate concentration, reaction time, and the addition of possible activators, such as $CaCl_2$ for some types of proteases. On the other hand, different experiments must be carried out to discover possible salts, ions and organic compounds that can inactivate the protease, as well as temperature and pH of stability of the enzyme.

It is a fact that the most important aspects of an enzyme for industrial application are stability and better catalytic performance (Iyer and Ananthanarayan, 2008). Thus, one of the major problems of the some proteases is their instability in alkaline pH and higher temperature (Griffin et al., 1992). This problem can be overcome by searching for new proteases with novel properties from different wild sources. The stability of an enzyme is evaluated by the residual activity and the catalytic performance is defined as the ability of an enzyme to catalyze a process. Both are affected by either physical (temperature and pH) or chemical (the presence of inhibitors or activators etc.) parameters (Iyer and Ananthanarayan, 2008; Naidu, T. Panda; Rana et al., 2003). Proteases need to be active and stable at high temperature and pH for viable industrial use (Manachini et al., 1998). Therefore, taking into consideration that these conditions are extremely relevant to most enzymes there is a growing demand for enzymes with improved stability (Iyer and Ananthanarayan, 2008).

For reasons of economy, a minimal number of enzyme purification steps and considerable knowledge of the biochemical properties of the enzyme are necessary for industrial application. For a truly and complete characterization of a protease, it must be completely pure. In most of the reports on protease purification the first step is the concentration of the proteins, using organic solvents or the addition of ammonium sulfate. After this step, a sequence of different chromatography steps is applied, which includes ion exchange, hydrophobic interaction, and gel filtration chromatography using different columns for each of this types of chromatography (Anbu 2013; Singh et al., 2012). The purification degree of the enzyme is in accordance with the application of the enzyme and in this case it must considered that the more purified the protease is more expensive is the process, which can make the application of the enzyme unviable.

Proteases have been extensively purified and characterized in terms of their properties of use in biotechnological applications. Homogeneity allows for the successful determination of their primary amino acid sequences and three-dimensional structures. Studies of the structure–function relationships of pure proteases have contributed to an understanding of the kinetic mechanisms of the enzyme actions (Duman and Löwe, 2010). In Table I.2, there are several reports on purification of *Bacillus* sp. protease with different properties.

Table I.2. Purification techniques applied to *Bacillus* sp. proteases purification and characteristics of the proteases

***Bacillus* sp. strain**	**Purification techniques**	**Molecular mass (kDa)**	**Optimal pH and temperature**	**pH and temperature of stability of the proteases**	**Reference**
Bacillus koreensis (BK-P21A)	Ammonium sulfate precipitation, Superdex 200 10/300 GL and Superdex 75 10/300 GL column chromatography	48	pH 9.0 and 60°C	pH 7-10 and 70°C for 1h of incubation	Anbu, 2013
B. cereus TKU022	Ammonium sulfate precipitation, DEAE-Sepharose CL-6B column, Phenyl Sepharose and Sephacryl S-100 chromatography.	45	pH 10 and 50 – 60°C	pH 7-10 and 60°C for 1h of incubation	Liang et al., 2012
Bacillus sp. SM2014	Ammonium sulfate precipitation and Sephadex-G100 column chromatography	71	pH 10 and 60°C	pH 7–12 and 80°C after 30 min of incubation	Jain et al., 2012
*B. megaterium*RRM2	Ammonium sulfate precipitation, Sephadex G100 and Q-Sepharose column chromatography	27	pH 10 and 60°C	pH 7-11 for 4h of incubation and 60°C for 2h of incubation	Rajkumar et al., 2011
Bacillus pseudofirmus SVB1	Acetone precipitation and CM650 Toyopearl column chromatography	85	pH 10 and 40°C	pH 8 – 10 for 1h of incubation and 35°C for 30 min of incubation	Sen et al., 2011
B. subtilis VSG-4	Ammonium sulfate precipitation and Sephadex G200 column chromatography	24	pH 9 and 50°C	pH 8-10 for 2h of incubation and 50°C for 3h of incubation	Giri et al., 2011
B. licheniformis MP1	Ultrafiltration, Sephadex G-100 and Mono Q-Sepharose column chromatography	30	pH 10 and 70°C	pH 7-12 for 1h of incubation and 50°C for 2h of incubation	Jellouli et al., 2011

An extracellular protease from the strain *B. licheniformis* NCIM-2042 was purified 39-fold by protein precipitation with ammonium sulfate followed by Sephadex G-100 (gel filtration chromatography) and the ion exchange chromatography using CM-Sepharose column. The molecular mass was estimated as 70 kDa by SDS-PAGE. The purified protease retained more than 93% of its initial activity after pre-incubation at 37°C for 30 min in the presence of 25% (v/v) different organic solvents, including methanol, ethanol, 2-propanol, benzene, toluene and hexane. Inhibition kinetic showed that this enzyme is a serine protease because it was competitively inhibited by antipain and aprotinin and these compounds are known to be competitive inhibitors of serine protease (Bhunia et al., 2013).

A protease from the *B. cereus* SIU1 strain was studied by the authors Singh et al. (2012). The enzyme was purified using a combination of Q-Sepharose and Sephadex G-75 chromatography and the pure enzyme showed molecular mass of 22 kDa. The Km, Vmax and kcat was calculated as to be 1.09 mg/mL, 0.909 mg/mL/min and 3.11 s^{-1}, respectively, towards a casein substrate. The protease presented higher activity and stability at pH 9.0 and a temperature range of 45–55°C. The compounds ethylenediaminetetraacetic acid, phenyl methyl sulfonyl fluoride and ascorbic acid were inhibitory with regard to enzyme activity, whilst cysteine, β-mercaptoethanol, calcium, manganese, magnesium and copper at concentration of 1.0 mM increased enzyme activity. At the concentrations of 0.1 and 1.0% sodium dodecyl sulfate, Triton X-100, Tween 80, hydrogen peroxide and sodium perborate significantly enhanced protease activity. The pure protease was considerably stable in the presence of 0.1 and 1.0% (w/v) detergents retaining 50–76% of activity. After an initial analysis of the circular dichroism spectrum in the ultraviolet range the authors observed that the protease is predominantly a β-pleated structure and a detailed structural composition showed approximately 50% of β-sheets. The circular dichroism based conformational evaluation of

the protease after incubation with metal ions, modulators, detergents and at different pH values, showed that the change in the β-content directly corresponded to the altered enzyme activity.

In the work of Lagzian and Asoodeh (2012) a protease from the *Bacillus* sp. MLA64 was purified with a 16.5-fold increase in specific activity and 93.5% recovery by (polyethylene glycol) precipitation and a sequence of chromatography techniques, such as Sephadex G-100 gel filtration, Q-Sepharose ion exchange chromatography and CM-Cellulose ion exchange chromatography. The molecular weight of the enzyme was estimated as 24 kDa, by SDS-PAGE. This purified protease was characterized and the authors observed that the enzyme was extremely stable and active over the temperature range from 40 to 100°C and in a wide range of pH from 6.0 to 12.5. The enzyme presented optimal activity at 95°C and at pH 9.5. The presence of calcium chloride did not enhance the enzyme activity, indicating that the enzyme is calcium-independent. The protease presented high stability towards non-ionic surfactants and anionic surfactant SDS and relatively stability in the presence of oxidizing agents. Taking into account the enzyme was inhibited by phenylmethylsulfonyl fluoride, but not by tosyl phenylalanyl chloromethyl ketone and tosyl lysinechloromethyl ketone, it suggests that the enzyme can be a subtilisin-like protease. Furthermore, the N-terminal sequencing of the first 20 amino acids of the purified protease showed less homology with other well-known bacterial peptidases.

Annamalai et al. (2013) isolated a protease producing marine bacterium, *B. halodurans* CAS6 from marine sediments. The steps applied to the purification of this enzyme were ammonium sulfate precipitation, DEAE-cellulose column and Sephadex G-50 gel filtration column. The enzyme was purified 7.96-fold and presented the molecular mass estimated as 28 kDa by SDS-PAGE. The purified enzyme retained 100% of its activity even at 70°C,

pH 10.0 and 30% NaCl for 1 h. The enzyme was stable when treated with ionic, non-ionic and commercial detergents, as well as with organic solvents.

I.4. Genetic improvement of *Bacillus* sp. strains

There are very interesting promising techniques of molecular biology for improvement of one or more properties of microbial enzymes focusing on the industrial application of this biocatalyst, including direct evolution. It is possible to obtain enzymes with increased activity and stability in different conditions that include extreme pH, temperature and non-conventional media, as well as altered specificity towards specific substrates. In addition, the production of protease can be considerably improved. The production of enzymes from organisms able to adapt to extreme conditions, eventually in combination with directed evolution approaches, has been reported to be an important source of new biocatalysts (Stemmer, 1994; Adams and Kelly, 1998). These strategies are in dependence of the efficient transformation of the mutated genes and the expression of the enzymes of interest in a suitable host, for instance *Escherichia coli*. In the case of proteases, this has proved to be difficult owing to the lysis of the host. Thus, they are usually expressed in *B. subtilis*. Despite the fact that this bacterium is naturally competent, obtaining the high number of transformants necessary for directed evolution strategies is highly difficult (Shafikhani et al., 1997). Based on that, the site-specific modification of enzymes by protein engineering can be considered a useful alternative and has been applied successfully to alter the properties of enzymes (Rubingh, 1997). However, molecular biology techniques are not always efficient or worthwhile, taking into consideration the long period of the studies that in many cases does not generate positive results. Therefore, in some studies some traditional techniques can be more effective, including the improvement of fermentation parameters and immobilization of the enzyme. Certainly, combination of all techniques can be applied, but always pondering

the cost of the process when the industrial application of the protease is considered.

Van den Burg et al. (1999) applied modeling, mutant design and site-directed mutagenesis to obtain an extremely stable variant of the thermolysin-like protease from *Bacillus stearothermophilus*. They observed that this variant is much more resistant to denaturing conditions, such as denaturing agents and temperature than the enzyme obtained from the wild strain. It was concluded that the constructed protease variants could be suitable alternatives to proteases that are currently applied in industries.

Martinez et al. (2012) studied the directed evolution campaign of a protease from *Bacillus gibsonii* based on the SeSaM random mutagenesis method, aiming to increase the protease activity at 15°C for the applications in laundry and dish washing detergents. The authors used an optimized microtiter plate expression system in *B. subtilis* and classical proteolytic detection methods adapted for high throughput screening. Additionally, they screened libraries for increased residual proteolytic activity after incubation at 58°C. Three interative rounds of directed protease evolution resulted in a set of protease variants with increased thermal resistance and increased specific activity (Kcat) at 15°C. Recombination of both groups of amino acid substitutions resulted in variant MF1 with over 100 fold prolonged half-life at 60°C and a 1.5-fold increased specific activity (15°C). The authors concluded that the thermal stability improvements were achieved by substitutions to negatively charged amino acids in loop areas of the protease surface and it probably fostered ionic and hydrogen bonds interactions.

The expression of a protease produced by an alkalophilic *Bacillus* sp. Y in *B. subtilis* by gene engineering methods has been studied by Tobe et al. (2006). The gene encoding protease was first cloned from *Bacillus* sp. Y, and the expression vector pTA71 was constructed from the amylase promoter of *B. licheniformis*, DNA fragments encoding the open reading frame of protease

BYA, and pUB110. The protease was secreted at an activity level of 5100 U/mL. After that, the authors substituted the amino acid residue Ala29 next to catalytic Asp30 by one of three uncharged amino acid residues (Val29, Leu29, Ile29), and each mutant enzyme was expressed and isolated from the culture medium. Val29 mutant enzyme was secreted at an activity level greater than 7000 U/mL with its specific activity 1.5-fold higher than that of the enzyme from the wild strain.

I.5. Applications of proteases from *Bacillus* sp.

Several *Bacillus* sp. strains, as already mentioned, are capable of producing a wide number of industrially interesting alkaline and neutral proteases. Different *Bacillus* sp. strains present remarkable properties leading to a broad range of applications as showed in the Table I.3.

Table I.3. Biotechnological applications of *Bacillus* sp. proteases

***Bacillus* sp. strain**	**Industry**	**Application**	**Reference**
B. cereus	Detergent industry	Blood stain removal from fabrics	Saleem et al., 2012
B. megaterium RRM2			Rajkumar et al., 2011
B. subtilis VSG-4			Giri et al., 2011
Commercial protease Bioplase from *B. subtilis*	Food industry	Xanthan gum purification	Armentrout et al., 1999
B. amyloliquefaciens FSE-68		Bioactive peptides obtaining	Cho et al., 2007
Commercial protease from *B. subtilis*		Cheese ripening	Ozcan and Kurdal, 2012
Commercial protease from *B. licheniformis*	Pharmaceutical industry	Amino acids resolution	Zhao et al., 2006
B. licheniformis BBE11-1	Textile industry	Prevent shrinkage and eliminate fibres of wool	Liu et al., 2013
B. megaterium RRM2		Dehair goat skin	Rajkumar et al., 2011

I.5.1. Detergent industry

The industry of detergents is a field in which some enzymes, such as proteases, lipases and amylases, are applied to remove stains from fabrics that is difficult to remove with common detergents. These stains include proteins, pigments and lipids. It is possible to remove most types of stains using detergents at high temperatures and vigorous mixing, however the cost of heating the water is high and the long mixing or beating can shorten the quality of the fabrics. In this context the use of proteases for removal of stains of proteins, mainly blood stains allows lower temperatures to be employed and shorter periods of agitation are needed, often after a preliminary period of soaking. Proteases from *Bacillus* sp. strains find in detergent industry one the most important field of application. This is due to the alkalophilic nature of these enzymes and the remarkable stability they present in contact with surfactants, detergents and oxidizing compounds (Lagzian e Asoodeh, 2012; Hmidet et al., 2009).

Joshi and Satyanarayana (2013) cloned and expressed the protease from *Bacillus lehensis* in *Escherichia coli*. The enzyme is a monomeric protein of 39 kDa with optimal activity at pH 12.8 and 50°C. The protease activity had the activity increased by SDS, Co^{2+}, Ca^{2+}, β-mercaptoethanol, and inhibited by Hg^{2+} and phenylmethylsulphonyl fluoride. The enzyme was compatible with commercial detergents such as Surf, Excel, Safe, Wash, Rin, Tide, Ariel, Ezee and Condite.

A protease produced by *Bacillus* sp. strain isolated from sea water was described by Sinha and Khare (2012). The protease was purified and presented optimal activity at pH 9.0, and 60°C. The protease was completely stable in Tween 80, Triton-X 100, and compatible with commercial detergents like Wheel, Henko, Rin, Vim and Pril. The enzyme was applied in removal of blood stain and the treatment of the cloth pieces caused maximum increase in reflectance and whiteness index in comparison to the control.

The protease from the *Bacillus pumilus* MCAS8 strain presented optimal activity at pH 9.0 and 60°C and its wash performance analysis was studied. The authors observed that the protease could effectively remove blood stains from cotton fabric, making it, therefore, suitable to use as an effective detergent additive. More specifically the protease from *B. pumilus* removed the blood stain completely from the fabrics without the aid of any of the detergents (Jayakumar et al., 2012).

The crude protease from *Bacillus* sp. SM2014 was studied for the removal of bloody stains, tomato sauce and turmeric paste in cotton fabrics. It was observed that when the enzyme was applied in combination with commercial detergents, blood stain removal was more efficient than when only the detergent was used. The optimal conditions for the removal of blood stains were at 50°C, but at 60°C for turmeric paste stain. The best protease concentration and wash period was 1069U and 30 min, respectively. After scan electron microscopy analysis, it was observed that the combination of detergent with the protease did not deteriorate the fabric (Jain et al. 2012).

I.5.2. Food industry

Proteases from *Bacillus* sp. strains, which can be commercial preparations or obtained from native strains, find a myriad of applications in food industry. Some of these applications can be considered for more than one specific industrial field, for instance, bioactive peptides can be applied in food and pharmaceutical industry. Here are described some relevant works and applications of *Bacillus* sp. protease on food industry.

I.5.2.1. Bioactive peptides

Several types of proteins can be limited hydrolyzed resulting in peptides, also called protein hydrolysates with remarkable biological

activities, such as antihypertensive, immunostimulating, antimicrobial and antioxidant activities (Danquah and Agyei, 2012). These peptides can be obtained from the application of proteases in proteins from milk (casein and whey), egg (albumin), soy, among others. They can be produced from precursor proteins using enzymatic hydrolysis by digestive enzymes, fermentation of foods such as milk with proteolytic starter cultures, proteolysis by enzymes derived from microorganisms or plants (Korhonen and Pihlanto, 2006).

There are several reports in the literature on the obtaining of bioactive peptides using *Bacillus* sp. proteases with interesting results. In the work of Daroit et al (2012), antioxidant and antimicrobial activities of ovine sodium caseinate, after using the protease from *Bacillus* sp. P45, were studied. It was observed that the hydrolysis exposed aromatic amino acids to the aqueous media, following a slight decrease in the surface hydrophobicity, indicating exposure of hydrophobic groups on the surface of the generated peptides. The time for the hydrolysis resulted in different functional properties of the peptides. The peptides presented an increase in the antioxidant properties, evaluated by the 2,2'-azino-bis-(3- ethylbenzothiazoline)-6-sulfonic acid method after three to seven hours of hydrolysis. One-hour hydrolysates inhibited the growth of different microorganisms, including *Salmonella enteritidis, Escherichia coli, Corynebacterium fimi, Listeria monocytogenes,* and showing antifungal activity as well.

Protein hydrolysates from tuna (*Thunnus thynnus*) heads with different degrees of hydrolysis were produced using protease from *Bacillus mojavensis* A21 and Alcalase (*B. licheniformis*). All protein hydrolysates produced by the A21 proteases presented higher antioxidant activity than that of the Alcalase protein hydrolysates. The highest DPPH radical-scavenging activity was observed with a degree of hydrolysis (DH) of 15%. The protein hydrolysates obtained with Alcalase (DH = 12%) and A21 (DH = 15%) contained glutamic

acid/glutamine and arginine as the major amino acids, followed by lysine, aspartic acid/asparagine, histidine, valine, phenylalanine, and leucine. Furthermore, the peptides had a high percentage of essential amino acids, which composed 50.47 and 50.52% of the protein hydrolysates obtained by the Alcalase and A21 proteases, respectively. The results indicated that these protein hydrolysates can be used as a promising source of bioactive peptides (Bougatef et al., 2012).

Corrêa et al. (2011) studied the antioxidant, antimicrobial, and angiotensin I-converting enzyme (ACE)-inhibitory activities of ovine milk caseinate hydrolysates obtained with a protease from *Bacillus* sp. P7. Antioxidant activity evaluated using the 2,2'-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid method increased with hydrolysis time up to 2 h, remaining stable for up to 4 h. The peptides presented low 2,2-diphenyl-1-picrylhydrazyl radical-scavenging abilities, with higher activity (31%) reached after 1 h of hydrolysis. Fe^{2+}-chelating ability was maximum for 0.5 h hydrolysates (83.3%), decreasing thereafter; and the higher reducing power was observed after 1 h of hydrolysis. Regarding the ACE-inhibitory activity of the peptides it was observed an increase up to 2 h of hydrolysis (94% of inhibition), declining afterwards. The peptides obtained after 3h of hydrolysis presented capacity of inhibition of the growth of *B. cereus, Corynebacterium fimi, Aspergillus fumigatus and Penicillium expansum.*

Oyster peptides produced by the proteolytic hydrolysis using the protease from *Bacillus* sp. SM98011 were studied in different scales by Wang et al. (2010). The authors studied the antitumor activity and immunostimulating effects of the oyster hydrolysates in mice and observed that the growth of transplantable sarcoma-S180 was clearly inhibited in a dose-dependent manner in mice given the oyster hydrolysates. In this case, mice receiving 0.25, 0.5 and 1 mg/g of body weight by oral gavage had 6.8%, 30.6% and 48% less tumor growth, respectively. The weight coefficients of

the thymus and the spleen, the activity of natural killer cells, the spleen proliferation of lymphocytes and the phagocytic rate of macrophages in S180-bearing mice significantly increased after administration of the peptides. It can be concluded that oyster hydrolysates produced strong immunostimulating effects in mice, which might result in its antitumor activity.

I.5.2.1 Food manufacture

There is a major focus on the use of proteinases and peptidases in proteolysis that is a key process during food ripening (Law and Wigmore 1982). Proteins are partially hydrolyzed by microbial proteases resulting into lower-molecular-weight compounds. These compounds are further broken down by peptidases into various nitrogenous substances, such as proteose, peptone, amino acids, and amines that can contribute directly to the cheese flavour (Fox et al., 1996).

Bacillus sp. proteases have been applied to the ripening of different types of food. The authors Ozcan and Kurdal (2012) studied the effects of neutral protease from *B. subtilis* and lipase from *Mucor miehei* alone and combined with a starter culture on ripening properties of traditional Turkish Mihalic cheese. They observed that when the protease was applied in combination with the lipase better flavour and texture with accelerated ripening were obtained. The gross compositions of the cheeses were changed by the type of enzymes and ripening time. When protease was added to cheeses, it resulted in bitterness and crumbly textural properties due to the intense breakdown of β-casein, which was not observed at same intensity in lipase-added cheeses.

The commercial protease from *B. subtilis*, Neutrase was applied in the evolution of some nitrogen fractions during the ripening of a traditional dry fermented sausage (chorizo). It was observed that when the enzyme was

applied, myofibrillar proteins were affected, resulting in a higher loss of solubility than that produced in the sarcoplasmic proteins. The treated sausages presented an increase in the nonprotein nitrogen fraction higher than that for sausages without enzyme treatment. The fraction α-NH_2 nitrogen increase in the peptide was higher in the product with Neutrase (78.29%) compared to the product without enzyme (20.93%). Subtle differences in the texture parameters were observed after sensory analysis and some of them were not correlated to product acceptability (Zapelena et al., 1997a).

The same authors in another work studied the amino acid profiles from the peptide and free amino acid fractions during the ripening of fermented sausage after the addition of the Neutrase. When the enzyme was applied, a slight decrease in the amino acids Asp, Thr, Ala, Met, and Lys was found in the product between the third and ninth days what did not occur in the control (with no enzyme added) that presented continuous increment along the ripening. The product treated with the commercial *B. subtilis* protease resulted in a more intense total increase of the peptide fraction amino acids during the elaboration. The overall acceptability significantly correlated with some flavor parameters presented a slight improvement that was observed after the sensory analysis (Zapelena et al 1997b).

I.5.5. Pharmaceutical industry

Organic synthetic catalysts, which are widely used in the synthesis of peptides, for example, possess advantages in large scale production with high yields. Yet, these chemical methods also show problems, including a requirement for excessive amounts of coupling agents and solvents and protection of amino acid side chains, and more importantly, resolution processes are required due to formation of racemic mixtures. The use of enzymes such as microbial proteases and lipases have several advantages, such as mild reactions conditions and a reduced number of reaction steps due

to the high specificity of these enzymes. Proteases reactions are frequently carried out in organic systems in which the equilibrium is favorable to synthesis rather than hydrolysis (Poppe and Novak, 1992). Additionally, when proteases are applied a high substrate solubility, simple purification procedures for products, and reduced contamination with organic solvents can be achieved.

Shin et al., (2003) studied the stereoselective protease produced by *Bacillus amyloliquefaciens* KCCM 12091 and applied it in the stereoselective enzymatic synthesis of an aspartame precursor.

In the work of Caille et al. (2002) the protease from *Bacillus lentus* was studied for the selective hydrolysis of the *R*-enantiomer of 2-ethoxy-carbonyl-3,6-dihydro-2H-pyran yielding *S*-2-ethoxycarbonyl-3,6-dihydro-2H-pyran in excellent optical purity. These compounds are intermediates for the synthesis of a protein kinase C inhibitor for the treatment of retinopathy and nephropathy in patients with diabetes mellitus. Furthermore, in another work, the production of several key chiral intermediates in the synthesis of anti-inflammatory, anti-viral and anti-leukaemic agents, using several enzymes such as a serine-type protease from an alkalophilic *Bacillus* sp. to resolve racemic N-substituted lactams was studied (Mahmoudian, 2000).

The commercial protease from *B. licheniformis* in the cross-linked enzyme aggregates immobilized form (Alcalase-CLEA®) was applied in different non-conventional biotransformations like CC bond formation processes such as aldol, Henry and Mannich reactions showing the promiscuous behaviour of this enzyme. On the other side, this protease presented remarkable specificity as demonstrated in the Bayllis–Hillman reaction between 4-nitrobenzaldehyde and methyl vinyl ketone (Lõpez-Iglesias, 2011).

In the work of Zheng et al. (2011) a sequence of enzymatic methods were applied to synthesize the metoprolol–saccharide conjugates, taking into account the combinations of drugs and carbohydrates for parts of their therapeutic actions have extended a wide range of drugs. In that work the protease from *B. subtilis* catalyzed highly regioselective acylation of glucose, mannose, galactose, maltose and sucrose with N-(5-vinyloxycarbonylpentanoyl)metoprolol in anhydrous pyridine at 50°C resulting into metoprolol–saccharide conjugates in good yields.

Boeriu et al. (2010) reported a mild and efficient method for the conversion of C-terminal esters of side-chain protected peptides into an amide function using ammonolysis catalyzed by Subtilisin A, the alkaline serine protease from *B. licheniformis,* and ammonium carbamate as source of ammonia, in organic media with low water content. In the work, the authors optimized the reaction conditions for maximum amide yield and minimum secondary hydrolysis, at 30°C, using solvent composition of butanol/dimethylformamide 82.5:17.5 (v/v) containing 0.2% water (v/v) and molar ratio of ammonia source to peptide methyl ester of 10:1. The maximum yield of Z-Ala-Phe-NH_2 was 87% after 21 h for a quantitative substrate conversion.

The compound 6-O-dodecanoyl-2-O-α-D-glucopyranosyl-L-ascorbic acid has been synthesized from 2-O-α-D-glucopyranosyl-L-ascorbic acid and vinyl laurate by Tai et al. (2010) using a commercial protease from *B. subtilis* in dimethylformamide /dioxane with a low water content, yielding 38.1% at optimal conditions. This compound presents higher bioavailability and is a derivative of the 2-O-α-D-glucopyranosyl-L-ascorbic acid, which exhibit vitamin C activity in vitro and in vivo, after enzymatic hydrolysis to ascorbic acid by α-glucosidases (Yamamoto et al., 1992).

I.5.4. Textile industry

The proteases with keratinase activity receive special attention due to their capability of hydrolyzing keratin that is an insoluble fibrous protein. Keratin is a major constituent of hair, feather, wool, and nail. It possess a stable structure rich in α-helix or β-sheet cross-linked with cystine bridges, resulting in mechanical stability thus hardly degradable by subtilisin, papain and pepsin (Brandelli, 2008). Based on that, keratinases are applied in the textile industry, with different steps, for instance, in textiles processing replacing the conventional pollution-creating physicochemical methods and efficient ability to impart shrink-resistance and to improve handling properties (Lv et al. 2010). Wool is a natural animal fiber commonly used in fabrics, but requires physical and chemical processing treatment for such applications. Protease acting on wool fiber can be an alternative method for the traditional chlorination-resin anti-shrinkage finishing process, which results in environment pollution.

In the work of Liu et al. (2013) the keratinase from *B. licheniformis* in recombinant *B. subtilis* WB600 expression system was purified, characterized and applied in wool fiber processing. The authors observed that with the increasing concentration of keratinase, the shrinkage area decreased from 10.22 to 0.82%, while 1% of savinase (*B. lentus*) alone decreased the shrinkage area from 10.22 to 9.38 %. Yet, the concentration of keratinase must be controlled at an appropriate ratio, taking into account, the loss of strength also increased. The authors observed that the appropriate concentration of keratinase and savinase could effectively reduce shrinkage with less strength loss of wool fabrics, considering keratinase probably opens the disulfide bonds, and increases the efficacy of savinase treated with wool scales.

Infante et al. (2010) isolated proteolytic bacteria identified as *B. megaterium* L4 and *Bacillus thuringiensis* L11. The effect of isolate L11 on

wool-fiber and fabric was studied and the results showed that during bacterial growth, the wool-fiber lost weight but the fiber diameter has not changed. When wool-fabric was used instead of wool- fiber fabric weight loss was also found, but no significant differences were found in shrinkage.

The protease from *Bacillus* sp. SJ-121 was studied and applied to treat wool and silk. It was observed that by increasing the protease treatment time to 48h, the dyeing characteristics of the fabrics were enhanced, and the surfaces of the single yarns of the fabrics became smoother, due to the removal of soil and scale in them. Nevertheless, no mechanical changes were detected in the fabrics (Kim et al., 2005)

The leather industry is one of the most environment-impacting industrial activities. This industry uses hides as raw materials, resulting in high amounts of solid waste that are not converted into leather. Taking into consideration that the hide represents 7% of the cattle weight and 25% of this is effectively transformed into leather, the remaining process intermediates or by-products are considered of having a potentially high impact on the environment (Cooper et al., 2011). In addition, lime, sulphide and chromium are also pollutants of this industrial sector. Dehairing using proteases is being increasingly considered as a reliable alternative to the conventional lime-sulphide process, avoiding the problems created by sulphide pollution. As the main advantages of enzymatic dehairing are recovery of hair as a by-product for production of synthetic fibres, biogas, foaming agent for fire extinguishers and total elimination of lime and sulphide from the effluent. It must be stated that hydrolyzed hair is used as agricultural fertilizer, soil conditioner, compost, animal/poultry feed and also for production of cosmetics, pharmaceuticals and amino acids, like cysteine (Paliwal et al., 1994).

In the work of Dettmer et al. (2012) a protease from the strain of *B. subtilis* was applied in leather production mainly in hide unhairing and the inter-fibrillary removal. The optimal conditions for the proteolytic activity

was at pH 9 and 10, temperature between 37 and 55°C, and the enzyme showed good thermal stability up to 45°C. The treated hides presented few remaining hairs. The removal of inter-fibrillary proteins was approximately 4-fold for glycosaminoglycans and 6-fold for proteoglycans, when compared with the conventional unhairing process.

Kumar et al. (2011) reported a study of a serine alkaline protease from a strain of *B.* altitudinis GVC11. The enzyme was purified, characterized and presented remarkable dehairing ability of goat hide in 18 h without disturbing the collagen and hair integrity.

I.5.5. Other applications of proteases from *Bacillus* sp.

Xanthan is a polysaccharide produced by *Xanthomonas campestris,* presenting a unique structure and useful rheological properties and because of that has been successfully commercialized. The xanthan gum is used as gelling or viscosity control agent in food, pharmaceutical, agricultural and other industrial applications. The main problem with the use of this gum is that the bacterial cells used for xanthan production remain after processing and impart turbidity when used in various products. It is not essential to separate bacterial cells from polysaccharide for all commercial applications; yet, its removal is required where the transparency of the product is to be maintained. Thus, among the strategies to remove the *X. campestris* cells are centrifugation but due to its high viscosity, polysaccharide containing broth has to be diluted several times before centrifugation at high speed to sediment the biomass. The removal of cells from fermented broth by heat treatment particularly at high temperatures often causes thermal degradation of the gum. The highly alkaline pH treatment of microbial broth for cell digestion may also result in degradation of the gum (Margatis, 1985). In contrast, enzymatic treatment has been proved to be efficient to obtain clarified polysaccharide (Holding and Pace, 1981). The compounds in bacterial cell wall mureins

(peptido-glycans) can be lysed by some enzymes and protease from different sources have been used for the degradation of *X. campestris cells* to obtain clarified product, including *Trichoderma koningii* (Triveni and Shamala, 1999), *Pseudomonas* sp. (CL 1457) (Shastry and Prasad, 2002) and *Lysobacter* species (Pollock and Yamazaki, 1993).

There are some patents reporting the use of proteases from *Bacillus* species on the clarification of xanthan gum. Colegrove (1977) reported the clarification of xanthan gum produced by *X. campestris*. In the patent, fermentation broths and other aqueous suspensions containing dissolved xanthan gum and suspended solids resulting from the fermentation producing the xanthan gum were clarified by treatment with a minor amount of an alkaline protease from *B. subtilis, B. licheniformis, B. amyloliquefaciens* and *Bacillus pumilis*.

Kohler et al. (1984) patented the enzymatic clarification process for improving the injectivity and filterability of xanthan gums using protease from *B. licheniformis*. Armentrout et al. (1999) patented the process of purifying xanthan gum using an alkaline protease and lysozyme. It was described that the fermented broth was treated with alkaline protease from *B. subtilis* ("Bioplase"). After treatment with alkaline protease and lysozyme the xanthan broth presented a transmittance of 90%.

Pollock (1994) patented enzymatic clarification of polysaccharides. It was used acid and/or neutral proteolytic enzymes for the degradation of the cells of the genus *Xanthomonas campestris* as well as of those of other polysaccharide-producing organisms in order to clarify xanthan gum or other polysaccharide. The protease was elaborated by *B. subtilis* and *Aspergillus niger*, using 20 ppm of the enzymes. Transparency was measured at 600 nm for a 1 cm light path, with deionized water serving as a 100% transparent standard. The transparency for all of the enzyme-treated samples increased.

In the patent of Dimasi et al. (2003), a process is disclosed for clarifying an aqueous xanthan gum solution comprising treatment of the

xanthan solution with at least one chelating agent, surfactant, organic acid, or a mixture thereof, and with a protease enzyme or a lysozyme and a protease enzyme. Among the enzymes it was tested the neutral proteases from *B. amyloliquefaciens* and alkaline proteases from *B. subtilis*, *B. licheniformis*, and *B. pumilus*, among other proteases. After conducting the clarification of the xanthan solution, the gum was precipitated and separated from aqueous/isopropanol solution using conventional procedures, e.g., by filtration, de-watering, centrifugation, followed by drying. The high purity xanthan gum provided substantially clear viscosified compositions having most preferably a transmittance of at least 93% for solutions containing 1% by weight of the high purity xanthan gum.

As described above several patents report the use of *Bacillus* sp. protease on the clarification of xanthan gum. However, it is important to evaluate if the strain of *Bacillus* sp. does not produce enzymes that can degrade the xanthan. Hashimoto et al. (1998) studied the strain *Bacillus* sp. GL1 and observed that the enzyme was capable of producing xanthan-depolymerizing enzymes, taking into account when the microorganism grew in a medium containing xanthan as the carbon source, the viscosity of the medium decreased in association with growth. One of the xanthan-depolymerizing enzymes present in the medium was xanthan lyase. The authors purified the enzyme and observed that it cleaved the linkage between the terminal pyruvylated mannosyl and glucoronyl residues in the side of xanthan. Some other studies from the same research group report the production of xanthan lyase by this strain (Maruyama et al., 2007 and Maruyama et al., 2005). The detection of xanthan depolymerizing enzyme in crude extract of protease from *Bacillus* sp. and purification of protease preparation is important for the application of xanthan gum clarification.

Studies have indicated the use of *Bacillus* sp. proteases in deproteination of wastes for obtaining chitin (Gagne and Simpson, 1993). The proteases have showed advantages compared to the use of strong acids or

bases that can cause a partial deacetylation of chitin and hydrolysis of the polymer, resulting in final inconsistent physiological properties.

Some articles from the literature report interesting results of this application using *Bacillus* sp. proteases. In the work of Yang et al. (2000) the deproteinization was carried out fermenting untreated shrimp shell, crab shell, and lobster shell wastes with the strains of *B. subtilis* Y-108, resulting in protein removal of 88%, 67%, and 83%, respectively. These results are better than the ones obtained with the acid treated wastes that resulted in 76%, 62% and 56%, respectively. On the other hand, the work of Wang et al. (2006) studied the deproteinization of squid pen in the preparation of β-chitin using the strains *Bacillus* sp. TKU004. The optimized condition for protease production was found using culture medium composed of 2% squid pen powder (m/v), 0.1% K_2HPO_4, and 0.05% $MgSO_4$, at 30°C for 4 days, resulting in 73% of protein removal of squid pen.

I.6. Immobilization of protease from *Bacillus* sp. strains

Despite the fact that the protease immobilization is not one of the main goals in this review, the reusability and improvement stability of proteases for industrial use is truly necessary, such as in the case of the organic reactions using organic solvents that can be toxic for the free protease. Proteases have been immobilized using a wide range of methods including deposition or precipitation onto porous supports (Barros et al., 2000); and covalent attachment to activated preexisting supports (Guisán et al., 1991; Chapman and Hultin, 1975).

There are several works on the immobilization of commercial proteases, mainly Alcalase, which is a commercial preparation of Subtilisin, obtained from *B. licheniformis.* In the work of Corîci et al. (2011) the commercial protease Alcalase 2.4.L FG was immobilized by physical entrapment in glass sol–gel matrices using alkoxysilanes of different types

mixed with tetramethoxysilane. Alkyltrimethoxysilanes with large alkyl groups showed to be detrimental for the immobilization process, which resulted in lower entrapment yield. This happened probably due to the release of protein from the hydrogel. Better performance was achieved by increasing the number of methyl groups. Maximum activity was obtained with derivatives resulting from the encapsulation of protease in gels prepared with dimethyldimethoxysilane as precursor. Yet, the specific activity of this best performing immobilized enzyme represented only 30.2% of the native enzyme activity. One explanation is that this activity decreased due to the mass transfer limitation of substrate and/or product through the sol–gel matrix. The percentage of protein immobilization varied from 68 to 98%, and total amidation activity of the immobilized protease was up to 1.76 μmol/h/mg gel. Regarding the stability of the protease it was observed that the activity of free enzyme decreased much faster than of the immobilized form, with increasing incubation temperature. The native enzyme lost about 65% of its initial activity when incubated at 70°C, showing sensitivity to high temperatures. Under the same conditions, the immobilized enzyme retained 78% of its initial activity. A possible explanation for the improved thermostability of encapsulated protease is most probably due to multipoint attachments of enzyme molecules to the matrix that prevent them from conformational changes upon heating. The immobilized enzyme prepared retained 100% of the original activity after used in fourteen repeated cycles. Interestingly the thermal stability of the protease at 70°C increased 3-fold upon immobilization in sol–gel materials, and 2-fold under storage after 50 days at ambient temperature.

The partially purified protease from *Bacillus polymyxa* was immobilized by using different adsorbents. The immobilization yield of 5.8–96% and bound protease of 52.1–950 U/g solid was achieved using raw zeolite, chitin, chitosan, and alginate. When alginate was used as the support

the highest immobilization yield (96%) and the highest activity per gram carrier (950 U/g solid) was observed. Immobilization for 24 h showed considerable bound enzyme activity (good loading efficiency) and immobilization yield. This good loading efficiency for the immobilization might have been due to the formation of stable cross linking between the carrier (alginate) and the protease. Another important factor is storage stability of the protease that is a major concern in enzyme preservation. Protease immobilized on alginate and synthetic zeolite maintained their activities 64 and 62%, respectively. After storage for 20 days, the immobilized enzyme still maintained about 64% of its initial activity, whilst free native enzyme maintained only about 40% of the initial activity (Kirkkopru et al., 2006).

Ferreira et al. (2003) reported the covalent immobilization of Alcalase onto physiochemically characterized silica supports. They observed that silica with large pores presented higher relative specific activities compared to those with smaller pore sizes. The apparent Km value for the immobilized enzyme was lower than the free form, probably because of the partitioning effects of the substrate. The activity profiles of free and immobilized enzyme were very similar, including the range of optimal pH and temperature. Therefore, it indicates that substrate mass transfer is not limited in the immobilized enzyme.

I.7. Conclusions

As reported in this review, proteases are important enzymes that are broadly studied for industrial applications, and the *Bacillus* genus produces extracellular proteases with tremendous potential. Within the diversity of their properties, their activity, stability and selectivity are highlighted. Proteases from *Bacillus* genus are currently produced and applied in many industrial fields and some proteases are commercially available, including proteases from *B. licheniformis* and *B. subtilis.* However, some factors limit the

industrial application of *Bacillus* sp. protease, such as the high cost of enzyme production and a lack of proteases with the optimal range of catalytic specificities and required properties. These are common difficulties for the use of the majority of enzymes, thus several points must be studied and improved. The most important ones are fermentation technology, allowing for a feasible production process of the enzyme on a large scale, using low cost substrates. Within this context much effort should be given to SSF that has been demonstrated to be a very efficient fermentation technique for *Bacillus* sp. protease production and other enzymes, but the large scale technology still fails. In addition, the immobilization of *Bacillus* sp. cells for protease production should also be focused, considering it can decrease the cost of the process if the productivity is high and the reusability of the immobilized cells is considered industrially satisfactory. Protein engineering to obtain *Bacillus* sp. proteases with desired properties is another important field for obtaining enzymes with improved properties.

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I.9. References

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II. CAPITULO 2 - SCREENING OF *Bacillus* sp. STRAINS FOR PROTEASES PRODUCTION IN DIFFERENT MEDIA AND EVALUATION OF XANTHAN GUM CLARIFICATION AND STABILITY IN DETERGENT AND ORGANIC SOLVENTS

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RESUMO

Cinquenta e nove linhagens de *Bacillus* sp. foram testadas quanto a produção de protease por fermentação submersa, sendo selecionadas nove linhagens. Estas nove linhagens foram cultivadas por fermentação submersa e fermentação em estado sólido e as proteases apresentaram atividade ótima em pH 9,0 e 60°C. As linhagens de *Bacillus* sp. LBA 07 e *Bacillus* sp. LBA 46 produziram maior atividade de proteases, sendo obtidas 222 U/mL e 548 U/mL após fermentação submersa no meio n° 1 (caseína e sais) e meio n° 2 (melaço, soro de queijo, água de maceração de milho e extrato de levedura Prodex-Lac SD®), respetivamente. A linhagem de *Bacillus* sp. LBA 08 produziu 13480 U/gss após fermentação em estado sólido em meio de cultivo n° 3 de farelo de trigo. As proteases mostraram-se estáveis na faixa de pH de 5,0 a 9,0 por 24h a 4°C e em pH 7,0 a 50°C por uma hora. As proteases obtidas do cultivo da linhagem de *Bacillus* sp. LBA 46 nos meios n° 1, n° 2 e n° 3 foram as mais estáveis quando incubadas em detergente Ariel® líquido e sólido. Algumas preparações de proteases apresentaram mais de 60% de atividade após 24h de incubação em acetona (*Bacillus* sp. LBA 8 e 44), hexano (*Bacillus* sp. LBA 19, 29, 44, 46 e 60), clorofórmio (*Bacillus* sp. LBA 44 e 60) e etanol (*Bacillus* sp. LBA 60). As preparações de protease obtidas do cultivo da linhagem de *Bacillus* sp. LBA 48 nos meios nos meios n° 1, n° 2 e n° 3 e as proteases obtidas da linhagem *Bacillus* sp. LBA 46 cultivada nos meios n° 2 e n° 3 foram as mais eficientes na lise de células de *Xanthomonas campestris*, resultando num aumento de 30% ou mais na transmitância (620 nm) do caldo fermentado contendo goma xantana. A linhagem de *Bacillus* sp LBA 46 foi selecionada para estudos de produção de proteases, considerando-se a boa estabilidade da enzima em detergente e capacidade de lisar células de *X. campestris*.

Palavras-chave: *Bacillus* sp., proteases, goma xantana, estabilidade em detergentes, estabilidade em solvente orgânicos

ABSTRACT

Fifty nine *Bacillus* sp. strains were primarily fermented under submerged fermentation and nine strains were selected for protease production. These nine strains were cultivated under submerged and solid state fermentation. The proteases obtained from *Bacillus* sp. showed optimal activity at 60°C and pH 9.0. *Bacillus* sp. LBA 07 and *Bacillus* sp. LBA 46 produced the highest protease activity, 222 U/mL and 548 U/mL by submerged fermentation in the medium no. 1 (casein and salts) and medium no. 2 (Molasses, dried whey, corn steep liquor and yeast extract Prodex-Lac SD®), respectively. *Bacillus* sp, LBA 08 produced 13480 U/gds by solid state fermentation in medium no. 3 composed of wheat bran. The proteases were stable in the pH range from 5.0 to 9.0 for 24 hours at 4°C and pH 7.0 at 50°C for 1 hour. Proteases obtained from the cultivation of *Bacillus* sp. LBA 46 in medium no. 1, no. 2 and no. 3 were the most stable when incubated in solid and liquid detergent Ariel®. Some protease preparations presented more than 60% of activity after 24h in acetone (*Bacillus* sp. LBA 8 and 44), hexane (*Bacillus* sp. LBA 19, 29, 44, 46 and 60), chloroform (*Bacillus* sp. LBA 44 and 60) and ethanol (*Bacillus* sp. LBA 60). The protease preparations obtained from strain *Bacillus* sp. 48 cultivated in medium no. 1, no. 2 and no. 3 and the proteases from strain *Bacillus* sp. 46 cultivated in media no. 2 and no. 3 were the most efficient on the cell lysis of *Xanthomonas campestris*, increasing 30% or more the transmittance (620 nm) of the xanthan broth. The strain *Bacillus* sp. LBA 46 was selected for future studies of production of proteases, considering good stability of proteases in detergents and *X. campestris* lysis capability.

Keywords: *Bacillus* sp., proteases, xanthan gum, detergent stability, organic solvent stability

II.1. Introduction

Proteases (E.C. 3.4.21.14) are a highlighted group of enzymes that correspond to approximately 60% of the total worldwide enzyme sales, representing one of the most important enzymes due to their applications in the detergent, food, pharmaceuticals, chemicals, leather, paper, pulp and silk industries (Kuddus and Ramteke, 2012).

The immense biodiversity of microorganisms improves their biotechnological importance and justifies the search for new proteases. Literature reports several microorganisms capable of producing extracellular proteases, such as *Aspergillus oryzae* (Belmessikh et al., 2013), *Aureobasidium pullulans* (Chi et al., 2007), *Serratia marcescens* (Bach et al., 2012) and *Bacillus subtilis* (Helal et al., 2012). Currently, a large proportion of commercially available alkaline proteases are derived from *Bacillus* sp. strains due to its ability to secrete large amounts of alkaline protease, having significant proteolytic activity and stability at high pH and temperatures (Shah et al., 2010, Chauhan and Gupta, 2004).

An important application of proteases is in the clarification of xanthan gum for the removal of the *Xanthomas campestris* from the fermentative broth (Holding and Pace, 1981), considering centrifugation, filtration or heating are not efficient or present disadvantages (Margatis, 1985). The compounds mureins (peptidoglycans) of bacterial cells can be lysed by some enzymes, and proteases from different sources have been used for the degradation of *X. campestris cells* to obtain clarified product, including proteases from *Trichoderma koningii* (Triveni and Shamala, 1999) and some commercial proteases from *Bacillus* species as reported in some patents (Colegrove 1977, Kohler et al. 1984).

Detergent industry is a field in which some enzymes, such as proteases, lipases and amylases, are applied due to their efficiency in removing stains that commom detergents do not easily remove from fabrics. Alkaline

proteases correspond 2/3 shares of the enzyme market in detergent industry alone (Anwar and Saleemuddin, 2000). The proteases from *Bacillus* sp. strains find in detergent industry one the most important field of application. This is due to the alkalophilic nature of this enzymes and the remarkable stability they present in contact with surfactants, detergents and oxidizing compounds (Lagzian e Asoodeh, 2012). In addition, some proteases are stable in several hydrophilic and hydrophobic organic solvents, which is highly interesting for the use of *Bacillus* sp. proteases in synthetic reactions (Lõpez-Iglesias, 2011).

In this work, nine strains were selected from fifty nine *Bacillus* sp. strains and studied for protease production in three different media. The properties of the crude protease extracts of these strains were compared. The parameters for the selection of *Bacillus* sp. strains were the protease activity in the different media, biochemical properties of the crude enzyme preparations, the stability in commercial detergents and organic solvents and the capability of clarifying the fermented broth containing *X. campestris* cells.

II.2. Materials and methods

II.2.1. Microorganisms

Fifty nine strains of *Bacillus* sp. LBA from the culture collection of Food Biochemistry Laboratory, College of Food Engineering – University of Campinas, and the *X. campestris* ATCC 13591 strain were maintained in nutrient agar at 4°C, with sterile vaseline addition and transfers in every 2 months.

II.2.2. Preliminary screening of strains of *Bacillus* sp. LBA for production of proteases in cultivation medium composed of casein, yeast extract and salts

Using an inoculating loop, the *Bacillus* sp. strains were inoculated into 50 mL Erlenmeyer flasks containing 18 mL of a culture medium no. 1

composed of 10 g/L of casein (Synth, BR), 1 g/L of yeast extract (Oxoid, IN), 7g/L of $(NH_4)_2HPO_4$; 1.5 g/L of K_2HPO_4; 0.5g/L of $MgSO_4.7H_2O$; 0.3 g/L of $CaCl_2.2H_2O$; 1 g/L of $FeSO_4.7H_2O$; 1 g/L $MnCl_2.4H_2O$; 1 g/L of $ZnSO_4.7H_2O$; and pH adjusted to 7.0. The flasks were incubated at 200 rpm, 30°C for 48 h. The culture broths were centrifuged at 9600 x *g* for 15 min at 5°C and the supernatants were used as crude protease extracts.

II.2.3. Production of proteases by the selected *Bacillus* sp. LBA in three different media

The nine selected strains of *Bacillus* sp. (*Bacillus* sp. LBA 07, 08, 19, 39, 44, 46, 48, 50 and 60) were cultivated in three different media to evaluate their capability of production of extracellular proteases. Aliquots of 2 mL of *Bacillus* sp. cell suspension in saline solution (0.85%, m/v) (optical density at 600 nm between 0.49 and 0.51) were used as inoculum.

II.2.3.1. Fermentation of Bacillus sp. under submerged fermentation

The nine *Bacillus* sp. strains were inoculated into 250 mL Erlenmeyer flasks containing 48 mL of a culture medium with identical composition of the medium reported in the item II.2.2, such as casein, yeast extract, different salts and pH adjusted to 7.0. The medium no. 2 was composed of 35 g/L of sugar cane molasses (Fios de ouro®, BR), 20 g/L of corn steep liquor (Corn Products do Brasil, BR), 3 g/L of yeast extract Prodex-Lac SD® (Produtos Especiais para Alimentos S/A, BR), 20 g/L of dried whey (Alibra, BR) and pH adjusted to 7.0. The flasks were incubated at 200 rpm, 30°C for 48 h. The culture broths were centrifuged at 9,600 x *g* for 15 min at 5°C and the supernatants were used as crude protease extracts.

II.2.3.2.Fermentation of Bacillus sp. under solid state fermentation

The nine *Bacillus* sp strains were inoculated into 250 mL Erlenmeyer flasks containing medium no. 3 composed of 5g of wheat bran (Natu's, BR), 3 mL of distilled water and 2 mL of cell suspension prepared as previously described in the item II.2.3. The flasks were incubated at 30°C for 96h. After the fermentation, 50 mL of 0.1 M sodium phosphate buffer pH 7.0 was added to each one of the flasks and they were stirred at 150 rpm for 1 h. The mixtures were filtered against cotton and centrifuged at 9600 x *g* for 15 min at 5°C. The supernatants were used as crude protease extracts.

II.2.4. Determination of protease activity

The protease activity was determined as described by Charney & Tomarelli (1947) with modifications. The reaction mixture containing 0.5 mL of 0.5% (m/v) azocasein (Sigma, US) in 0.05 M phosphate buffer (pH 7.0) and 0.5 mL the enzyme solution was incubated at 50°C for 40 min. The reaction was stopped by adding 0.5 mL of 10.0% (m/v) trichloroacetic acid and the test tubes were centrifuged at 17,000 x *g* for 15 min at 25°C. A 1.0 mL aliquot of the supernatant was neutralized with 1.0 mL 5 M KOH. One unit of protease activity was defined as the amount of enzyme which caused an increase of 0.01 of absorbance at 428 nm per minute. The enzyme activities were expressed as U/mL of crude enzymatic extract and U/gds (gram of dried substrate) for the extracts obtained from submerged and solid state fermentation, respectively.

II.2.5. Characterization of crude proteases

II.2.5.1. Effect of pH and temperature on the activity of Bacillus sp. proteases

The effect of pH on protease activity was measured in pH range of 5.0 – 11.0 at 50°C using 0.1M acetate (pH 5.0), phosphate (pH 6.0 - 8.0) Tris–HCl (9.0) and carbonate-bicarbonate buffers (pH 10.0), and sodium bicarbonate-NaOH buffers (pH 11.0) buffers and azocasein as substrate. The effect of temperature on activity of the proteases was determined at different temperatures (30 – 80°C) and at optimal pH activity of each protease preparation.

II.2.5.2. Effect of pH and temperature on the stability of Bacillus sp. proteases

To evaluate the stability at different pH values the crude protease extracts were incubated in 0.2 M of acetate (pH 5.0), phosphate (pH 6.0 - 8.0) Tris–HCl (9.0) and carbonate-bicarbonate buffers (pH 10), at 4°C for 24h. To evaluate the stability in different temperatures, the crude protease preparations were diluted in phosphate buffer 0.2 M, pH 7.0 and kept at different temperatures (30 – 80°C) for 1h. The residual activity was determined and maximum activity was defined as 100%.

II.2.5.3. Study of stability of the proteases in commercial detergents

The stability of *Bacillus* sp. proteases towards liquid and solid detergent Ariel® was examined. Solutions of liquid (1%, m/v) and solid (0.7% m/v) detergent in tap water were heated at 100°C for 30 min to denature the enzymes presented in the detergent. The protease preparations were diluted in the detergent solution and incubated at room temperature (25°C) for 2 h,

followed by measuring the residual protease activity by standard assay procedure and compared with the control (enzyme diluted in tap water without detergent).

II.2.5.4. Study of stability of the proteases in different organic solvents

The stability of the *Bacillus* sp. proteases in the presence of organic solvents was evaluated by incubating the enzyme preparations with the organic solvents acetone, ethanol, chloroform and hexane at the concentration of 50 % (v/v) in water for 24h at 30°C with shaking of 150 rpm. Similar experiments were carried diluting the proteases extracts in water, with no addition of solvent, to compare the stability of the proteases in water against organic solvents in the same conditions. The enzymatic activity was assayed and the residual activity was calculated considering the initial protease activity with no treatment as 100%.

II.2.6. Production of xanthan gum

Xanthan gum was produced as described by Shastry e Prasad (2002). *X. campestris* ATCC 13591 was grown in conical flasks (250 mL) containing 50 mL medium composed of 45 g/L of sucrose, 4 g/L of peptone, 5 g/L of Na_2HPO_4, 3 g/L KH_2PO_4, 1 g/L $MgSO_4 \cdot 7H_2O$ and pH 7.0, at 200 rpm, 28°C. After 24h of incubation one aliquot of 5 mL of inoculum culture was added to 95 mL of fresh medium with the same composition in conical flasks (500 mL) and incubated on a 200 rpm rotary shaker for 72 h, at 28°C. The viscous *X. campestris* broth obtained was heated at 90°C for 30 min and used for the study for clarification of xanthan gum with crude protease extracts.

II.2.7. Application of the proteases on the clarification of xanthan gum

The fermented broth of *X. campestris* ATCC 13591 was used as substrate for crude protease extracts. Erlenmeyer flasks of 125 mL containing 10 mL of fermented broth diluted in water (1:1) were incubated at 50°C, with stirring and 400 µL crude protease extracts were added to start the lysis. Aliquots of 0.1 mL were withdrawn after each 30 minutes, diluted in distilled water and the transmittance (T) was determined at 620 nm. Control assays were also carried out adding distilled water instead of the protease extract.

II.2.8. Determination of xanthanase activity

The xanthanase activity in the crude protease extracts was determined using xanthan gum as substrate, according to Cadmus et al. (1982) with modifications. The reaction mixture containing 1.6 mL of 0.2% (m/v) of xanthan gum in 0.1M phosphate buffer pH 4.5 and 0.4 mL of crude protease preparation was incubated for 30 min at 50°C or 42°C and the reducing sugars were determined using Somogy method (Somogyi, 1945). A unit of xanthanase was defined as 1 µmol of apparent mannose liberated per min under the above assay conditions.

II.3. Results and Discussion

II.3.1. Preliminary screening of strains of *Bacillus* sp. LBA for production of proteases

Fifty nine strains of *Bacillus* sp. LBA were fermented under submerged fermentation in 50 mL Erlenmeyer flasks containing medium no. 1 composed of casein, yeast extract and salts and the enzymatic activity was measured using the substrate azocasein at pH 5.0 and 7.0. As it can be observed in the Figure II.1 all the fifty nine *Bacillus* sp. LBA strains were capable of

producing proteases that were more active at pH 7.0. The strains *Bacillus* sp. LBA 07, 08, 19, 39, 44, 46, 48, 50 and 60 were the best protease producers reaching 50, 100, 58, 49, 55, 49, 61, 51, and 43 U/mL of protease activity, respectively. These nine strains were selected for the following studies of production of proteases in 250 mL Erlenmeyer flasks in three different media.

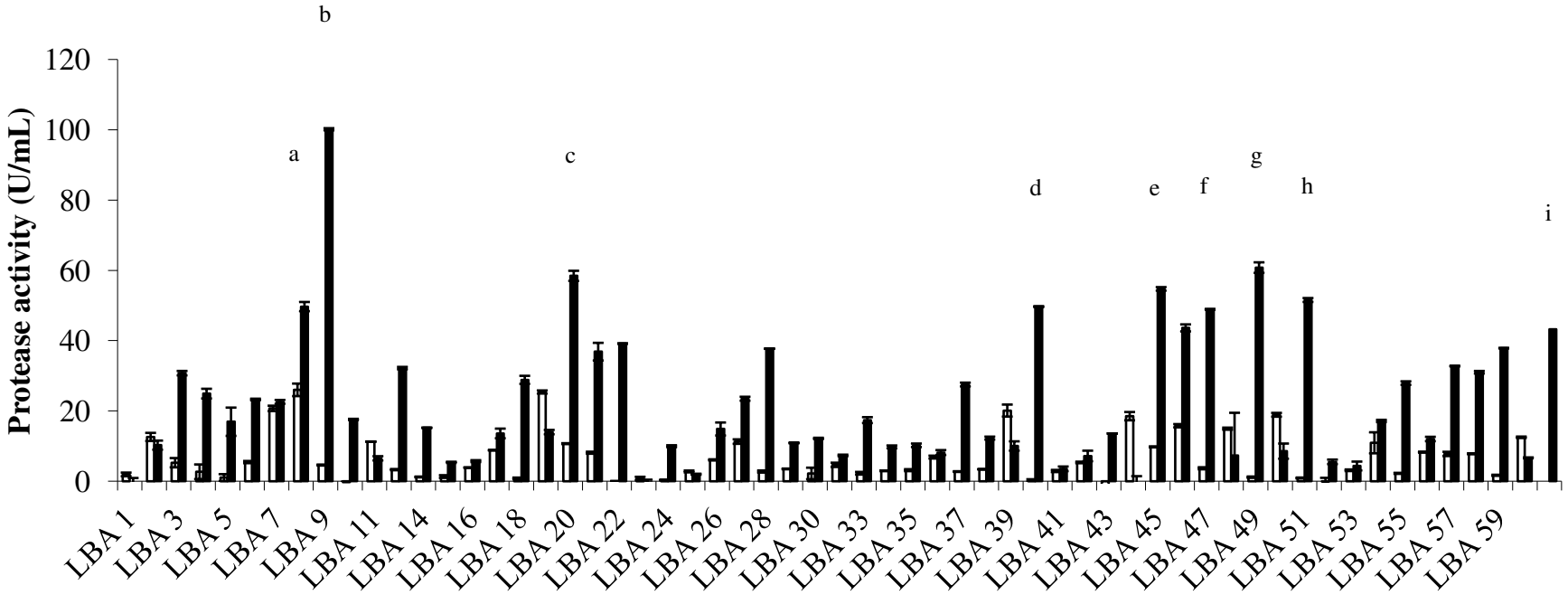

Figure II.1. Protease production from the fifty nine *Bacillus* sp. LBA strains in 50 mL Erlenmeyer flasks containing medium no. 1 composed of casein, yeast extract, $(NH_4)_2HPO_4$, K_2HPO_4, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, $FeSO_4.7H_2O$, $MnCl_2.4H_2O$, $ZnSO_4.7H_2O$ after 48h at 30°C.

Each data point represents the mean of three independent assays with the standard deviation. a- *Bacillus* sp. LBA 7, b- *Bacillus* sp. LBA 8, c- *Bacillus* sp. LBA 19, d- *Bacillus* sp. LBA 39, e- *Bacillus* sp. LBA 44, f- *Bacillus* sp. LBA 46, g- *Bacillus* sp. LBA 48, h- *Bacillus* sp. LBA 50, i- *Bacillus* sp. LBA 60. ■-pH 7,0 ▨-pH 5,0

II.3.2. *Bacillus* sp. proteases production in three different media

The crude protease extract from submerged fermentation of strains of *Bacillus* sp. LBA 7 and *Bacillus* sp. LBA 48 in medium no. 1 composed of casein (Synth, BR), yeast extract and salts presented 222 U/mL and 172 U/mL of protease respectively, while the protease extracts from strains LBA 19, 39, 44, 46, 50 and 60 presented less than 50 U/mL (Figure II.2a).

The crude protease extracts from strains LBA 46, LBA 48 and LBA 50 cultivated in medium no. 2 containing sugar cane molasses, corn steep liquor, food grade yeast extract (Prodex Lac-SD®) and dried whey presented the highest protease activity, reaching 548 U/mL, 250 U/mL and 438 U/mL, respectively (Figure II.2b).

Literature reports low cost substrates, such as sugar cane molasses as an efficient carbon source for protease production by *Bacillus* species. However, most of the works evaluate the combination of low and high cost substrates, which is different from this work that studied separately these two types of substrates. Moorthy and Baskar (2013) optimized the production of alkaline protease from the strain of *Bacillus* sp. BGS, observing that the highest protease activity was found (470 U/mL) using 16.82 g/L of peptone, 11.28 g/L of molasses, pH 11, and 2% (v/v) of inoculum size. In the work of Helal et al. (2012) the optimal conditions for protease production from *Bacillus subtillis* was possible using 20 g/L of corn steep liquor, 20 g/L of casein hydrolysate, 12 g/L of cane sugar molasses as a carbon source at pH 10 and 37°C, over 24h of incubation period, while in the work of Shikha and Darmwal (2007) the highest production of alkaline protease by a strain of *Bacillus pantotheneticus* was 242 U/mL in the medium containing only molasses as carbon source, which was comparable with molasses plus wheat bran (285 U/mL) as carbon and nitrogen sources, respectively.

There was no direct comparison between the results from submerged fermentation (media no. 1 and no. 2) and solid state fermentation (medium no. 3), due to the difference in the unit to express the enzymatic activity. When the *Bacillus* sp. LBA strains were grown under solid state fermentation using wheat bran (medium no. 3) the strains LBA 7, 8 and 48 presented 9720, 13480 and 8950 U/gds respectively (Figure II.2c).

Wheat bran is recognized as one of most studied substrate for microbial protease production under solid state fermentation. In the work of Rajkumar et al. (2011) wheat bran was the best substrate for *Bacillus megaterium* RRM2 protease production compared to other sources such as pigeon pea husk, black gram husk and rice bran, resulting in a protease activity of 985.6 U/g. Other works also reported the use of wheat bran for protease production using *Bacillus* sp. strains (Ramakrishna et al., 2012; Vijayaraghavan et al. 2013).

Other substrates are also reported for *Bacillus* sp. protease production with promising results. Madhuri et al., (2012) used castor husk waste as substrate for alkaline protease production by *Bacillus altitudinis* GVC11 in SSF observing the highest enzyme production of 419,293 U/g of husk.

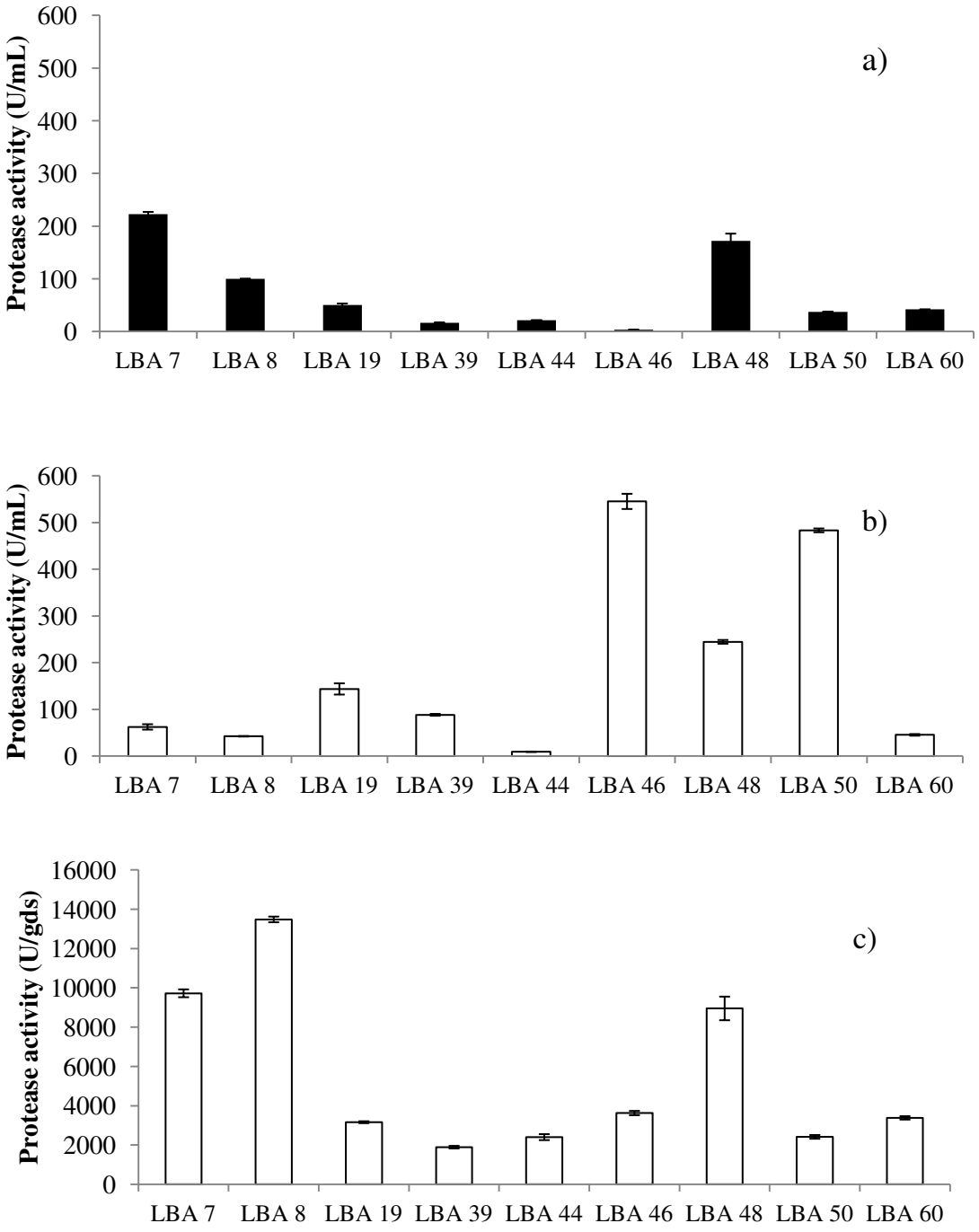

Figure II.2. Protease production from the *Bacillus* sp. LBA 7, 8, 19, 39, 44, 46, 48, 50 and 60 strains in medium no. 1 composed of casein and salts (a), medium no. 2 composed of sugar cane molasses, corn steep liquor, yeast extract Prodex-Lac SD® and milk whey (b) both in 48h of submerged fermentation and medium no. 3 composed of wheat bran and water under solid state fermentation (c) in 96h of fermentation.

Each data point represents the mean of three independent assays with the standard deviation.

II.3.3. Biochemical characterization of *Bacillus* sp. proteases

II.3.3.1. Effect of pH and temperature on the activity of Bacillus sp. proteases

The crude protease preparations exhibited optimal activity in 7-9 pH range (Figure II.3a, II.3b, II.3c). The crude proteases from strains sp. LBA 46, 48 and 50 produced in the media no. 2 and no. 3 had optimal activity at pH 9.0 (Figure II.3b and II.3c). All the crude protease extracts obtained from fermentation of microorganisms in the three different media showed optimal temperature of 60°C (Figure II.4a, II.4b and II.4c).

These results are in accordance with literature. The optimal activity of the protease from *Bacillus halodurans CAS6* was at pH 9.0 and 50°C (Annamalai et al., 2012), while the protease from a strain of *Bacillus* sp. showed optimum activity at alkaline pH 10 and 60°C (Jain et al., 2012).

The activity profile in different pH values and temperature of all the enzymes preparations in the three media were considerably similar, indicating that the different fermentation media have not influenced these properties of crude protease preparations.

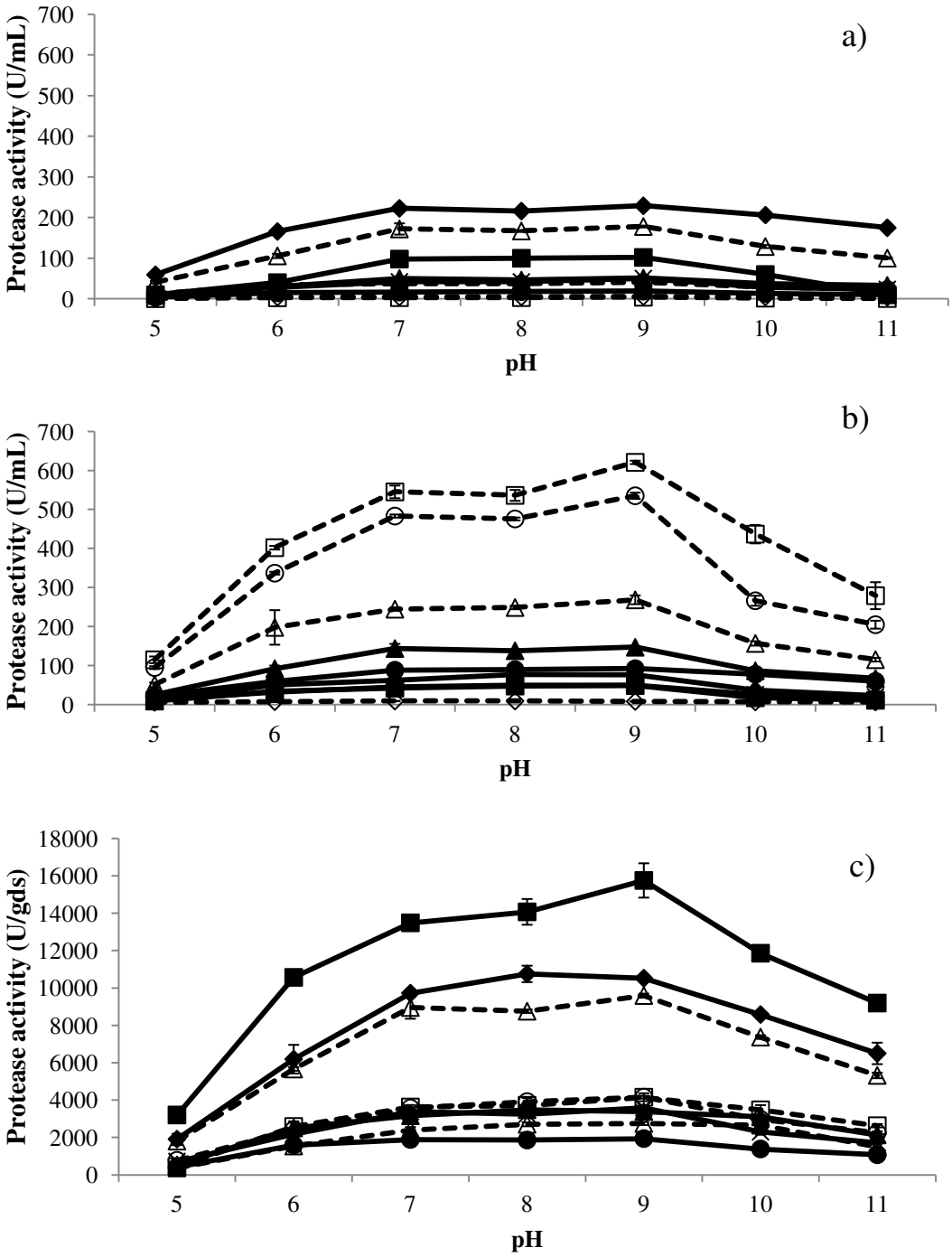

Figure II.3. Effect of pH on the activity of crude proteases extracts from the strains *Bacillus* sp. LBA 7, 8, 19, 39, 44, 46, 48, 50 and 60 obtained from medium no. 1 (a), medium no. 2 (b) and medium no. 3 (c).

Each data point represents the mean of three independent assays with the standard deviation. ♦ - LBA 7, ■ – LBA 8, ▲- LBA 19, ● - LBA 39, ◊ - LBA 44, □ - LBA 46, Δ - LBA 48, ○ - LBA 50, × - LBA 60

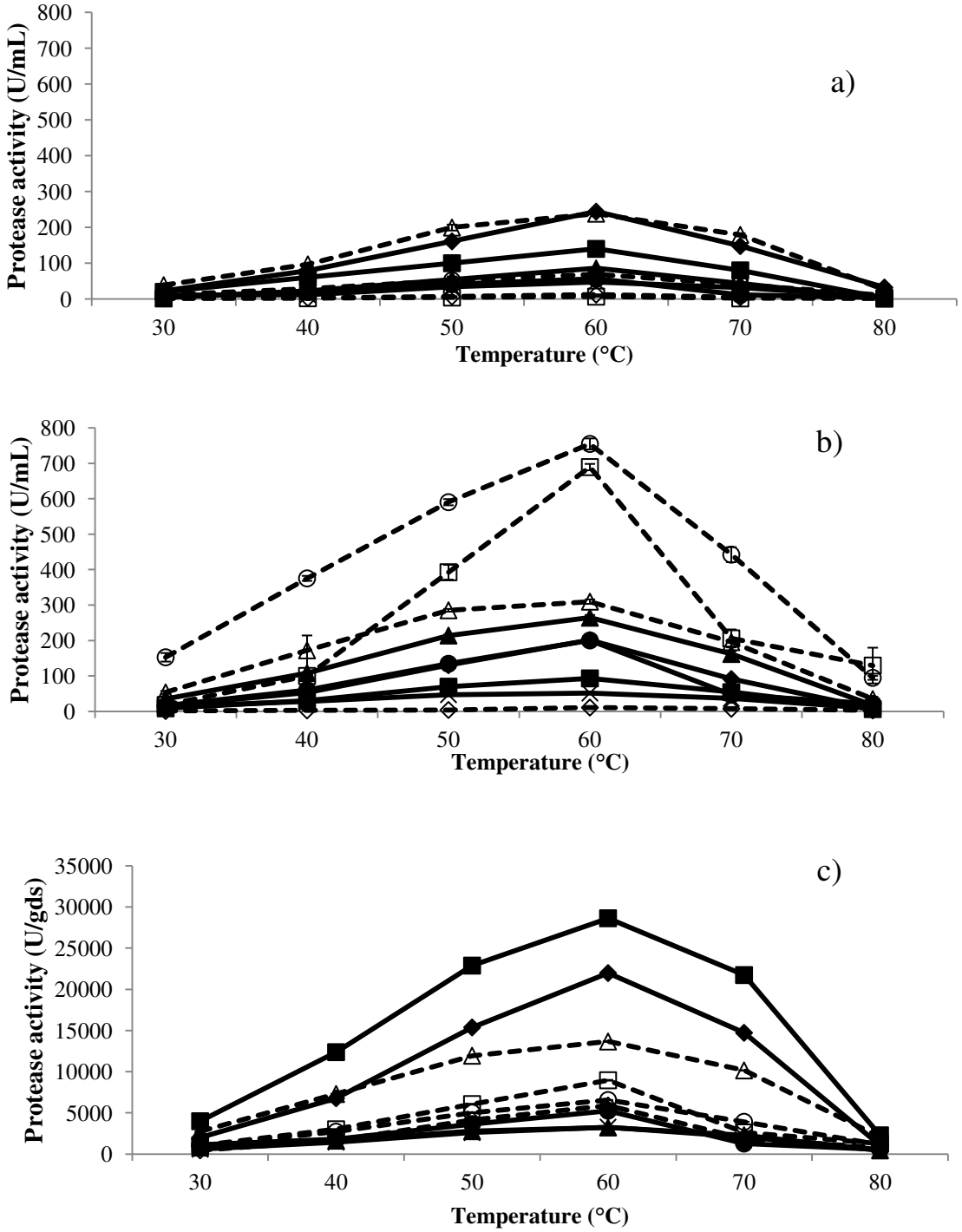

Figure II.4. Effect of temperature on the activity of crude proteases extracts from the strains *Bacillus* sp. LBA 7, 8, 19, 39, 44, 46, 48, 50 and 60 obtained from medium no. 1 (a), medium no. 2 (b) and medium no. 3 (c).

Each data point represents the mean of three independent assays with the standard deviation. ♦ - LBA 7, ■ – LBA 8, ▲- LBA 19, ● - LBA 39, ◊ - LBA 44, □ - LBA 46, Δ - LBA 48, ○ - LBA 50, × - LBA 60

II.3.3.2. Effect of pH and temperature on the stability of Bacillus sp. proteases

The crude protease extracts of the nine strains of *Bacillus* sp. LBA cultivated in the three different media were stable in 5-9 pH range (Figure II.5a, II.5b and 5c) after 24h at 4°C, except the crude proteases from the *Bacillus* sp. cultivated in medium no. 3 that were stable in pH 10. The protease extracts obtained from the cultivation of the strain *Bacillus* sp. LBA 8 in the three different media were also stable at pH 4.0 after 24h at 4°C.

The protease from *Bacillus* sp. MLA64 and a strain of *Bacillus* sp. presented 70% of residual activity after two hours incubation at pH 7.0 (Lagzian and Asoodeh, 2012), and less than 60% of residual activity at pH 6.0 and 7.0 (Jain et al., 2012), respectively.

The crude protease extracts from the strains LBA 7, 19, 39, 44, 46, 48, and 50 cultivated in media no.1 and no. 2 were stable after 1 hour incubation at 50°C and pH 7.0 (Figure II.6a and II.6b). On the other side, the protease extracts from strains LBA 08 and LBA 60 cultivated in medium no. 1 showed low stability, retaining 15% and 32% of residual activity after 1 hour incubation at 50°C and pH 7.0. The crude extracts of protease obtained from all the nine strains of *Bacillus* sp. LBA cultivated in medium no. 3 showed 70 - 90% of residual activity after 1h incubation at 50°C and pH 7.0.

It is reported that proteases from the strains of *Bacillus* sp. and *Bacillus pumilus* MCAS8 presented good stability at 60°C (Jain et al., 2012; Jayakumar et al., 2012).

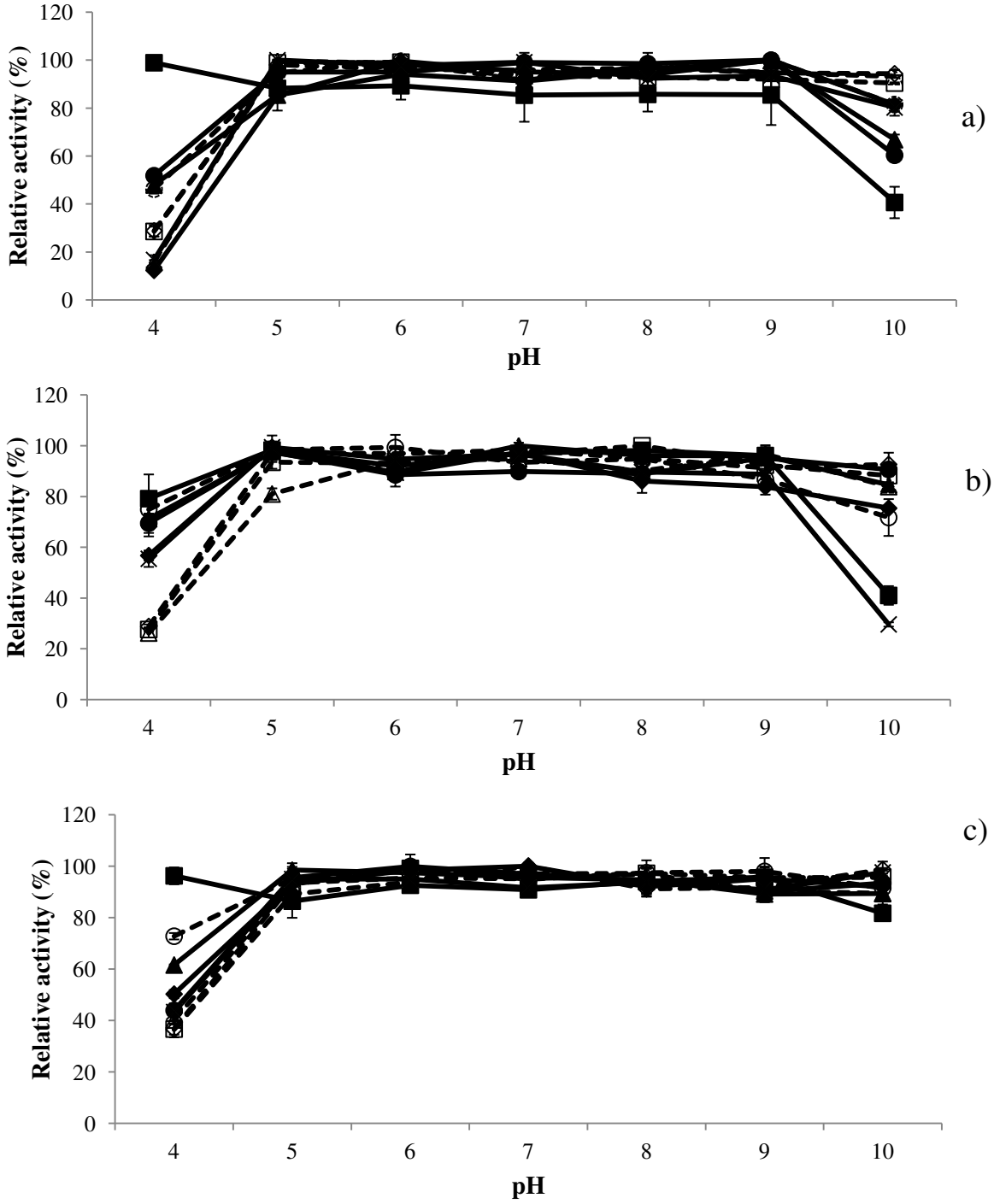

Figure II.5. Effect of pH on the stability of crude proteases extracts from the strains *Bacillus* sp. LBA 7, 8, 19, 39, 44, 46, 48, 50 and 60 obtained from the medium no. 1 (a), medium no. 2 (b) and medium no. 3 (c). The enzyme preparations were pre-incubated in different buffers at 4°C, for 24h.

Each data point represents the mean of three independent assays with the standard deviation. ♦ - LBA 7, ■ – LBA 8, ▲- LBA 19, ● - LBA 39, ◊ - LBA 44, □ - LBA 46, Δ - LBA 48, ○ - LBA 50, × - LBA 60.

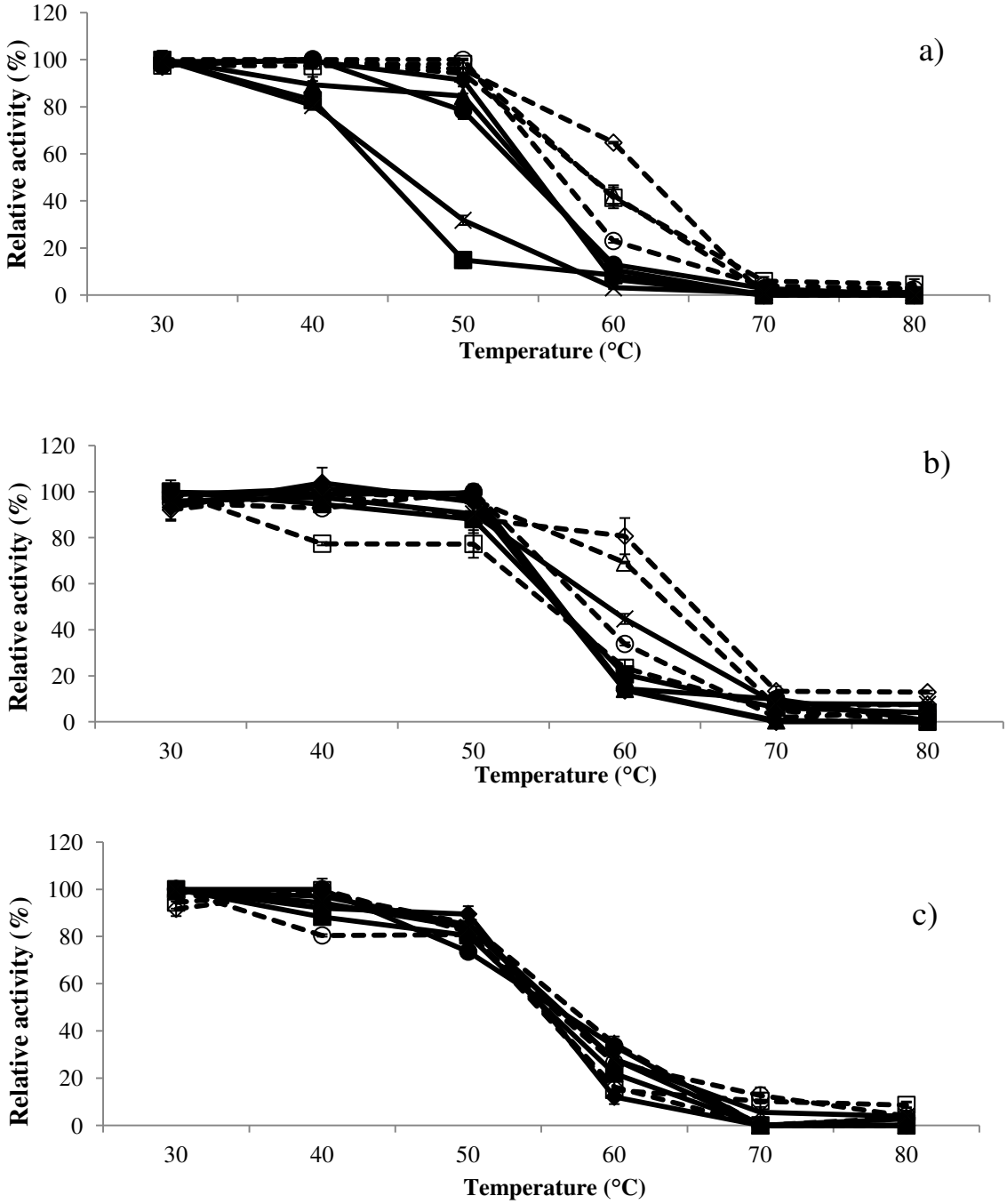

Figure II.6. Effect of temperature on the stability of crude proteases extracts from the strains *Bacillus* sp. LBA 7, 8, 19, 39, 44, 46, 48, 50 and 60 obtained from the medium no. 1 (a), medium no. 2 (b) and medium no. 3 (c). The enzyme preparations were pre-incubated at pH 7.0 for 1h, at different temperatures.

Each data point represents the mean of three independent assays with the standard deviation. ♦ - LBA 7, ■ – LBA 8, ▲- LBA 19, ● - LBA 39, ◊ - LBA 44, □ - LBA 46, Δ - LBA 48, ○ - LBA 50, × - LBA 60.

II.3.4. Effect of commercial detergents on the stability of *Bacillus* sp. proteases

It was observed that crude proteases from *Bacillus* sp. LBA strains cultivated in medium no. 1 showed higher stability in liquid and solid detergent Ariel® compared to preparations obtained from media no. 2 and no. 3 (Figure II.7a, II.7b and II.7c). Crude protease extracts from *Bacillus* sp. LBA 19 and 44 cultivated in medium no. 1 retained 70-80% of residual activity after 2h incubation with detergent Ariel® at room temperature and proteases preparations from strains LBA 39, 44 and 46 cultivated in medium no. 2, retained approximately 50 -60% of residual activity after 2h incubation with detergent Ariel® at room temperature (Figure II.7a and II.7b).

The crude protease extracts obtained from the cultivation of the microorganisms in medium no. 3 were less stable in the detergent solutions tested. However, the crude proteases from strain LBA 19 and LBA 46 cultivated in medium no. 3 retained 60% of residual activity after 2h incubation with detergent Ariel® at room temperature (Figure II.7c).

It is important to state that most of the commercial detergents contain hydrolytic enzymes, including proteases, which are considered green chemicals. They are widely applied in dishwashing, laundry, textile and other related industries (Mukherjee et al., 2009).

Singh et al. (2001) showed that the alkaline protease from *Bacillus* sp. SSR1 retained 37% of its initial activity after 1 h incubation at 40°C in the presence of Ariel® at a concentration of 5mg/mL, while in another study the protease from *Bacillus halodurans* CAS6 presented 88%, 79%, 85%, and 76% of activity after incubation in a solution of 1% of detergents Rin, Ariel, Henko and Tide, respectively, for 1h at 50°C (Annamalai et al., 2012). On the other hand, the protease from *B. laterosporus*-AK1 presented 75 % of activity with Ariel, 63% with Henko, 43% with Surf 43 % and 38 % with Tide (Arulmani et al., 2006).

The crude proteases obtained from fermentation of *Bacillus* sp. LBA 19 and 46 in media no. 1, no. 2 and no. 3 retained 60% of residual activity after incubation with detergent Ariel®, presented activity and stability at alkaline pH and can be considered for future studies of application in detergent for removal of protein stains including blood stains.

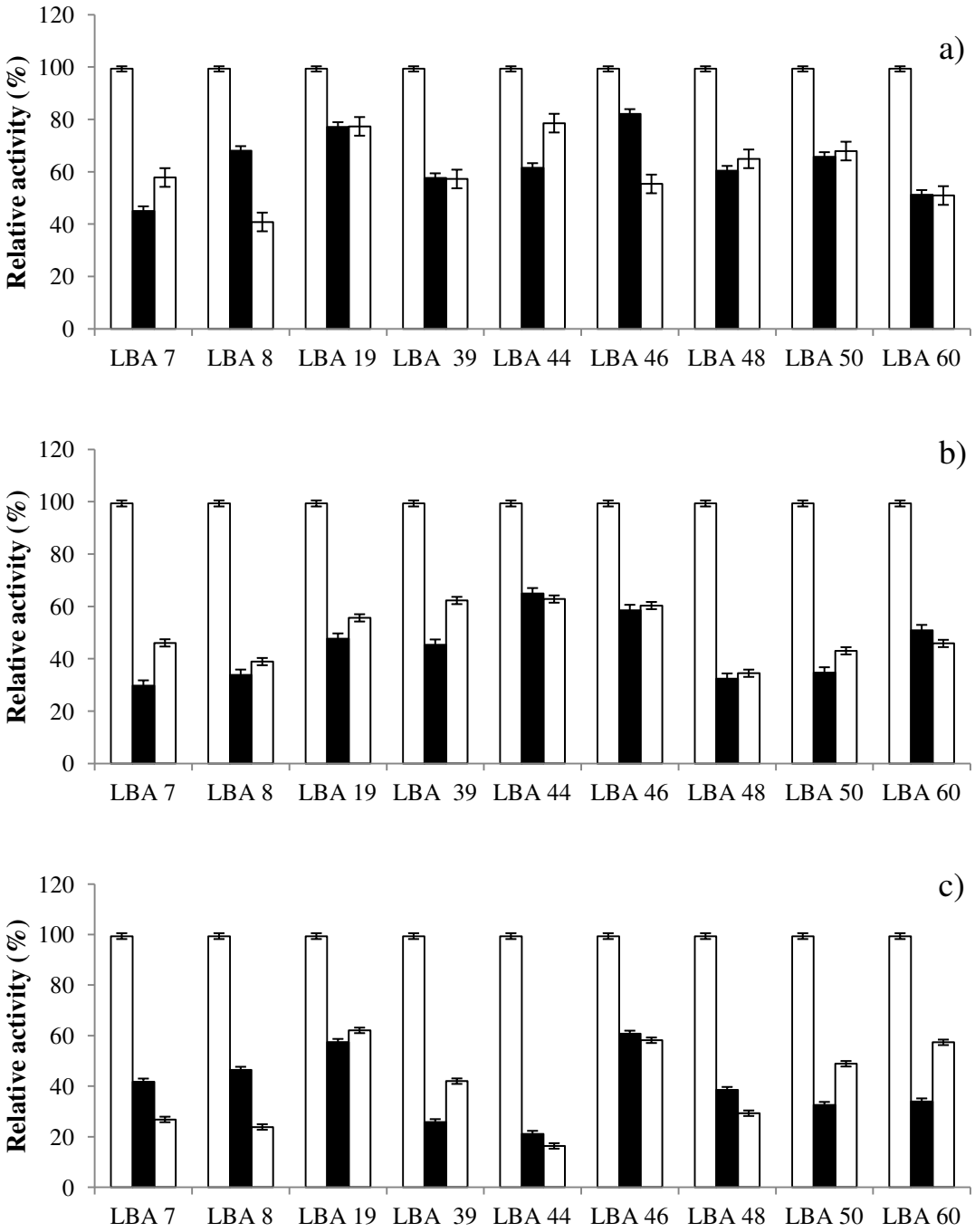

Figure II.7. Stability of the crude proteases extracts, from the strains *Bacillus* sp. LBA 7, 8, 19, 39, 44, 46, 48, 50 and 60, obtained from the medium no.1 (a), medium no. 2 (b) and medium no. 3 (c), in the presence of commercial detergent Ariel® solid (0.7%, m/v) and liquid (1%, m/v) after pre-incubation for 2 h at 25°C.

Each data point represents the mean of three independent assays with the standard deviation. □ – Control, ■ – Solid Ariel®, ⊟ - Liquid Ariel®

II.3.5. Effect of organic solvents on the stability of *Bacillus* sp. proteases

It is well established that the synthesis of different compounds, including peptides, using proteases, could be carried out by the use of a biphasic system composed of organic solvents and water as the reaction mixture (Gupta, 1992). However, proteases can be inactivated or denaturated in the presence of such solvents. Figure II.8 shows the stability of the crude proteases extracts from the nine strains of *Bacillus* sp. LBA in mixtures of different organic solvents and water (50%, m/v) or in water alone, with no organic solvent addition, after 24h incubation, at 30°C and 150 rpm. The protease activity decreased compared to the control test (protease extracts with no incubation). Most of proteases were unstable in ethanol, and only a few proteases were stable in chloroform (Figure II.8a, II.8b and II.8c). In many cases the proteases preparations were more stable in the organic solvents than in only water. One of the reasons is the possible self-digestion or the inactivation of the enzyme when exposed to 30°C for 24h at 150 rpm in water. This is very interesting because in some cases the solvents positively affected the proteases turning the enzyme extracts more stable than the absence of the organic solvents.

The Figure II.8a illustrates that the crude protease extracts from the *Bacillus* sp. LBA 7 and 8 cultivated in medium no. 1 were the most stable preparations in acetone, retaining more than 50% and almost 100%, respectively, while the same enzyme preparations were unstable in water alone. The proteases from strains LBA 19, 44 and 46 when cultivated in medium no. 1 presented good stability in hexane and water (more than 60%) and the proteases from *Bacillus* sp. LBA 44 presented more than 60% of initial activity in chloroform, as well (Figure II.8a).

Most of crude protease extracts from the *Bacillus* sp. strains cultivated in medium no. 2 were not stable in the mixture of water with ethanol, acetone,

chloroform, hexane or water alone, except the proteases from *Bacillus* sp. LBA 44 which presented more than 70% of residual activity in acetone (Figure II.8b).

Most of proteases produced in medium no. 3 were stable in water (more than 60% of activity) except the proteases from *Bacillus* sp. LBA 7 and 8. The proteases from *Bacillus* sp. LBA 19 and 39 presented more than 60% of residual activity in hexane and the protease from *Bacillus* sp. LBA 60 was stable in ethanol (Figure 8c).

These results revealed that proteases could be stable or activated by the solvents that have higher values of log P (coefficient partition), which means they are more hydrophobic, as the solvents acetone, ethanol, chloroform and hexane had log P equal to -0,042, -0,18, 2.0, 3.7, respectively. Organic solvents with lower values of log P are considered to be extremely denaturing because of their higher degree of partitioning into the aqueous phase leading to the lost of activity of the enzyme, considering the reduction in the structural flexibility. It happens because of the disruption of hydrogen bonding and hydrophobic interactions that deprive the water hydration shell of the protein. Thus, one of the possible reasons for enzyme stability in presence of such organic solvents could be the presence of greater number of negatively charged acidic amino acids compared to few basic amino acids on their surface. It is thought that these negative charges can keep the protein soluble either by forming a hydrated ion network with cations or by preventing the protein aggregation through electrostatic repulsive charges at the protein surface (Jain et al., 2012).

The half-life of the protease from *Bacillus cereus* BG1 in the absence of organic solvents was about 40 days at 30°C, at pH 8.0 with shaking at 180 rpm. The enzyme showed good stability in the presence of dimethyl sulfoxide, methanol, ethanol and isopropanol, since the half-lives were longer than that in the absence of organic solvent. On the other side, the half-life of the enzyme in the presence of dimethylformamide was shorter than that in the

absence organic solvents. In addition, protease activity was rapidly decreased by acetonitrile with an estimated half-life of 5 days (Ghorbel et al., 2003). Rahman et al. (2007) verified the stability of the protease from *Bacillus pumilus* 115b and observed that in organic solvents with log P lower than 3.1 the stability of the enzyme decreased massively. When the protease was incubated for 30 min at 37°C and 150 rpm, with 25% of ethyl acetate (logP 0.68) and 1-heptanol (logP 2.4) the enzyme presented 10% and 54% of protease activity, respectively.

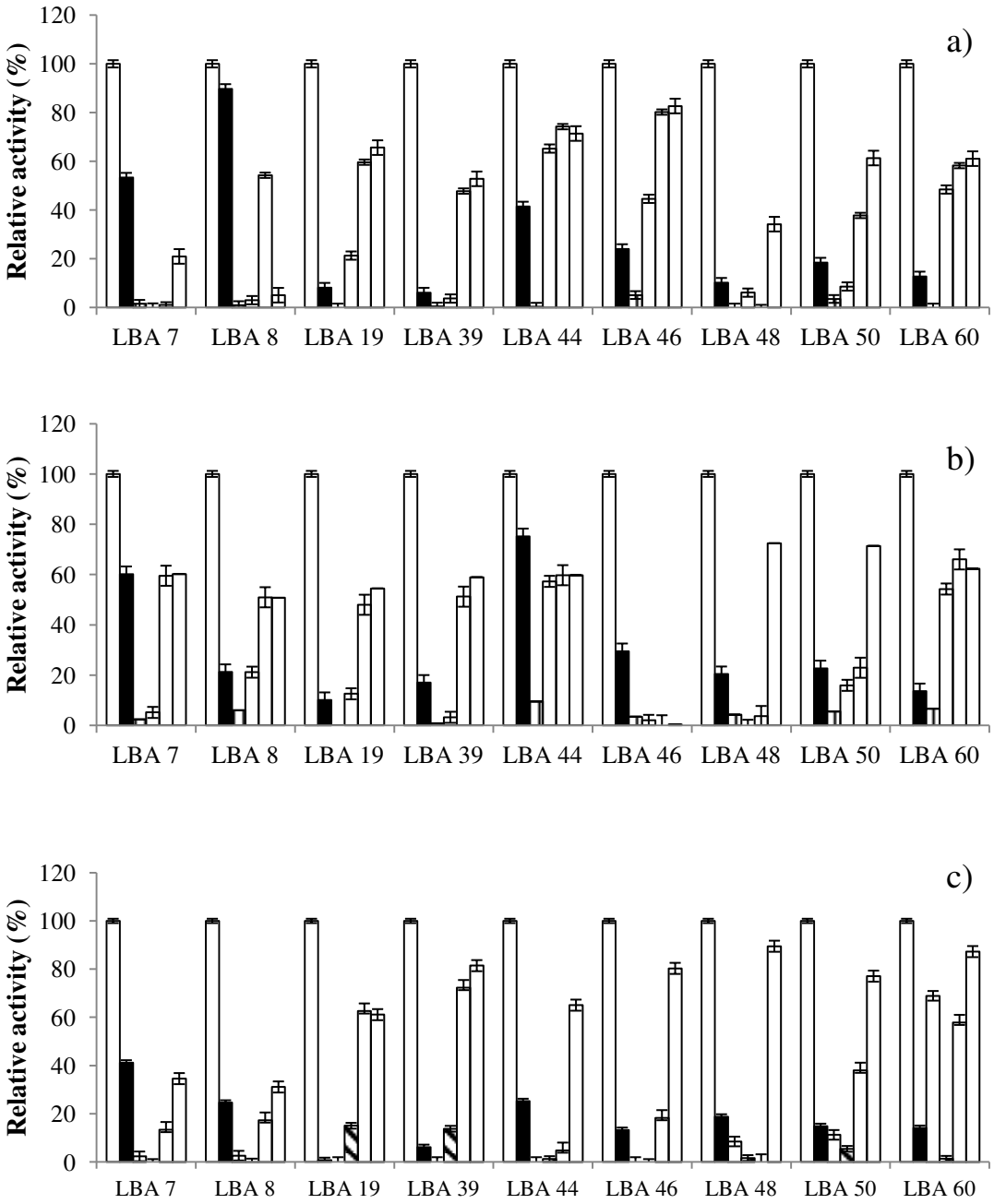

Figure II.8. Stability of the crude proteases extracts, from the strains *Bacillus* sp. LBA 7, 8, 19, 39, 44, 46, 48, 50 and 60 obtained from the medium no. 1 (a), medium no. 2 (b) and medium no. 3 (c), in the presence of different organic solvents and water (50%, v/v/) or only water for 24 h at 150 rpm and 30°C.

Each data point represents the mean of three independent assays with the standard deviation. □ – Control, ■ – Acetone, ▯ - Ethanol, ▨ - Chloroform, ▭ - Hexane, ▩ - Water

II.3.6. Clarification of xanthan gum using proteases from *Bacillus* sp. strains

The cell lysis of *X. campestris* using proteases is an important step for xanthan gum clarification. In this work the proteases extracts from *Bacillus* sp. LBA 07, 08, 19, 39, 44, 46, 48, 50 and 60 were applied in the clarification of the xanthan broth containing cells of *X. campestris* and it was evaluated based on the increase of the transmittance (Trans $_{620nm}$) of the xanthan broth (Figure II.9). Most of the crude protease extracts from *Bacillus* sp. strains cultivated in medium no. 3 were capable of clarifying the xanthan broth (Figure II.9c). The protease extracts from the *Bacillus* sp. LBA 48 obtained after cultivation in the three different media were efficient in the clarification, resulting in an increase of more than 30% in the T_{620nm} after two hours of reaction. Additionally, the protease preparations from the strains LBA 46 obtained from the cultivation in media no. 2 and no. 3 also presented promising results for xanthan broth clarification showing an increase of more than 35% in the T_{620nm} of the broth after 2h. All the other strains were also capable of clarifying the xanthan broth, but with less efficiency. The crude proteases from *Bacillus* sp. LBA 46 and 48 showed the highest capability of clarification of *X. campestris* broth compared to the other crude extracts and showed potential for *X. campestris* cell lysis and xanthan gum clarification, an important step for xanthan gum purification. Furthermore, these extracts from *Bacillus* sp. 46 and 48 neither presented xanthanase activity at 50°C nor at 42°C, indicating that they did not degrade the xanthan from the fermented broth.

Although some patents are found in the literature, for the best of our knowledge there are few scientific articles on the study of the lysis of *X. campestris* cells using enzyme extracts from *Bacillus* sp. strains. Proteases from a few different sources have been used for the degradation of *X. campestris* cells to obtain clarified product, including *Trichoderma koningii*

(Triveni and Shamala, 1999), *Pseudomonas* sp. (CL 1457) (Shastry and Prasad, 2002) and *Lysobacter* species (Pollock and Yamazaki, 1993).

Kohler et al. (1984) patented the enzymatic clarification process for improving the injectivity and filterability of xanthan gums using protease from *B. licheniformis*. On the other side, Armentrout et al. (1999) patented the process of purifying xanthan gum using an alkaline protease and lysozyme. In this last patent it is described that the fermented broth was treated with alkaline protease from *B. subtilis* ("Bioplase"). After treatment with alkaline protease and lysozyme the xanthan broth presented a transmittance of 90%. In the patent of Dimasi et al. (2003), a process is disclosed for clarifying an aqueous xanthan gum solution. Neutral proteases from *B. amyloliquefaciens* and alkaline proteases from *B. subtilis*, *B. licheniformis*, and *B. pumilis*, among other proteases were tested. The high purity xanthan gum provided substantially clear viscosified compositions having most preferably, a transmittance of at least 93%.

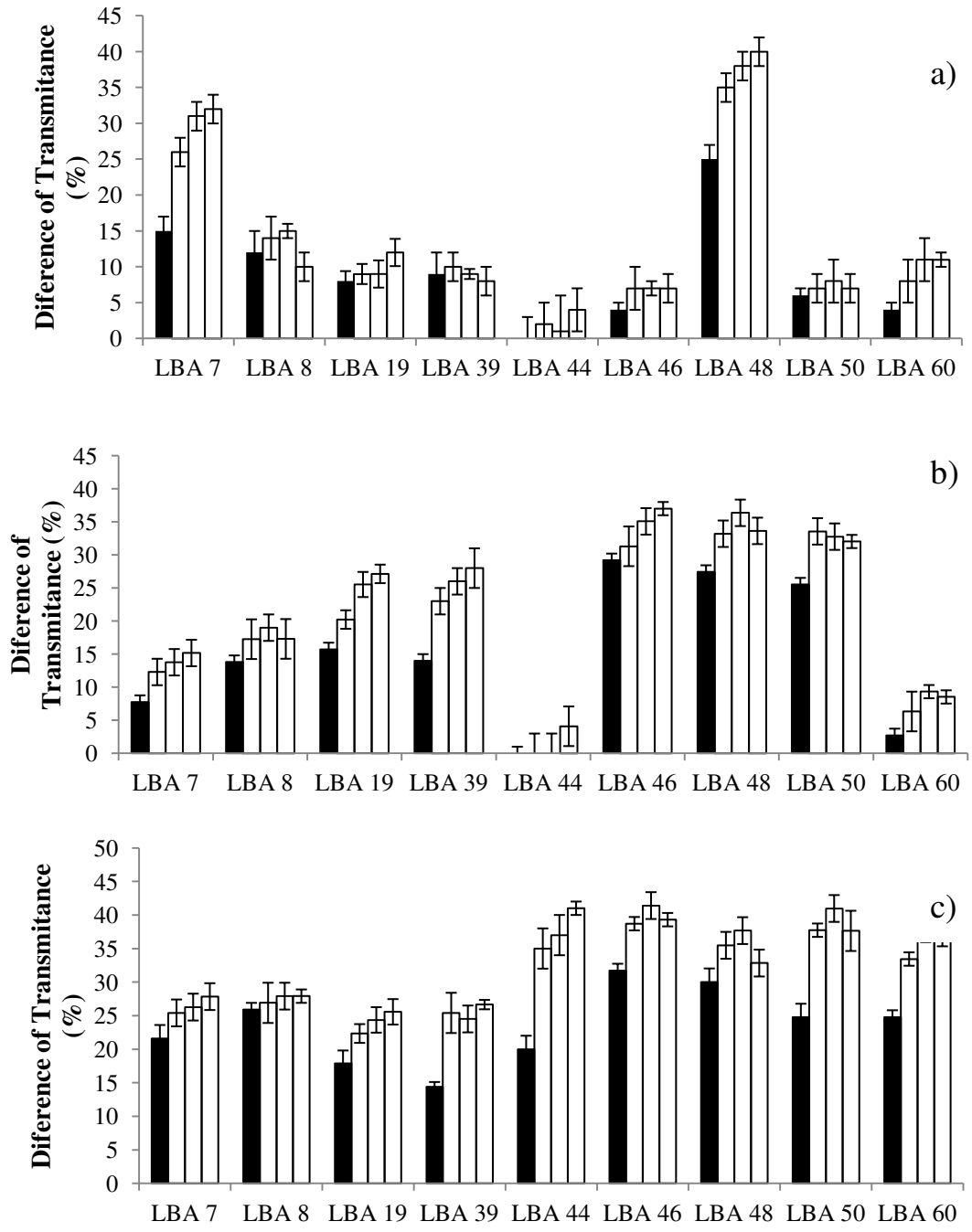

Figure II. 9. Effect of the crude protease extracts obtained from fermentation of *Bacillus* sp. LBA 7, 8, 19, 39, 44, 46, 48, 50 and 60 in medium no.1 (a), medium no. 2 (b) and medium no. 3 (c) on clarification of *X. campestris* cells broth estimated by difference of Trans $_{620\ nm}$ before and after the application of the crude protease extracts.

Each data point represents the mean of three independent assays with the standard deviation. ■ – 30 min, ▬ - 60 min, □ – 90 min, ▨ - 120 min.

II.4. Conclusions

Among fifty nine strains of *Bacillus* sp. tested the strains LBA 7, 8, 19, 39, 44, 46, 48, 50 and 60 were selected due to the higher production of protease in media composed of casein, yeast extract and salts. After cultivation of these nine *Bacillus* sp. LBA strains in three different media with different composition, it was observed that the strains LBA 7, 8, 46, 48 and 50 were the best protease producers.

The strains *Bacillus* sp. LBA 7 and 48 produced respectively 222 U/mL and 172 U/mL of protease after fermentation in Erlenmeyer flasks containing medium no. 1 composed of 10g/L of casein, 1 g/L of yeast extract and salts at 30°C, 200 rpm during 48h. The strains LBA 46, 48 and 50 produced lower activity of protease (3 U/mL, 172 U/mL and 37 U/mL) after fermentation in medium no. 1, respectively.

The crude extracts of proteases from *Bacillus* sp. LBA 46, 48 and 50 cultivated in Erlenmeyer flasks (250 mL) with agitation, containing medium no. 2 composed of 20 g/L of sugar cane molasses, 20 g/L of corn steep liquor, 3 g/L of yeast extract Prodex-Lac®, 20 g/L of dried whey (medium no. 2) presented protease activity of 548 U/mL, 244 U/mL and 483 U/mL, respectively.

The protease activity amounts of crude protease extracts obtained after the fermentation of *Bacillus* sp. LBA 7, 8, 46, 48 and 50 under solid state fermentation in medium no. 3 composed of wheat bran were 9720, 13480, 3625, 8950, and 2413 U/gds, respectively.

Regarding the biochemical properties, the proteases from the strains of *Bacillus* sp. LBA 7, 8, 19, 39, 44, 46, 48, 50, 60 presented optimal activities at 60°C and in 7-9 pH range.

The crude protease extracts from *Bacillus* LBA 8, 19 and 39 were stable in 5-9 pH range at 4°C after 24h, while the extracts from *Bacillus* sp.

LBA 7, 44, 46, 50 and 60 were stable in 5-10 pH range. The crude protease extracts from all the nine *Bacillus* sp. LBA strains obtained from the fermentation of the microorganisms in the culture medium no. 2 were stable at 50°C for one hour at pH 7.0.

The crude protease extracts from *Bacillus* sp. LBA 19, 44, 46, 48, and 50 obtained from the fermentation of the microorganisms in culture medium no. 1 retained approximately 60-80% of the initial activity after incubation with liquid and solid Ariel® at 25°C for 2h. Furthermore the protease extracts from *Bacillus* sp. LBA 44 and 46 obtained from the cultivation of the microorganisms in culture medium no. 2 retained approximately 60% of the initial activity after incubation with the detergent.

The crude protease extracts from *Bacillus* sp. LBA 44 and 46 obtained from the fermentation of the microorganisms in medium no. 1 retained approximately 60-80% of the initial activity after incubation with the solvents chloroform and hexane.

The crude protease extracts from *Bacillus* sp. LBA 46 and 48 presented higher capacity of clarification of the fermented broth of *X. campestris* compared to the extracts from the other *Bacillus* sp. strains and therefore showed potential to the lysis of cells of *X. campestris* and clarification of xanthan gum.

The *Bacillus* sp. LBA 46 was selected for the next studies of protease production based on the protease activity amounts, compatibility with detergent and capability of lysing of *X. campestris* cells.

II.5. Acknowledgements

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III. CAPITULO 3 - OPTIMIZATION OF THE PRODUCTION OF PROTEASES FROM *Bacillus* sp. UNDER SUBMERGED FERMENTATION AND EVALUATION OF REMOVAL OF STAINS FROM FABRICS

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RESUMO

A produção de protease pela linhagem de *Bacillus* sp. LBA 46 foi estudada utilizando substratos agroindustriais de baixo custo, usando metodologia de superfície de resposta (MSR). Inicialmente foi observada maior atividade de protease (2200 U/mL) e crescimento bacteriano após 96h de fermentação submersa, em meio de cultura composto de 35g/L de melaço de cana de açúcar, 20 g/L de água de maceração de milho, 3 g/L de extrato de levedura Prodex-Lac SD® e 20 g/L de soro de queijo. Após a utilização de MSR foi observado que as melhores condições para a produção de protease foram obtidas utilizando-se meio de cultura composto de 20 g/L de melaço de cana de açúcar, 6 g/L de água de maceração de milho, 2 g/L de extrato de levedura Prodex-Lac SD® resultando em atividade de protease igual a 600 U/mL, após 96h de fermentação a 30°C e 200 rpm. Estudos posteriores utilizando diferentes concentrações de soro de queijo mostraram que as condições ótimas de produção de protease de *Bacillus* sp. LBA 46 foram obtidas utilizando-se meio n° 4 composto de 40g/L de melaço de cana de açúcar, 6 g/L de água de maceração de milho, 2g/L de extrato de levedura Prodex-Lac SD® e 20g/L soro de queijo, resultando em atividade de protease de 3000 U/mL após 96h de fermentação a 30°C e 200 rpm. O extrato bruto de protease obtido do cultivo da linhagem de *Bacillus* sp. LBA 46 no meio otimizado de fermentação submersa foi aplicado na remoção de manchas de sangue e molho de tomate com carne moída em tecidos de algodão. Quando os tecidos foram lavados utilizando a combinação de enzima com o detergente, as manchas de sangue foram completamente removidas. Com relação aos tecidos manchados de molho de tomate com carne moída, os melhores resultados de remoção de manchas foram observados utilizando a combinação de proteases com detergente para a lavagem de roupas, comparado com os testes utilizando somente o detergente.

Palavras-chave: proteases, *Bacillus* sp., fermentação submersa, remoção de manchas, substratos de baixo custo

ABSTRACT

Bacillus sp. LBA 46 strain was studied for protease production using low cost agro-industrial substrates utilizing response surface methodology (RSM). Initially, it was observed that the highest protease activity (2200 U/mL) and bacterial growth was achieved after 96h of submerged fermentation (SmF) in a medium composed of 35 g/L of sugar cane molasses, 20 g/L of corn steep liquor, 3 g/L of yeast extract Prodex-Lac SD® and 20 g/L of dried whey. After using RSM, the best conditions for protease production were obtained using 20 g/L of sugar cane molasses, 6 g/L of corn steep liquor, 2 g/L of yeast extract Prodex-Lac SD® reaching protease activity of 600 U/mL, at 30°C, 200 rpm, after 96h of fermentation. Further univariable studies with different concentrations of dried whey showed that the best conditions for protease production by *Bacillus* sp. LBA 46 was found using medium no. 4 composed of 40 g/L of sugar cane molasses, 6 g/L of corn steep liquor, 2 g/L of yeast extract Prodex-Lac SD® and 20 g/L of dried whey resulting in 3000 U/mL of protease activity, after 96h of fermentation, at 30°C and 200 rpm. The crude protease extract from the *Bacillus* sp. LBA 46 obtained from the cultivation in optimized medium under SmF was applied in the removal of blood and tomato sauce with ground beef stains in cotton fabrics. When the fabrics were washed using the combination of the enzymes with laundry detergent, the blood stains were completely removed. In addition, better results in the removal of tomato sauce with ground beef stains were observed using the combination of the proteases with laundry detergent when compared to the use of detergent alone.

Keywords: proteases, *Bacillus* sp., submerged fermentation, stain removal, low cost substrates

III.1. Introduction

Proteases are one of the most used enzymes in several industrial fields, which include laundry detergents, protein recovery, meat tenderization and organic synthesis, among others (Gupta et al., 2002a). Among the microorganisms studied, *Bacillus* sp. are recognized as one of the most important sources for protease production due to secreting large amounts of alkaline proteases, having significant proteolytic activity and stability at high pH and temperatures (Shah et al., 2010, Chauhan and Gupta, 2004).

Another important aspect of *Bacillus* sp. strains as protease producers is the viability of production of this enzyme under submerged fermentation (SmF) and solid state fermentation (SSF). The former is the classic technique and is responsible for the majority of the enzymes industrially produced and shows some advantages over SSF, including reduction of contamination due to relatively short growth period, lower capital investment compared to continuous processes for same bioreactor volume, among other aspects (Gupta et al., 2002b).

Regarding the fermentation processes, media compositions have major influence on the protease production and are different for each microorganism (Tari et al., 2006). In addition, they represent approximately 30–40 % of the production costs (Kirk et al., 2002). Thus, the required constituents and their concentrations have to be optimized accordingly and industrial wastes or byproducts, including sugar cane molasses (Younis et al., 2009), corn steep liquor (Puri et al., 2002) and wheat bran (Renganathan et al., 2011) are reported as good candidates. One highlighted technique for this purpose is the use of response surface methodology (RSM) that is applied to identify the relationships between the variables and the response, generally resulting in the optimization of the process and simultaneously limiting the number of experiments.

Enzymes find in detergent industries their main consumers, in terms of both volume and value (Mitidieri et al., 2006). Proteases present in detergent formulations enhance their ability to remove proteins stains, including blood stains, making the detergent environmentally safe. Proteases are important due to the fact that they prevent the redeposition of hydrophobic proteins, therefore providing a whiteness benefit of fabrics. *Bacillus* sp. strains are the most studied due to the production of proteases with good stability in alkaline solutions and oxidizing compounds present in commercial detergents (Jellouli et al., 2011). Hence, the aim of this work was to optimize the production of protease by the strains of *Bacillus* sp. LBA 46, under SmF using central composite design (CCD) and RSM. The application of the proteases obtained from the cultivation of *Bacillus* sp. LBA 46, under optimized conditions, in the removal of stains from fabrics was also investigated.

III.2. Materials and methods

III.2.1. Microorganism

The strains of *Bacillus* sp. LBA 46 from culture collection of Food Biochemistry Laboratory, College of Food Engineering – UNICAMP was maintained in nutrient agar at 4°C, with sterile vaseline addition and transfers in every 2 months.

III. 2.2. Molecular identification and phylogenetic analysis of *Bacillus* sp. LBA 46

The DNA extraction of the *Bacillus* sp. LBA 46 was carried out as described by Neumann et al., 1992 and Cheng and Jiang, 2006 with minor modifications. The genomic DNA extraction was quantified by NanoDrop 2000c Spectrophotometer (Thermo Scientific) at 260 nm. Possible protein contamination was evaluated based on the ratio of the wavelengths 260/280 nm.

The 16S rDNA sequence was amplified using the set of initiator oligonucleotides p27f (5' AGAGTTTGATCMTGGCTCAG 3') and p1492r (5' TACCTTGTTACGACTT 3') (Lane, 1991). The reactions (50 μL) contained 100 ng of genomic DNA, 2 U of Taq polymerase (Thermo Scientific), 0.2 mmol L-1 of dNTP mix, and 0.2 μmol L–1 of each primer, in 1X Taq buffer. Amplifications were carried out by PCR using initial cycle of denaturation at 94°C for 3 minutes, followed by 30 cycles at 94°C for 40 seconds, 55°C for 40 seconds, 72°C for 2 minutes and one final extension cycle at 72°C for 10 minutes. The reaction product of PCR was separated by electrophoresis in agarose gel 1% (m/v) in buffer TAE 1X, at 90 V for 1 hour and analyzed after staining with ethidium bromide. The band (with approximately 1,500 bp) was excised from the gel and purified with the purification kit GFX™ PCR DNA and Gel Band Purification (GE Healthcare). The reaction product of the purified PCR was applied to sequencing reaction using the oligonucleotides mentioned above plus the oligonucleotide 907r (5' CCGTCAATTCMTTTRAGTTT 3') (Lane, 1991). The sequencing reactions were carried out using the reagent BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and were analyzed in the DNA sequenciator DNA ABI PRISM 377 Genetic Analyzer (Applied Biosystems). The sequences obtained were analyzed using the software Geneious version 6.1.5 (Biomatters) and compared with 16S rRNA sequence data from some types of strains available at the public database Genbank (http://blast.ncbi.nlm.nih.gov) by using the BLAST N sequence match routines. The sequences were aligned using the CLUSTAL W2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2) and phylogenetic and molecular evolutionary analyses were conducted with the program MEGA version 6.0 (Tamura et al., 2013) using the substitution model of the nucleotide Kimura 2-P (Kimura, 1980) and the neighbor-joining algorithm (Saitou e Nei, 1987), with bootstrap values calculated from 1,000 replicate runs.

III.2.3. Production of proteases by *Bacillus* sp.

III.2.3.1. Influence of fermentation time in protease production by Bacillus sp. LBA 46

Aliquots of 2 mL of cell suspension of *Bacillus* sp. LBA 46 in saline solution (0.85% NaCl, m/v) (optical density at 620 nm between 0.49 and 0.51) were inoculated into 250 mL Erlenmeyer flasks containing 48 mL of a culture medium no. 2 composed of 35 g/L of sugar cane molasses (Fios de ouro®, BR), 20 g/L of corn steep liquor (Corn Products do Brasil, BR), 3 g/L of yeast extract Prodex-Lac SD® (Produtos Especiais para Alimentos S/A, BR), 20 g/L of dried whey (Alibra, BR) and pH adjusted to 7.0. The flasks were incubated at 30°C and 200 rpm. Samples were collected in different periods of time during the fermentation process and they were centrifuged at 9.600 × *g* for 15 min at 5°C and the supernatant was used to measure the pH and protease activity. For cell growth estimation aliquots of 1.0 mL of the fermentation were also added into Eppendorf tubes and centrifuged under the same conditions and the precipitate was washed twice with 0.85% NaCl (m/v). After centrifugation, 1 mL of 0.85% NaCl (m/v) was added to Eppendorf tubes of 2 mL and the absorbance (Abs_{620nm}) was determined.

III.2.3.2. Optimization of production of proteases by Bacillus sp. LBA 46 under submerged fermentation

The production of protease from *Bacillus* sp. LBA 46 under SmF has been optimized using CCD and RSM. A 2^3 central composite design (2^3-CCD) with 2 axial points (+\–) at a distance of α=1.68 was used, with three replicates at the center point, resulting in a total of 17 runs. Aliquots of 2 mL of cell suspension of *Bacillus* sp. LBA 46 in saline solution (0.85% NaCl, m/v) (optical density at 620 nm between 0.49 and 0.51) were used as inoculum. The *Bacillus* sp strains were inoculated into 250 mL Erlenmeyer flasks containing 48 mL of culture medium composed of sugar cane molasses,

corn steep liquor and yeast extract Prodex-Lac SD® as the three independent variables (Table III.1). The flasks were incubated at 200 rpm, 30°C for 96h. The samples were centrifuged at 9600 × *g* for 15 min at 5°C. The supernatants were used as crude protease extracts and the dependent variable was the protease activity. The statistical analyses were carried out using software Statistica 8.0®. After the optimization of the concentration of the three variables, some univariable tests were carried out in order to verify the optimal conditions for protease production. Afterwards, other tests were carried out fermenting the *Bacillus* sp. LBA 46 in the defined medium with different concentrations of dried whey (0 – 40 g/L) as an attempt to improve protease production.

III.2.4. Determination of protease activity

The protease activity was determined as described by Charney & Tomarelli (1947) with modifications. The reaction mixture containing 0.5 mL of 0.5% (m/v) azocasein (Sigma, US) in 0.05 M phosphate buffer (pH 7.0) and 0.5 mL of the enzyme solution was incubated at 60°C for 40 min. The reaction was stopped by adding 0.5 mL of 10.0% (m/v) trichloroacetic acid and the test tubes were centrifuged at 17000 x *g* for 15 min at 25°C. A 1.0 mL aliquot of the supernatant was neutralized with 1.0 mL 5 M KOH. One unit of protease activity was defined as the amount of enzyme which caused an increase of 0.01 of absorbance at 428 nm per minute. The enzyme activity was expressed as U/mL of crude enzymatic extract.

III.2.5. Evaluation of the addition of crude protease from *Bacillus* sp. LBA 46 on washing performance

Clean cotton cloth pieces (5cm × 5cm) previously washed were soiled with 0.1 mL defibrinated sheep blood and commercial tomato sauce with ground beef and then dried for 48h. For this experiments the commercial solid detergents Omo® and Tixan® in tap water (7mg/mL), or liquid detergent Ariel® in tap water (1%, m/m) were used. They were bought in commercial Brazilian market and before use they were previously heated at 95°C for 30 minutes to remove any enzymatic activity presented in detergent. The stained cloth pieces were placed in separate Erlenmeyer flasks of 250 mL, with 50 mL as final volume, as described: tap water only; tap water and commercial detergents; tap water and crude protease extract (1000 U) from *Bacillus* sp. LBA 46 (after the optimization of the culture medium); tap water, commercial detergent and crude enzyme extract from *Bacillus* sp. LBA 46 diluted to two different enzymatic activity amounts (100 and 1000 U). The flasks were incubated at 40°C for 2 hours under agitation (200 rpm). After incubation, cloth pieces were taken out, washed with tap water for 30 min, 200 rpm, at

40°C and then dried. Visual examination of various pieces showed the effect of preparations in the removal of stains.

III.3. Results and Discussion

III.3.1. Identification of the microorganism and phylogenetic comparison

The 16S rDNA partial sequence of the strain of *Bacillus* sp. LBA 46 was compared to sequences of organisms represented in the database GenBank. As observed in the Figure III.1 *Bacillus* sp. LBA 46 and its nearest neighbors, *Bacillus licheniformis* strain BCRC 11702, *Bacillus licheniformis* strain NBRC 11702 and *Bacillus licheniformis* strain ATCC 14580 shared a 16S rDNA gene sequence similarity of 99%, indicating that the *Bacillus* sp. LBA 46 strain probably is a strain of *Bacillus licheniformis*. These results are in accordance with the literature, taking into consideration that several strains of *B. licheniformis* are well recognized as proteases producers (Hanlon et al., 1982; Jellouli et al., 2011).

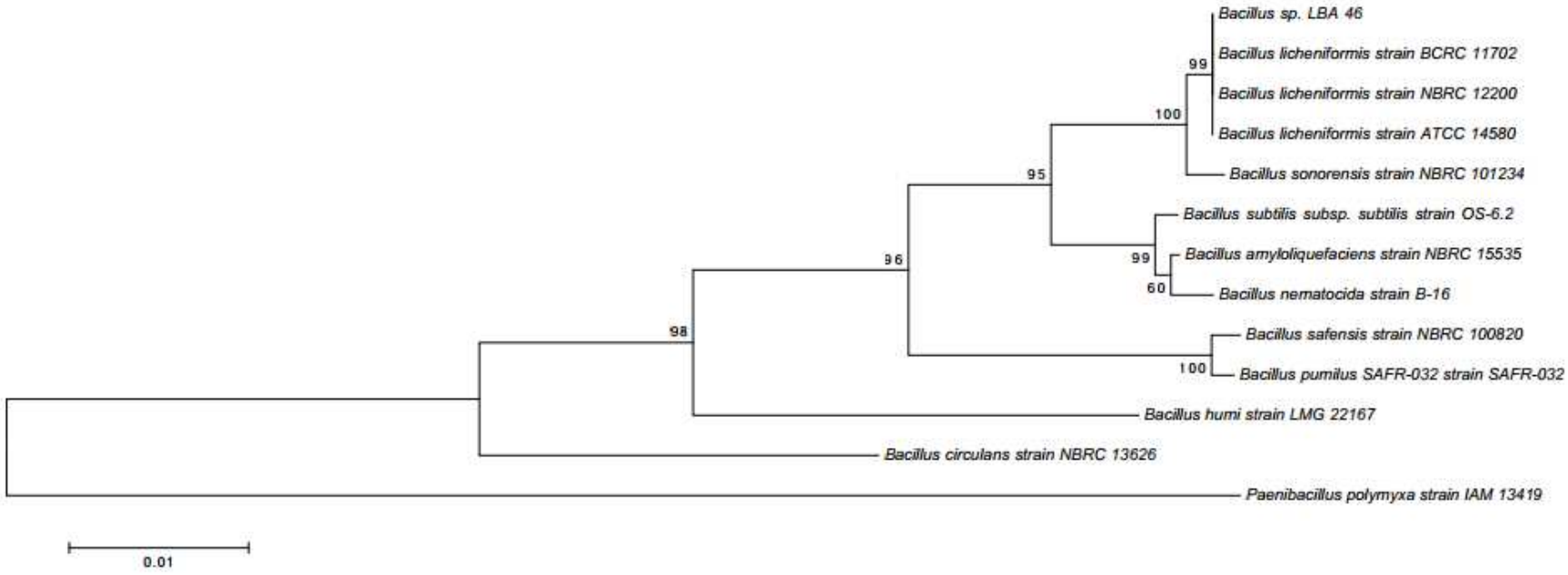


Figure III.1. Neighbor-joining tree based on 16S rDNA sequences showing the relationships between the isolate *Bacillus* sp. LBA 46 and strains of phylogenetically closely related members of the genus *Bacillus*.

III.3.2. Production of proteases by *Bacillus* sp.

III.3.2.1. Influence of time on protease production by Bacillus sp. LBA 46

The influence of the time on the production of protease from *Bacillus* sp. LBA 46 in the medium no. 2 composed of 35 g/L of sugar cane molasses, 20 g/L of corn steep liquor, 3 g/L of yeast extract Prodex-Lac SD® and 20 g/L of dried whey in different fermentation time is illustrate in the Figure III.2. Maximum protease production occurred after 96 h reaching 2200 U/mL, but after 120h the protease activity decreased considerably to 1500 U/mL. It is also observed that the enzyme production had a similar profile to the bacterial growth, once it started to grow considerably after 60 h of and reached the maximum growth in 72h of fermentation. In 120h of fermentation the growth density decreased. The pH of the fermented medium increased during the fermentation reaching 8.2 after 60h of fermentation. After this period of time the pH of the medium remained stable and after 120h of fermentation the pH was 7.9. The rise in pH could be attributed to consumption of organic acids and amino acids released.

Joo and Chang (2006) studied the time course of the protease production under the SmF of *Bacillus clausii* I-52 in a medium composed of 20 g/L of soybean meal, 10 g/L of wheat flour, 25 g/L of liquid maltose, 4 g/L of K_2HPO_4, 1 g/L of Na_2HPO_4, 0.1 g/L of $MgSO_4 \cdot 7H_2O$, 4 g/L of NaCl, 0.5 g/L of $FeSO_4 \cdot 7H_2O$, g/L of Na_2CO_3, at 37°C observing maximum range activity between 24h to 72h of fermentation with maximum in 48h. The initial pH was approximately 11.5, the pH decreased to 8.0 after 12h of fermentation and maintained increasing for 72h of fermentation reaching a pH value above 9.0. The authors also observed that the bacterial growth was similar to protease production reaching the maximum value in 18h of fermentation and kept stable.

In the study of Moorthy and Baskar (2013) the *Bacillus* sp. BGS strain was studied for protease production in a medium composed of 16.827 g/L of peptone, 11.28 g/L of molasses, pH 11, and 2% (v/v) of inoculum size, showing higher protease activity (3000 U/mL) after 56h of fermentation and a decrease in enzyme production after this time. The bacterial growth was considerable after 24h of fermentation, increasing drastically until 48h, reaching stability after this time of fermentation.

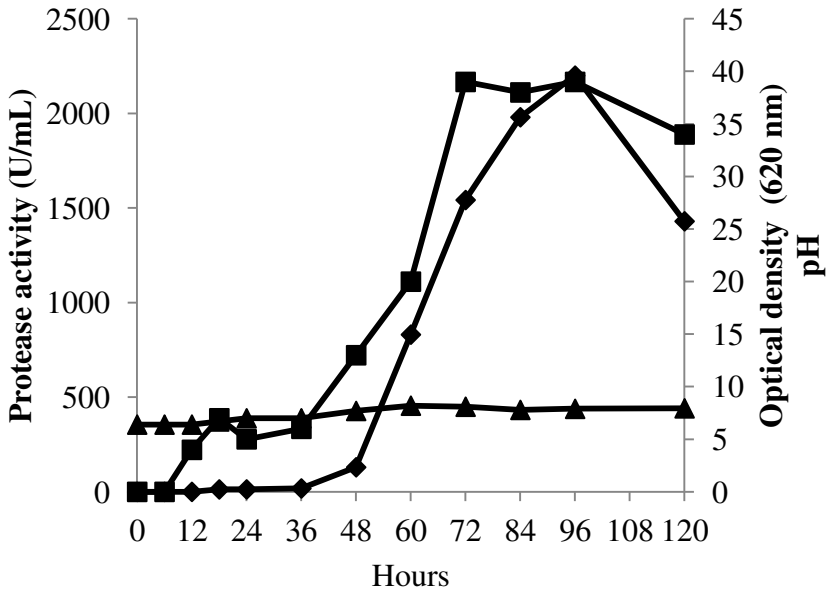


Figure III.2. Influence of the fermentation time on protease production, pH and growth of *Bacillus* sp. LBA 46 under submerged fermentation in a medium composed of 35 g/L of sugar cane molasses, 20 g/L of corn steep liquor, 3 g/L of yeast extract Prodex-Lac SD® and 20 g/L of dried whey, at 30°C and 200 rpm.

The symbols are: Protease activity (♦) Bacterial growth (■) pH (▲)

III.3.2.2. Optimization of production of proteases by Bacillus sp. LBA 46 under submerged fermentation

Table III.1 shows the results of the CCD applied for protease production by *Bacillus* sp. LBA 46 under 96h of SmF and it was observed the highest protease activity (868 U/mL) using 32g/L of sugar cane molasses, 6g/L of corn steep liquor and 2g/L of yeast extract Prodex-Lac SD® (run 2). In the run 7 lower quantity of molasses and higher concentrations of corn steep liquor and yeast extract were used resulting in the lowest protease activity (36.5 U/mL). These results indicate that using high concentration of molasses in the medium, high protease production was obtained (runs 2 and 4), but using high concentrations of corn steep liquor and yeast extract the protease production decreased considerably (run 7).

Table III.2 shows the regression coefficient of the analysis of the CCD using 90% of confidence level ($p < 0.1$). Based on this data, the variable molasses linear was statistically significant and had a positive effect in the protease production, while the variable yeast extract Prodex-Lac SD® linear was also statistically significant but had a negative effect in protease production. The variable corn steep liquor had a p value of 0.16 which was relatively closed to 0.1 and due to the fact that this substrate is rich in nutrients and is an agro-industrial waste it was also considered statistically significant. The equation 1 is the mathematical model obtained after the analysis of variance (ANOVA) and was validated based on the F-test (Table III.3). The R^2 was equal to 0.81 that is also acceptable for biological process.

Table III.1. Central composite design 2^3 coded for the study of the influence of sugar cane molasses, corn steep liquor, yeast extract Prodex-Lac SD® on protease production by *Bacillus* sp. LBA 46, under 96h of submerged fermentation, at 30°C and 200 rpm.

Runs	Variables			U/mL
	Sugar cane molasses (g/L)	Corn steep liquor (g/L)	Yeast extract Prodex-Lac SD® (°C)	
1	-1 (8)	-1 (6)	-1 (2)	248.0
2	+1 (32)	--1 (6)	-1 (2)	868.0
3	-1 (8)	+1 (24)	-1 (2)	358.0
4	+1 (32)	+1 (24)	-1 (2)	600.0
5	-1 (8)	-1 (6)	+1 (8)	246.0
6	+1 (32)	-1 (6)	+1 (8)	326.0
7	-1 (8)	+1 (24)	+1 (8)	36.5
8	+1 (32)	+1 (24)	+1 (8)	182.5
9	-1.68 (0)	0 (8)	0 (5)	48.0
10	+1.68 (40)	0 (15)	0 (5)	546.0
11	0 (20)	-1.68 (0)	0 (5)	448.0
12	0 (20)	+1.68 (30)	0 (5)	426.0
13	0 (20)	0 (15)	-1.68 (0)	430.0
14	0 (20)	0 (15)	+1.68 (10)	286.0
15	0 (20)	0 (15)	0 (5)	328.0
16	0 (20)	0 (15)	0 (5)	326.0
17	0 (20)	0 (15)	0 (5)	386.0

Table III.2. Regression coefficients of the analysis of the 2^3 CCD for protease production by *Bacillus* sp. LBA 46 under 96h of submerged fermentation at 30°C and 200 rpm.

	Regression coefficients.	Standard error	t(7)	P	-90,%	+90,%
Mean/Interc.	358.17	24.38	14.69	0.000000	314.72	401.63
(1) Molasses (L)	141.05	27.21	5.18	0.000228	92.54	189.55
(2) Corn (L)	-40.15	27.21	-1.47	0.165796	-88.66	8.34
(3) Yeast (L)	-111.75	27.21	-4.10	0.001455	-160.26	-63.25
1L by 3L	-79.50	35.54	-2.23	0.045058	-142.84	-16.15

Table III.3. Analysis of variance of the effect of sugar cane molasses, corn steep liquor, yeast extract Prodex-Lac SD® on protease production under 96h of submerged fermentation, at 30°C and 200 rpm.

Source of variation	Sum of squares	Degrees of fredom	Mean square	F - Value
Regression	514466	4	128617	12.72
Residues	121272	12	10106	
Lack of fit	118949.1	10	11894.91	
Pure error	2322.7	2	2322.7	
Total	635738.0	16		

$R^2 = 0.81$; $F_{0.90;4;12} = 2.48$

Equation 1: Protease Activivity = 358.17 + 141.05.Molasses – 40.15.Corn – 111.75.Yeast – 79.50.Molasses.Yeast

Figure III.3 shows the response surfaces of the production of protease using the three agro-industrial byproducts. It is interesting to observe that, as already indicated in the Table III.1, high values of sugar cane molasses and low values of corn steep liquor and yeast extract Prodex-Lac SD® are necessary for high protease activity. In fact, according to the surfaces the highest protease activity (>1000 U/mL) would be obtained if 40 g/L of molasses and neither corn steep liquor nor yeast extract was used. However, posterior experiments showed that small quantities of corn steep liquor and yeast extract are necessary for protease production. In this case, when the fermentation was carried out using 40 g/L of sugar cane molasses as the only substrate for fermentation, no bacterial growth and protease activity was observed after 96h of reaction at 30°C and 200 rpm. Nevertheless, when the fermentation was carried out using 40 g/L of sugar cane molasses, 6 g/L of corn steep liquor and 2 g/L yeast extract Prodex-Lac SD® as substrate it was observed protease activity of 600 U/mL. This is because nitrogenous sources are required and there is no nitrogen in molasses. Therefore, these

concentrations were defined as the best conditions for protease production until this point.

Hanlon et al., (1982) verified that complex carbon and nitrogen sources constitute better substrates for protease production than simple sugars that induce catabolic repression. In addition, alkaline protease production is heavily dependent on the availability of both carbon and nitrogen sources within the medium. Both present regulatory effects on enzyme synthesis. The nitrogen sources also functioned as inducers of enzyme production.

Molasses is considered a very useful carbon source for protease production of several *Bacillus* sp. strains. Shikha et al. (2007) studied different carbon sources and concluded that molasses was found to be the best source for the protease production by a mutant of alkalophilic *Bacillus pantotheneticus*. When glucose was used there was an appreciable loss in protease production from 48 to 72 h of incubation. Regarding the nitrogen sources, wheat bran was found to be the best source for protease production, while the combination of peptone and yeast extract was comparable. The authors also studied some oil cakes and mustard oil cake was found to be appreciably optimal over 48 h of incubation (100U/mL). On the other hand, the addition of inorganic nitrogen sources in the medium resulted in lesser enzyme production in comparison to organic sources. In another study MR-VP medium was previously selected as the best medium for *Bacillus* sp. BGS protease production. MR-VP medium was supplemented individually with five different substrates and molasses resulted in relatively higher alkaline protease production (859.25U/mL) (Moorthy and Baskar, 2013).

Corn steep liquor has also been studied for *Bacillus* sp. protease production. Reddy et al. (2008) screened some variables for *Bacillus* sp. RKY3 protease production and observed that corn step liquor had a positive effect together with corn starch and enzyme production increased in a linear fashion with the concentrations of these compounds. The optimal conditions

for protease production were l0g/L of corn starch, 30 g/L of corn steep liquor, and 2.99% (v/v) of inoculum size, resulting in the protease activity of 873 U/mL. On the other side, corn steep liquor can inhibit protease production as in the case of *Pseudomonas aeruginosa* strain K (Zalih et al., 2005).

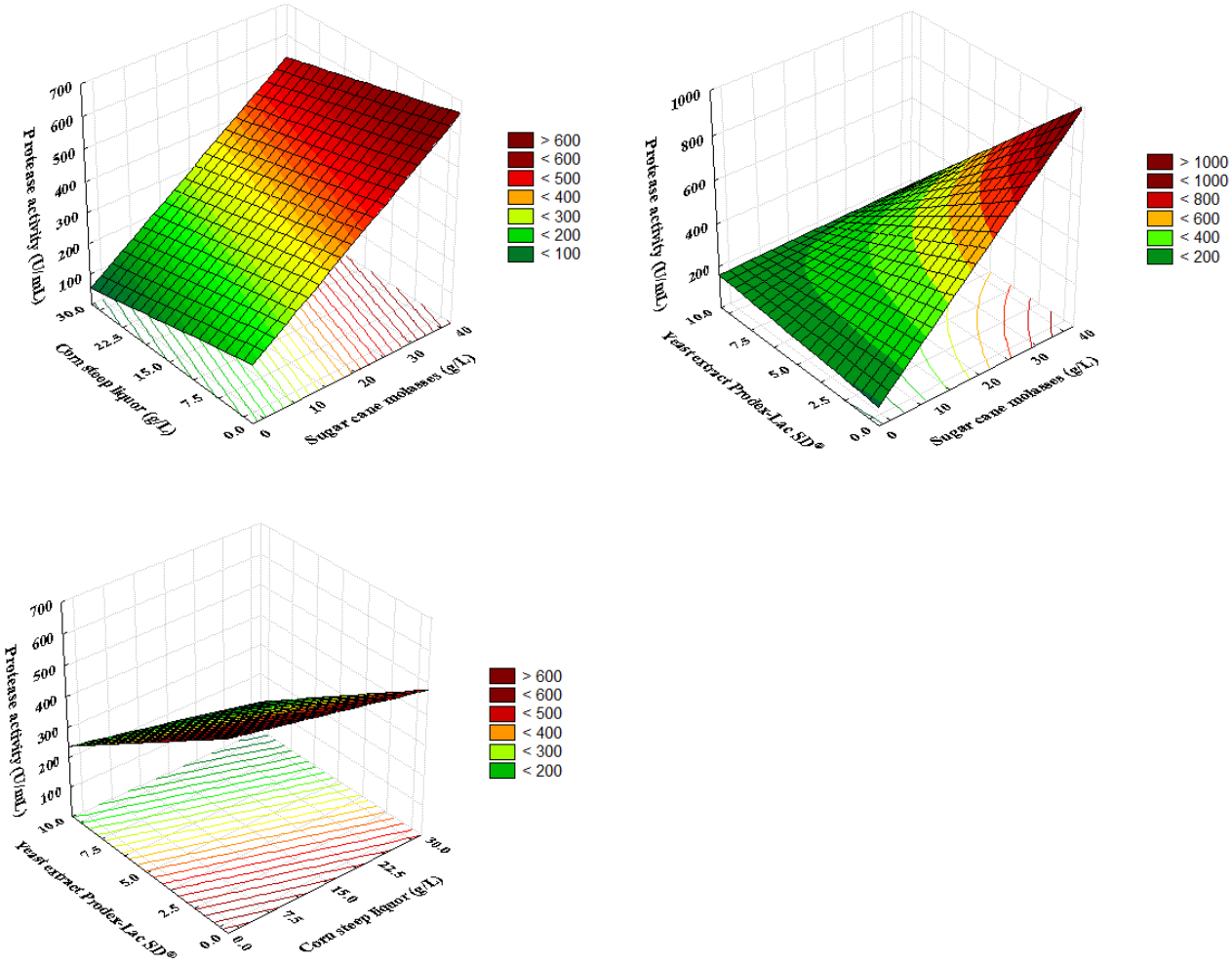


Figure III.3. Response surface of the effect of concentrations of sugar cane molasses, corn steep liquor, yeast extract Prodex-Lac SD® on the protease production by *Bacillus* sp. LBA 46 after 96h of submerged fermentation, at 30°C and 200 rpm.

After studying the concentration of molasses, corn steep liquor and yeast extract, the influence of dried whey on protease production by *Bacillus* sp. LBA 46 was evaluated. The dried whey was not considered for the CCD

due to the fact that it presents a high concentration of carbohydrates. In combination with sugar cane molasses, which also presents a high concentration, this could interfere with the statistical analysis. In the Figure III.4 it can be observed that the presence of this substrate was extremely important for improving protease production. The best protease activity (~3000 U/mL) was observed using 20 g/L of dried whey plus 40 g/L of molasses, 6 g/L of corn steep liquor and 2g/L of yeast extract Prodex-Lac SD®. When the fermentation was carried out using higher quantities of dried whey, no increase in protease production was observed, indicating that the optimal concentration of dried whey has been found. Therefore, the protease production was considerably improved comparing the initial medium no. 2 (35 g/L of sugar cane molasses, 20 g/L of corn steep liquor, 3 g/L of yeast extract Prodex Lac SD® and 20 g/L of dried whey) to the optimized medium no. 4 composed of 40 g/L of sugar cane molasses, 6 g/L of corn steep liquor, 2 g/L of yeast extract Prodex-Lac SD® and 20 g/L of dried whey. This is economically interesting once the concentration of corn steep liquor has been decreased after the optimization studies and these results are higher than the protease production prior optimization (2200 U/mL).

Dried whey can be very interesting as a component of fermentation medium taking into account it presents more than 70% of lactose and more than 10% of proteins, which can be important compounds for protease production, in addition to its low-cost, compared to other sources of carbon and nitrogen, including peptone, casein, or isolated lactose. However, there are few articles reporting the use of this interesting substrate for protease production from *Bacillus* sp. (Khan et al., 2012). Maal et al. (2009) studied the production of protease from *Bacillus cereus* and *Bacillus polymyxa* using whey as substrate obtaining proteases activity amounts of 418 U/mL and 455 U/mL, respectively.

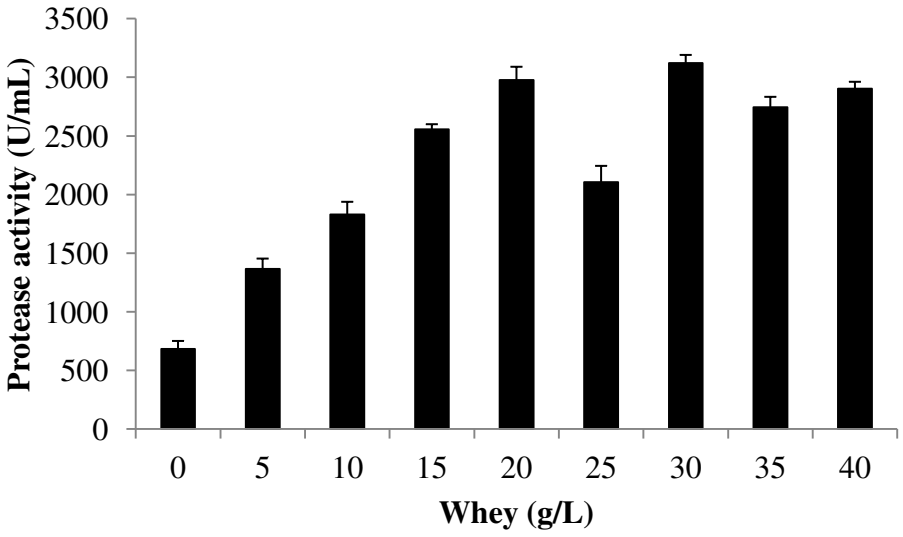


Figure III.4. Influence of concentration of dried whey on protease production by *Bacillus* sp. LBA 46 under 96h of submerged fermentation, at 30°C and 200 rpm. The medium was composed of 40 g/L of sugar cane molasses, 6g/L of corn steep liquor and 2 g/L of yeast extract Prodex-Lac SD® and different concentrations of dried whey.

Each data point represents the mean of three independent assays with the standard deviation.

III.3.3. Evaluation of the addition of crude protease from *Bacillus* sp. 46 on washing performance

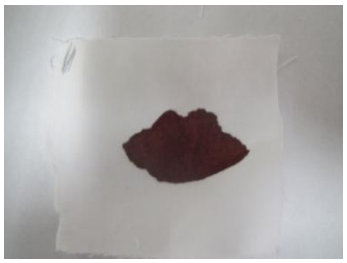
The cotton fabrics stained with blood or tomato sauce with ground beef were washed with only water, detergents (pre-heated), only crude protease extract from *Bacillus* sp. LBA 46 or with the combination of detergent and crude protease extract at 40°C for 2h. Complete blood stain elimination was observed when it was used only 1000 U of crude protease extract or in the tests using protease extract (100 U and 1000 U) in combination with the detergent Omo® (Figure III.5). It was observed that the stains of tomato sauce with ground beef from cotton fabrics could not be removed after washing with only water or crude protease extract (1000U). In addition to that, although it

was observed considerable cleaning in the fabrics stained with tomato sauce with ground beef when washed with detergent only, much better cleaning was observed when the fabrics were washed with the combination of the detergent with the crude protease extract (100 and 1000 U) (Figure III.5). It must be stated that the tomato sauce is rich in starch, pigments and fat and poor in proteins. The latter is mainly from the ground beef. Therefore, it was not observed the complete removal of the stain of tomato sauce with ground beef using the combination of detergent with the crude extract of protease from *Bacillus* sp. LBA 46 in comparison with the fabrics stained with blood that is rich in proteins where the proteins were efficiently removed. These results show the efficiency of *Bacillus* sp. LBA 46 protease in proteinaceous stain removal which is in agreement with an earlier report on laundry performance of Subtilisin proteases (Wolff et al., 1996). The protease acts synergistically with the detergent to effectively remove the stain due to the hydrolysis of a large insoluble protein fragments strongly adhered to the fabric.

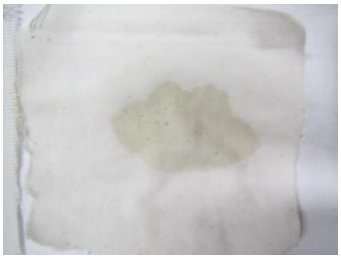
Hmidet et al. (2009) applied the crude enzymatic extract of *Bacillus licheniformis* NH1 containing proteolytic and amylolytic activity in blood, chocolate and barbecue sauce stains removal from fabrics and observed limited washing performance with detergent only. Yet, the treatment of stains with detergent supplemented with the enzymatic extract gave a better stain removal. In other work, as reported by Sinha and Khare (2013) when the protease from *Bacillus* sp. EMB9 was applied to removal of blood stain it could be observed that the enzyme treatment caused maximum increase in reflectance and whiteness index of cloth piece in comparison to the fabrics with no enzymatic treatment.

According to the work of Jellouli et al. (2011) the proteins of the blood stain were initially removed from the cotton fabric surface either by components of the detergent matrix, or by water alone. Nonetheless, a better blood stain elimination was observed when the crude protease from *Bacillus*

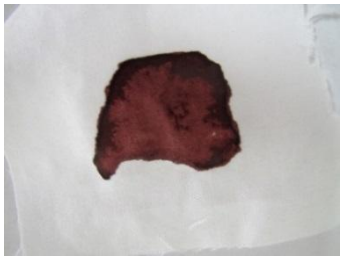
sp. EMB9 was added in only water wash solution. Moreover, the authors observed that crude enzyme gave complete blood stain elimination when supplemented with laundry detergent solution.



(1)



(1a) – Cotton fabric stained with blood and washed with tap water only



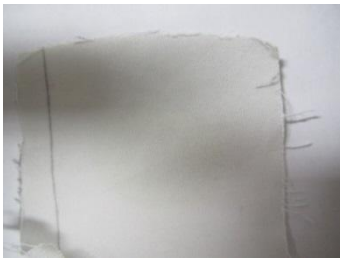
(1)

(1b) – Cotton fabric stained with blood and washed with Omo® without crude protease extract

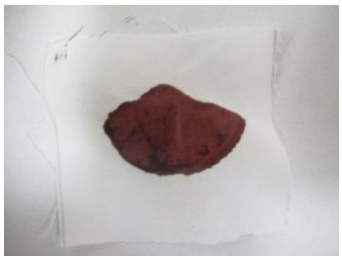
(1)

(1c) – Cotton fabric stained with blood and washed with Omo® plus crude protease extract (100U)

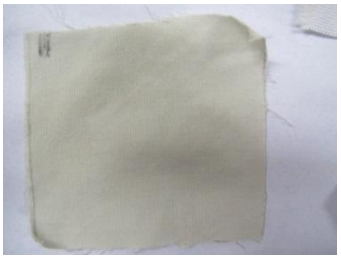
(1)



(1d) – Cotton fabric stained with blood and washed with Omo® plus crude protease extract (1000U)



(1)

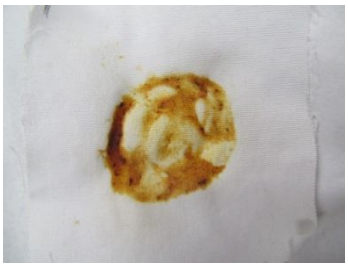


(1e) – Cotton fabric stained with blood and washed with crude protease extract (1000U) without Omo®

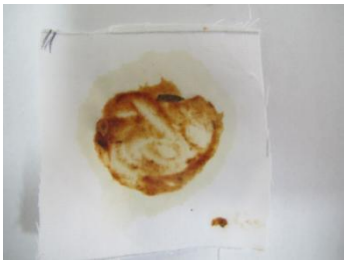
Figure III.5. Effect of the crude protease extract from *Bacillus* sp. LBA 46 on remotion of stains from fabrics in the presence or absence of the commercial detergent Omo® (after denaturation of the enzymes present in the detergent).

(1) Cloth stained with blood before washing test; (1a – 1e) Cloth stained with blood after the washing tests.

(2)

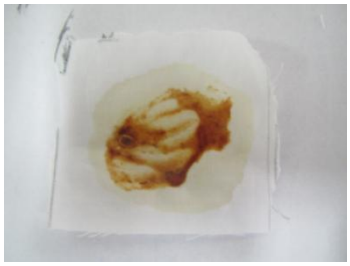


(2a) – Cotton fabric stained with tomato sauce with ground beef and washed with tap water only



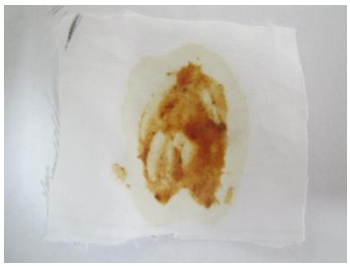
(2)

(2b) – Cotton fabric stained with tomato sauce with ground beef and washed with Omo® without crude protease extract



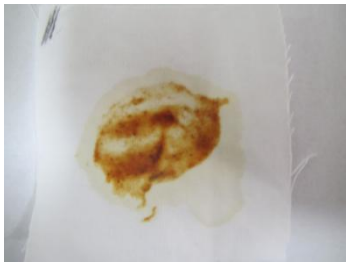
(2)

(2c) – Cotton fabric stained with tomato sauce with ground beef and washed with Omo® plus crude protease extract (100U)

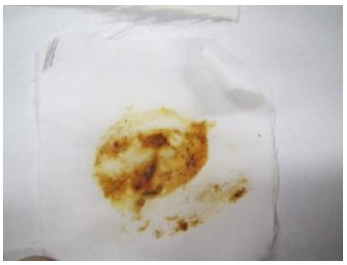


(2)

(2d) – Cotton fabric stained with tomato sauce with ground beef and washed with Omo® plus crude protease extract (1000U)



(2)



(2e) – Cotton fabric stained with tomato sauce with ground beef and washed with crude protease extract (1000U) without Omo®

Figure III.6. Effect of the crude protease extract from *Bacillus* sp. LBA 46 on remotion of stains from fabrics in the presence or absence of the commercial detergent Omo® (after denaturation of the enzymes present in the detergent).

(2) Cloth stained with tomato sauce with ground beef before washing test; (2a - 2e) Cloth stained with tomato sauce with ground beef after washing tests.

4. Conclusions

The time course of the production of proteases from *Bacillus* sp. LBA 46 has been studied using medium composed of 35 g/L of sugar cane molasses, 20 g/L of corn steep liquor, 3 g/L of yeast extract Prodex-Lac SD®, 20 g/L of dried whey and the best fermentation time, at 30°C, for protease production under SmF (2200 U/mL) and bacterial growth was 96 h.

The production of proteases by Bacillu sp. LBA 46 under SmF was optimized using RSM and the best conditions for the production of proteases were obtained using medium no. 4 containing 40 g/L of sugar cane molasses, 6 g/L of corn steep liquor, 2 g/L of yeast extract Prodex-Lac SD® and 20 g/L of dried whey protein, resulting in approximately 3000 U/mL, which is 36% higher than the results before the optimization.

The crude protease extract from *Bacillus* sp. LBA 46 obtained from the cultivation in the optimized medium under SmF was applied in the removal of stains from fabrics. When fabrics with blood stains were washed with 1000 U of enzyme in water or the combination enzyme and laundry detergent, the stains were completely removed what did not occur with the fabrics washed with detergent or only water. In addition, when the fabrics stained with tomato sauce with ground beef were washed, better results were observed using the combination of 100 U or 1000 U of crude protease extract with the detergent.

5. Acknowledgements

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IV. CAPITULO 4 - USE OF RESPONSE SURFACE METHODOLOGY FOR CHARACTERIZATION OF CRUDE AND PARTIALLY PURIFIED PROTEASE FROM *Bacillus* sp. AND OPTIMIZATION OF LYSIS OF *Xanthomonas campestris* FOR XANTHAN GUM CLARIFICATION: A COMPARATIVE STUDY

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RESUMO

A protease da linhagem de *Bacillus* sp. LBA 46 foi parcialmente purificada utilizando sulfato de amônio seguido de cromatografia de filtração em gel Sephadex G-100. As preparações de protease bruta e parcialmente purificada foram caracterizadas utilizando metodologia de superfície de resposta (MSR). Ambas as preparações apresentaram atividade ótima em pH 7,5 e 55°C e foram consideravelmente estáveis na faixa de pH 5,7-9,3 após 1 h de incubação a 30-36°C, mantendo cerca de 95% de atividade residual. As preparações bruta e parcialmente purificada mantiveram acima de 78.9% de atividade residual após 1h de incubação em pH 7,5 e 50°C. A aplicação de ambas as preparações de protease na lise de células de *Xanthomonas campestris* presentes no meio fermentado contendo goma xantana foi otimizada utilizando MSR. Observou-se que as condições ótimas foram 42 U de protease/mL de suspensão de células de *X. campestris* e temperaturas superiores a 60°C resultando no aumento de 20% e 30% na transmitância a 620 nm (Trans $_{620\ nm}$) do meio fermentado de goma xantana, quando utilizado extrato bruto e protease parcialmente purificada, respectivamente. Foi observado, posteriormente, aumento de quase 40% na Trans $_{620\ nm}$ após 2h de reação utilizando 42U de protease bruta/mL de suspensão de células de *X. campestris.*

Palavras-chave: protease, *Bacillus* sp. clarificação da goma xantana, *Xanthomonas campestris*, metodologia de superfície de resposta.

ABSTRACT

The protease from the strain *Bacillus* sp. LBA 46 were partially purified using ammonium sulfate and Sephadex G-100 gel chromatography and crude and partially purified protease were characterized using response surface methodology (RSM). Both protease preparations presented optimal activity at 55°C and pH 7.5 and were considerably stable in the range of pH 5.7 – 9.3 after 1 hour incubation at 30 – 36°C, retaining above 95% of residual activity. The crude and partially purified protease retained above 78.9% of residual activity after 1h incubation at pH 7.5 and 50°C. Afterwards, the application of both protease preparations in *Xanthomonas campestris* lysis in order to clarify the fermented broth containing xanthan gum was optimized using RSM and the best conditions for crude and partially purified protease preparations were observed using the amount of proteases equivalent to 42 U/mL of cell suspension of *X. campestris* and temperatures higher than 60°C resulting in an increase of more than 20% and 30% of transmittance at 620 nm (Trans $_{620\ nm}$) of the fermented broth containing xanthan gum, respectively. Furthermore, an increase of almost 40% of Trans $_{620\ nm}$ was observed after 2h of reaction using 42 U of crude protease/ mL of cell suspension of *X campestris*.

Keywords: protease, *Bacillus* sp. xanthan gum clarification, *Xanthomonas campestris*, response surface methodology

IV.1. Introduction

Proteases are highly important enzymes with remarkable properties that guarantee highlighted uses in several important industrial fields. These properties include thermostability and stability regarding a wide range of pH (Lagzian and Asoodeh, 2012). Depending on the properties of the proteases they are studied for different applications. For instance, acidic proteases, such as the protease from *Aspergillus oryzae*, are preferable for using in food industry, including the production of antioxidant protein hydrolysates (Castro and Sato, 2014). On the other hand, proteases from strains from *Bacillus* sp. tend to be alkaline with high activity and good stability in alkaline conditions and, as a consequence, they are explored for their use in detergent industry for better removal of proteins stains (Hmidet et al., 2009).

Proteases from *Bacillus* sp. strains showing different biochemical properties have been described in the literature. The protease from *Bacillus megaterium* was purified by ammonium sulfate precipitation, ion exchange chromatography on DEAE-Cellulose and gel filtration chromatography on Sephadex G-200 and maximum activities for two purified fractions were attained at 50°C, pH 7.5 and both fractions were more stable over pH 7.0–8.5 for 30 minutes (Asker et al., 2013). On the other side, the protease from *Bacillus koreensis* (BK-P21A) has been partially purified by ammonium sulfate precipitation and again by Superdex 200 10/300 GL and Superdex 75 10/300 GL chromatography. As result this enzyme found to be most active at pH 9.0 and 60°C (Anbu, 2013).

Xanthan is a polysaccharide produced by *Xanthomonas campestris* that due to its unique structure and useful rheological properties has been successfully commercialized. However, some aspects regarding xanthan gum purification must be studied, considering some industrial areas including food and pharmaceutical industry require purified xanthan gum with low levels of turbidity. This can be a difficult step taking into consideration that

conventional techniques are not effective or can degrade the gum, including centrifugation, filtration or heating (Margatis, 1985). The compounds in bacterial cell wall mureins (peptido-glycans) can be lysed by some proteases produced by different sources, including *Trichoderma koningii* (Triveni and Shamala, 1999) and some commercial proteases from *Bacillus* species as reported in some patents (Colegrove, 1977).

The purification and characterization of enzymes is crucial for the application of proteases in order to guarantee the best conditions for their industrial use. In this context, response surface methodology (RSM) is applied to identify the relationships between the variables and the response, generally resulting in the optimization of the process and simultaneously limiting the number of experiments. This is a very interesting and efficient technique for the characterization and application of proteases. Therefore, in this work the protease from *Bacillus* sp. LBA 46, produced in a medium composed of agro-industrial byproducts, was partially purified and the biochemical characterization of the crude and partially purified enzyme was carried out using RSM. In addition, the application of both crude and partially purified protease in *X. campestris* cells lysis was carried out using RSM.

IV.2. Materials and methods

IV.2.1. Microorganisms

The strain of *Bacillus* sp. LBA 46, from the culture collection of Food Biochemistry Laboratory, College of Food Engineering – University of Campinas, and the strain *X. campestris* ATCC 13591 were maintained in nutrient agar, at 4°C, with sterile vaseline addition and transfers in every 2 months.

IV.2.2. Production of proteases by *Bacillus* sp. LBA 46

Aliquots of 2 mL of cell suspension of *Bacillus* sp. LBA 46 in saline solution (0.85%, m/v) (optical density at 620 nm between 0.49 and 0.51) were inoculated into 250 mL Erlenmeyer flasks containing 48 mL of the culture medium no. 4 composed of 40 g/L of sugar cane molasses (Fios de ouro®, BR), 6 g/L of corn steep liquor (Corn Products do Brasil, BR), 2 g/L of yeast extract Prodex-Lac SD® (Produtos Especiais para Alimentos S/A, BR), 20 g/L of dried whey (Alibra, BR) and pH adjusted to 7.0. The flasks were incubated at 200 rpm, 30°C for 96h and the fermented medium was centrifuged at 9.600 × *g* for 15 min at 5°C. The supernatant was used as the crude protease extract.

IV.2.3. Determination of protease activity

The protease activity was determined as described by Charney & Tomarelli (1947) with modifications. The reaction mixture containing 0.5 mL of 0.5% (m/v) azocasein (Sigma, US) in 0.05 M phosphate buffer (pH 7.0) and 0.5 mL of the enzyme solution was incubated at 50°C for 40 min. The reaction was stopped by adding 0.5 mL of 10.0% (m/v) trichloroacetic acid and the test tubes were centrifuged at 17000 x *g* for 15 min at 25°C. A 1.0 mL aliquot of the supernatant was neutralized with 1.0 mL 5 M KOH. One unit of protease activity was defined as the amount of enzyme which caused an increase of 0.01 of absorbance at 428 nm per minute. The enzyme activity were expressed as U/mL of crude enzymatic extract.

IV.2.4. Protein estimation

Protein content of the enzyme during the purification steps was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

IV.2.5. Purification of proteases

Ammonium sulfate was added to the crude protease extract to obtain a 80% saturation (m/v) and allowed to stand overnight at 4°C. The precipitates were collected by centrifugation at 9600 x *g* for 15 min, dissolved in 0.05M phosphate buffer (pH 7.0) and dialyzed against distilled water (4°C) for 48 h. The dialyzed protein was loaded onto Sephadex G-100 column (1 x 100 cm) and eluted with 50 mM phosphate buffer (pH 7.0) at a flow rate of 0.3 mL/min. Fractions were collected and analyzed for protease activity and used as partially purified protease for further studies.

IV.2.6. Biochemical characterization

IV.2.6.1. Effect of pH and temperature on protease activity

The effect of pH and temperature on protease activity was carried using a 2^2 central composite design (2^2-CCD) with 2 axial points (+\–) at a distance of α=1.41 with three replicates at the center point, resulting in a total of 11 runs for each of the two protease preparations (crude and partially purified), using a 90% of confidence level. The independent variables were pH and temperature and the dependent variable was relative protease activity, considering the highest protease activity as 100%. Both protease preparations were incubated in pH range of 5.0 – 10.0 using acetate, phosphate, Tris–HCl, and carbonate-bicarbonate buffers (0.1M) for 40 minutes in different temperatures (30 – 80°C) (Table IV.2). The statistical analyses were carried out using software Statistica 8.0®.

IV.2.6.2. Effect of pH and temperature on protease stability

Both crude and partially purified proteases were pre-incubated in different buffer solutions (0.2M, acetate, phosphate, Tris–HCl, and carbonate-bicarbonate buffers) with different pH values and different temperatures (Table IV.5) for 1 hour and afterwards the protease activity was measured as described in the item IV.2.3. The effect of pH and temperature on protease stability was carried using a 2^2 central composite design (2^2-CCD) with 2 axial points (+\–) at a distance of α=1.41, with three replicates at the center point, resulting in a total of 11 runs for each of the two protease preparations (crude and partially purified). The independent variables were pH and temperature of the solution where the proteases were pre-incubated and the dependent variable was the relative protease activity, considering the highest activity as 100%. The statistical analyses were carried out using software Statistica 8.0®.

IV.2.7. Production of xanthan gum

Xanthan gum was produced as described by Shastry e Prasad (2002). *X. campestris* ATCC 13591 was grown at 200 rpm, 28°C in Erlenmeyer flasks (250 mL) containing 50 mL medium composed of 45 g/L of sucrose, 4 g/L of peptone, 5 g/L of Na_2HPO_4, 3 g/L KH_2PO_4, 1 g/L $MgSO_4 \cdot 7H_2O$ and pH 7.0. After 24h of incubation one aliquot of 5 mL of inoculum culture was added to 95 mL of fresh medium with the same composition in conical flasks (500 mL) and incubated on a 200 rpm rotary shaker for 72 h, at 28°C. The viscous *X. campestris* broth obtained was heated at 90°C for 30 min and used for the study for clarification of xanthan gum with crude and partially purified protease extracts.

IV.2.8. Optimization of the lysis of *X. campestris* cells for xanthan gum clarification

The fermented broth of *X. campestris* ATCC 13591 was used as substrate for crude protease extracts. Erlenmeyer flasks of 125 mL containing 10 mL of fermented broth diluted in water (1:1) were incubated at different temperatures, with stirring and different amounts of crude and partially purified protease preparations were added to start the lysis (Table IV.8). Aliquots of 0.1 mL were withdrawn after 30 minutes and the transmitance (T) at 620 nm was determined. Control assays were also carried out adding distilled water instead of the protease extract. The effect of temperature and protease amount on lysis of *X. campestris* was investigated using a 2^2 central composite design (2^2-CCD) with 2 axial points (+\–) at a distance of α=1.41, with three replicates at the center point, resulting in a total of 11 runs for each of the two protease preparations (crude and partially purified). The independent variables were temperature and protease amount/mL of cell suspension of *X. campestris* (U/mL) and the dependent variable was the difference of the value of transmitance before and after 30 min of reaction expressed in percentage. The statistical analyses were carried out using software Statistica 8.0®. After optimization the time course of *X. campestris* lysis using crude protease preparation in optimal conditions was investigated analyzing the transmittance at 620 nm ($Trans_{620\ nm}$) of the fermented broth in different reaction periods.

IV.3. Results and discussions

IV.3.1. Purification of proteases

The protease from *Bacillus* sp. LBA 46 produced in a medium no. 4 composed of agro-industrial byproducts (sugar cane molasses, corn steep liquor, yeast extract Prodex-Lac SD® and dried whey) was purified 3.69 fold using ammonium sulfate at 80% of saturation, dialysis and Sephadex G-100

chromatography (Table IV.1). The fraction 13 (6 mL) containing the highest protease activity (~180 U/mL) was used for characterization and application of partially purified protease (Figure IV.1).

Although the protease activity decreased considerably comparing the crude and partially purified (Table IV.1), the protein concentration decreased considerably as well, indicating the removal of several proteins that could interfere on protease activity.

The literature reports several protocols for *Bacillus* sp. protease purification including different techniques for proteins precipitation and chromatographic resins. Bhunia et al. (2013) purified the protease from *Bacillus licheniformis* NCIM-2042 using precipitation with ammonium sulfate followed by gel filtration with Sephadex G-100 column and cationic exchange chromatography with CM-Cellulose resulting in a purified protease with molecular mass of 70 kDa.

As reported by Lagzian and Asoodeh (2012) the protease from *Bacillus* sp. MLA64 was purified by precipitation with polyethylenoglicol, Sephadex G-100 column, Q-Sepharose and CM-Cellulose. The protease activity of purified enzyme was not enhanced in the presence of $CaCl_2$. The enzyme was stable in contact with non-ionic and anionic surfactants. Additionally, the enzyme was relatively stable with respect to oxidizing agents.

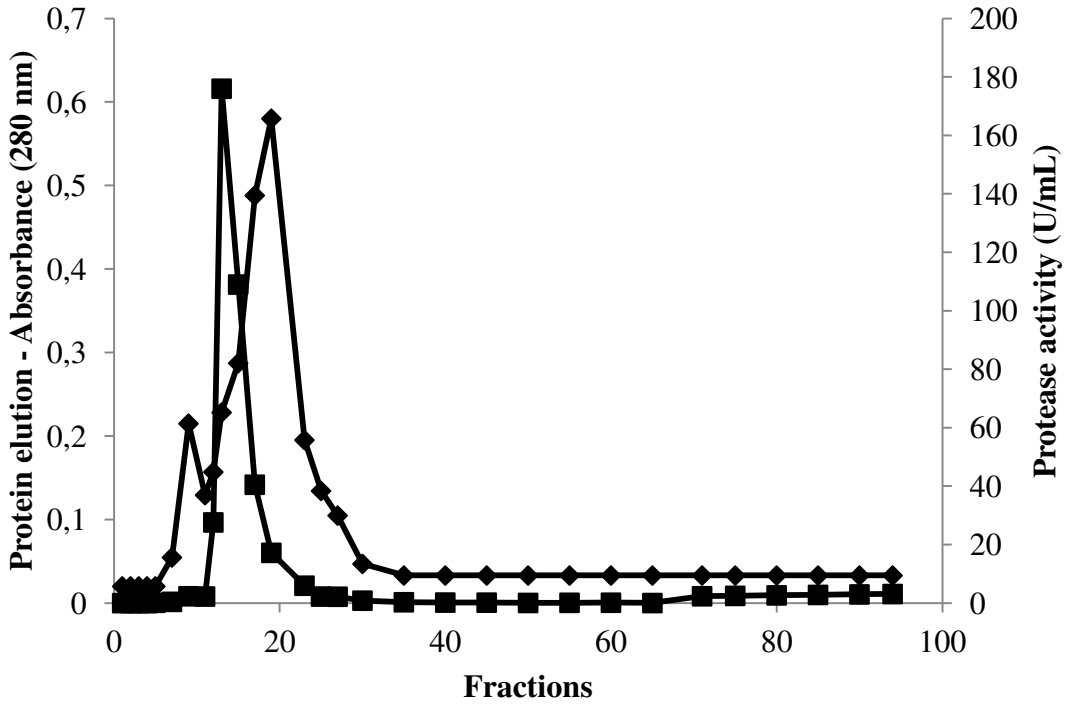

Figure IV.1. Elution profiles of proteins and protease activity in Sephadex G-100 gel chromatography.

The symbols are: protease activity (■) and protein elution ($A_{280\ nm}$) (♦)

Table IV.1. Partial purification of protease from *Bacillus* sp. LBA 46

Purification Step	Total activity (U)	Proteins (mg/mL)	Specific Activity (U/mg)	Purification Fold	Yield (%)
Crude Protease Extract	635000	8.30	76.50	1	100
Ammonium Sulfate (60 %)	199500	8.69	229.48	2.99	31.7
Sephadex G100	1074	0.62	283.01	3.69	10.7*

* - Calculation based on the volume (5 mL) that was applied in the column (Sephadex – G100), correspondent to 9975 U that was defined as 100%.

IV.3.2. Biochemical characterization

IV.3.2.1. Effect of pH and temperature on protease activity

The crude and partially purified protease showed higher activity at pH 7.5 and 55°C (center points). However, the lowest relative activity were obtained at pH 5.7 and 37°C (run 1) resulting in 8.7 % and 11.1 % for crude and partially purified protease respectively. At pH 10.0 and 55°C (run 6) the crude and partially purified protease showed 83.3% and 93.6% of activity, respectively (Table IV.2).

The regression coefficients are presented in the Table IV.3 and the quadratic variables pH and temperature were statistically significant with a 90% confidence level ($p < 0.1$) and had negative effects for crude and partially purified protease preparations. Yet, in the case of crude protease the interaction of the two variables pH and temperature presented p value of 0.13, and considering it is close to 0.1 and presented negative effect, it was also considered in the mathematical model (equation 1). The models (equation 1 and 2) can be obtained according to the analysis of variance analysis (ANOVA) and F test (Table IV.4). In addition, the R^2 values were equal to 0.82 and 0.77.

Table IV.2. Central composite design 2^2 coded for the study of the influence of pH and temperature on activity of crude and partially purified protease from *Bacillus* sp. LBA 46.

Runs	Variables		Protease activity - CP (%)	Protease activity – PP (%)
	pH	Temperature (°C)		
1	-1 (5.7)	-1 (37)	8.7	11.1
2	1 (9.3)	-1 (37)	32.4	33.8
3	-1 (5.7)	1 (73)	71.9	59.3
4	1 (9.3)	1 (73)	32.4	33.0
5	-1.41 (5)	0 (55)	30.7	33.6
6	1.41 (10)	0 (55)	83.3	93.6
7	0 (7.5)	-1.41 (30)	10.5	13.1
8	0 (7.5)	1.41 (80)	13.1	14.3
9	0 (7.5)	0 (55)	99.1	100.0
10	0 (7.5)	0 (55)	100.0	96.8
11	0 (7.5)	0 (55)	95.6	94.4

CP - Crude protease extract; PP - Partially purified protease

Table IV.3. Regression coefficients of the analysis of the 2^2 CCD for the effect of pH and temperature on activity of protease from *Bacillus* sp. LBA 46.

	Regression coefficients.	Standard error	t(7)	P	-90%	+90%
Mean/Interc.[a]	98.22	10.84	9.05	0.000041	77.67	118.76
(1) pH (Q)[a]	-20.14	7.93	-2.53	0.03	-35.18	-5.11
(2) T°C (Q)[a]	-42.88	7.93	-5.40	0.001	-57.91	-27.84
1L by 2L[a]	-15.80	9.39	-1.68	0.13	-33.59	1.99
Mean/Interc.[b]	97.08	11.20	8.66	0.00002	76.24	117.91
(1) pH (Q)[b]	-17.84	8.20	-2.17	0.06	-33.09	-2.59
(2) T°C (Q)[b]	-42.94	8.20	-5.23	0.0007	-58.19	-27.69

a- Statistical analysis for crude protease; b- Statistical analysis for partially purified protease

Table IV.4. Analysis of variance of the effect of pH and temperature on stability of protease from *Bacillus* sp. LBA 46.

Source of variation	Sum of squares	Degrees of fredom	Mean square	F – Value
Regression[a]	11659.13	3	3886.37	11
Residues[a]	2469.35	7	352.76	
Lack of fit[a]	2458.54	5	491.70	
Pure error[a]	10.81	2	5.40	
Total[a]	14128.48	10		
Regression[b]	10506.26	2	5253.12	14
Residues[b]	3013.50	8	376.68	
Lack of fit[b]	2997.71	6	499.61	
Pure error[b]	15.79	2	7.89	
Total[b]	13519.76	10		

a- Statistical analysis for crude protease; $R^2 = 0.82$; $F_{0.1;3;7} = 3.26$

b- Statistical analysis for partially purified protease; $R^2 = 0.77$; $F_{0.1;2;8} = 3.11$

Equation 1: $\text{Relative Activity}^1 = 98.22 - 20.14.pH^2 - 42.88.T^2 - 15.80.pH.T$

Equation 2: $\text{Relative Activity} = 97.08 - 17.84.pH^2 - 42.94.T^2$

The response surfaces of the effect of pH and temperature on activity of both crude and partially purified proteases were obtained after the statistical analysis (Figure IV.2a and IV.2b, respectively). It can be observed that the optimal conditions for both enzymatic preparations were at 55°C and pH 7.5. According to the surfaces, considerable protease activity amounts can be obtained in pH 5.0 and 10.0 at 55°C. However, when the temperature is under 40°C and above 70°C the protease activity of the preparations were low.

Using RSM an efficient analysis of the optimal conditions for protease activity of the two preparations could be obtained and for the best of our knowledge there are few articles using RSM for the optimal biochemical characterization of proteases from *Bacillus* sp. that is an industrially important enzyme producer.

These results are very interesting taking into account that the protease from *Bacillus* sp. LBA 46 presented better activity in lower pH values than other proteases described in literature. In the work of Jain et al. (2012) the protease from *Bacillus* sp. SM2014 was completely inactive in pH 6.0. Moreover the enzyme presented optimal activity at 60°C. Lagzian and Asoodeh (2012) reported that the purified protease from *Bacillus* sp. MLA64 showed optimal activity at 95°C and pH 9.5 and low activity in the pH range from 5.0 to 7.0 (less than 60% of relative activity). The protease from *Bacillus halodurans* CAS6 showed optimal activity at 50°C and pH 9.0. In pH values lower than 7.0 the activity was lower than 60% (Annamalai et al., 2013).

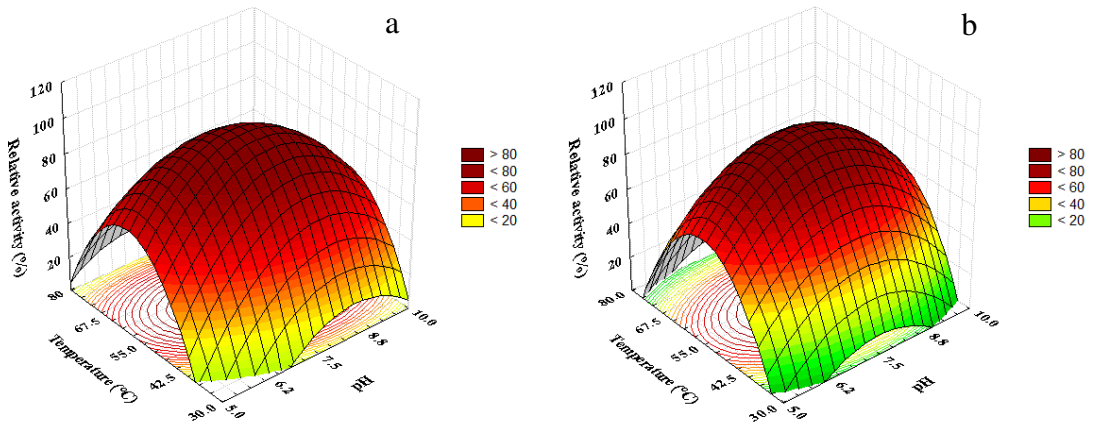

Figure IV.2. Response surfaces of the effect of pH and temperature on activity of protease from *Bacillus* sp. LBA 46: a- crude protease extract; b- partially purified protease

IV.3.2.2. Effect of pH and temperature on protease stability

Table IV.5 shows the relative enzymatic activity of crude and partially purified proteases, after carrying out the CCD. The two protease preparations presented similar stability profile when incubated for 1 hour at different

temperatures and in some buffers with different pH values. Both preparations were more stable at lower temperatures (runs 1, 2 and 7).

The statistically significant variables with 90% of confidence level ($p < 0.1$) were pH quadratic and linear and quadratic temperature, with all presenting negative effects for both enzyme preparations (Table IV.6). After the ANOVA and F test (Table IV.7), the two equations for both crude and partially purified enzyme preparations were obtained (Equations 3 and 4). The R^2 values were equal to 0.90 and 0.93, respectively.

Table IV.5. Central composite design 2^2 coded for the study of the influence of pH and temperature on the stability of crude and partially purified protease from *Bacillus* sp. LBA 46.

Runs	Variables		Relative activity - CP (%)	Relative activity - PP (%)
	pH	Temperature (°C)		
1	-1 (5.7)	-1 (36)	95.43	95.27
2	1 (9.3)	-1 (36)	100	98.44
3	-1 (5.7)	1 (64)	7.14	0
4	1 (9.3)	1 (64)	5.46	0
5	-1.41 (5)	0 (55)	1.39	1.82
6	1.41 (10)	0 (55)	38.23	41.59
7	0 (7.5)	-1.41 (30)	95.72	100
8	0 (7.5)	1.41 (70)	0	0
9	0 (7.5)	0 (50)	81.52	89.87
10	0 (7.5)	0 (50)	81.13	89.80
11	0 (7.5)	0 (50)	78.94	86.15

CP- Crude protease extract; PP- Partially purified protease

Table IV.6. Regression coefficients of the analysis of the 2^2 CCD for effect of pH and temperature on the stability of protease from *Bacillus* sp. LBA 46.

	Regression coefficients.	Standard error	t(7)	P	-90%	+90%
Mean/Interc.[a]	80.45	8.85	9.08	0.00004	63.67	97.23
(1) pH (Q)[a]	-25.86	6.48	-3.99	0.005	-38.14	-13.58
(2) T°C (L)[a]	-39.84	5.43	-7.33	0.0001	-50.13	-29.55
(2) T°C (Q)[a]	-11.75	6.48	-1.81	0.11	-24.03	0.52
Mean/Interc.[b]	88.55	8.36	10.58	0.00001	68.76	108.34
(1) pH (Q)[b]	-30.38	6.12	-4.96	0.001	-44.86	-15.90
(2) T°C (L)[b]	-41.96	5.13	-8.17	0.007	-54.09	-29.82
(2) T°C (Q)[b]	-16.15	6.12	-2.63	0.03	-30.63	-1.67

a- Statistical analysis for crude protease; b- Statistical analysis for partially purified protease

Table IV.7. Analysis of variance of the results of effect of pH and temperature on stability of protease from *Bacillus* sp. LBA 46

Source of variation	Sum of squares	Degrees of fredom	Mean square	F – Value
Regression[a]	16518	3	5506.10	23.39
Residues[a]	1647	7	235.30	
Lack of fit[a]	1643.29	5	547.76	
Pure error[a]	3.87	2	1.28	
Total[a]	18165.46	10		
Regression[b]	19543	3	6514.34	31.00
Residues[b]	1471	7	210.07	
Lack of fit[b]	1461.46	5	487.15	
Pure error[b]	9.06	2	3.01	
Total[b]	21013.54	10		

a- Statistical analysis for crude protease; $R^2 = 0.90$; $F_{0.1;3;7} = 3.07$
b- Statistical analysis for partially purified protease; $R^2 = 0.93$; $F_{0.1;3;7} = 3.07$

Equation 3: Relative Activity = $80.45 – 25.86.pH^2 – 39.84.T - 11.75.T^2$

Equation 4: Relative Activity = $88.55 – 30.38.pH^2 - 41.96.T – 16.15.T^2$

The crude and partially purified protease showed stability after 1 hour incubation in pH range 5.7-9.3 and 30-36° C retaining above 95.12% of residual activity. The crude and partially purified protease retained respectively 78.12% - 81.52% and 86.15 – 89.87% of residual activity after 1 hour incubation at pH 7.5 and 50° C but were inactivated after 1 hour incubation at pH 7.5 and 70° C (Table IV.5, Figure IV.3)

Jain et al. (2012) reported in a univariable study that the protease from *Bacillus* sp. SM2014 were not stable at pH values equal or lower than 8.0 when incubated for 30 min. Furthermore, in the work of Lagzian and Asoodeh (2012) the purified protease from *Bacillus* sp. MLA64 presented 70% of relative activity after incubation at pH 5.0.

Haddar et al. (2010) purified the protease from *Bacillus mojavensis* A21 and observed that it was stable from pH 6.0 to 11 but presented 20 and 70% of activity when incubated at pH 4.0 and 5.0 respectively, for 1 hour. The

enzyme was also stable at 50°C for 1h, however when incubated at 55°C the relative activity decreased to less than 60% of relative activity. The purified protease from *Bacillus* sp. B001 presented good stability in a wide range of pH, but less than 40% of activity when incubated at 60°C for 1 hour (Deng et al., 2010).

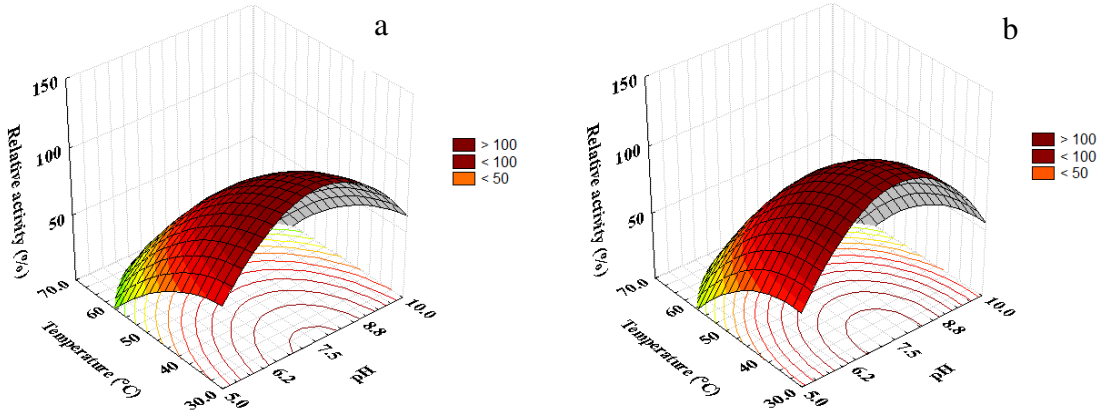

Figure IV.3. Response surfaces of the effect of pH and temperature on stability of protease from *Bacillus* sp. LBA 46, after incubation for 1 hour: a- crude protease extract; b- partially purified protease

IV.3.3. Optimization of the lysis of *X. campestris* cells for xanthan gum clarification

The *X. campestris* cells lysis is a very relevant application of proteases, taking into account it is part of the purification of xanthan gum that is required in some fields, including pharmaceutical and food industries. In addition to that, RSM can lead to the optimization of the *X. campestris* cells lysis, which is economically relevant from an industrial point of view. Table IV.8 shows the results of the CCD applied to evaluate the influence of protease amount and temperature on lysis of the cells after 30 minutes of reaction. The highest increase of Trans $_{620\ nm}$ of the fermented broth containing xanthan gum was

found using 47.2U of crude protease/mL of cell suspension, at 64°C (run 4) and 25.5 U of partially purified protease/ mL of cell suspension, at 70°C (run 8). For both enzyme preparations the worst result was in the run 7 corresponding to 31.5 U of protease/mL of cell suspension, at 30°C. It is interesting to note that when the highest temperature was utilized (70°C), the best results of *X. campestris* lysis (27% increase in Trans $_{620\ nm}$) was obtained using partially purified proteases.

Table IV.9 shows the regression coefficients of CCD and it was observed that the statistically significant variables for crude protease using 85% of confidence interval (0.15) were both linear and quadratic protease amount and temperature. While for the partially purified, the statistically significant variables with 90% of confidence level ($p < 0.1$) were protease amount quadratic and linear and quadratic temperature. The ANOVA and F-test confirmed the validation of the models (equations 5 and 6) and the R^2 were equal to 0.79 and 0.92 for crude and partially purified proteases, respectively (Table IV.10).

Table IV.8. Central composite design 2^2 coded for the study of the influence of temperature and amount of crude and partially purified protease (*Bacillus* sp. LBA 46) on *X. campestris* lysis, after 30 minutes of reaction.

Runs	Variables		Difference in Trans $_{620\ nm}$ (Crude protease) (%)[c]	Difference in Trans $_{620\ nm}$ (Partially purified protease) (%)[c]
	Enzyme (U/mL)	Temperature (°C)		
1	-1 (15.8[a];13.8[b])	-1 (36)	5.7	6.6
2	1 (47.2[a];37.2[b])	-1 (36)	16.3	9.6
3	-1 (15.8[a];13.8[b])	1 (64)	9.9	22.6
4	1 (47.2[a];37.2[b])	1 (64)	24.8	24.1
5	-1.41 (9.3[a];9.0[b])	0 (50)	5.9	12.2
6	1.41 (53.7[a];42.0[b])	0 (50)	12.9	25.4
7	0 (31.5[a];25.5[b])	-1.41 (30)	4.6	3.9
8	0 (31.5;25.5[b])	1.41 (70)	14.9	27
9	0 (31.5;25.5[b])	0 (50)	17.5	20.9
10	0 (31.5;25.5[b])	0 (50)	18.0	20.1
11	0 (31.5;25.5[b])	0 (50)	16.2	22.5

a - Amount of crude protease preparation; b - Amount of partially purified protease preparation; c. Difference of Trans $_{620\ nm}$ of tests after and before 30 min of reaction.

Table IV.9. Regression coefficients of the analysis of the 2^2 CCD for the effect of the amount of protease from *Bacillus* sp. LBA 46 and temperature on the lysis of *X. campestris* cells

	Regression coefficients.	Standard error	t(7)	P	-90%	+90%
Mean/Interc.[a]	17.21	2.10	8.16	0.0001	13.73	20.69
(1) U (Q)[a]	4.43	1.29	3.43	0.01	2.30	6.56
(1) U (Q)[a]	-2.76	1.54	-1.79	0.12	-5.30	-0.21
(2) T°C (L)[a]	3.41	1.29	2.64	0.03	1.28	5.54
(2) T°C (Q)[a]	-2.58	1.54	-1.67	0.14	-5.13	-0.04
Mean/Interc.[b]	19.73	1.08	18.21	0.00	17.17	22.29
(1) U (L)[b]	2.89	0.91	3.17	0.01	0.74	5.05
(2) T°C (L)[b]	7.90	0.91	8.67	0.00005	5.75	10.06
(2) T°C (Q)[b]	-2.77	1.04	-2.66	0.03	-5.23	-0.31

a- Statistical analysis for crude protease; b- Statistical analysis for partially purified protease

Table IV.10. Analysis of variance of the effect of the amount of protease from *Bacillus* sp. LBA 46 and temperature on the lysis of *X. campestris* cells

Source of variation	Sum of squares	Degrees of fredom	Mean square	F – Value
Regression[a]	312	4	77.98	5.85
Residues[a]	80	6	13.32	
Lack of fit[a]	78.20	4	26.06	
Pure error[a]	1.72	2	0.57	
Total[a]	391.86	10		
Regression[b]	613	3	204.28	30.79
Residues[b]	46	7	6.63	
Lack of fit[b]	43.44	5	14.48	
Pure error[b]	2.98	2	0.99	
Total[b]	659.29	10		

a- Statistical analysis for crude protease; $R^2 = 0.79$; $F_{0.15;4;6} = 3.18$; b- Statistical analysis for partially purified protease; $R^2 = 0.92$; $F_{0.05;3;7} = 4.35$

Equation 5: Relative Activity = $17.21 – 4.43.U – 2.76.U^2 + 3.41.T – 2.58.T^2$

Equation 6: Relative Activity = $19.73 – 2.89.U + 7.90.T – 2.77.T^2$

The response surfaces show that in the studied range, the optimal temperature for lysis of *X. campestris* cells was found for crude protease corresponding to 65°C (Figure IV.4a), while 70°C was the best temperature for partially purified protease (Figure IV.4b). Temperatures lower than 50°C decreased considerably the capability of lysing the *X. campestris* cells by both protease preparations. Regarding the protease amount, it could be observed in the Figure IV.4 that higher increase in Trans $_{620\ nm}$ can be obtained using higher enzyme amount for both enzyme preparations. Yet, when 42 U of protease/mL of cell suspension of enzyme at 65°C was used, the increase of Trans $_{620\ nm}$ was approximately 20% using crude protease, while in the same conditions it was almost 30% using the partially purified enzyme. Interestingly, considerable increase in Trans $_{620\ nm}$ values (~20%) would be obtained using lower amounts of partially purified protease (10 U/mL) at

70°C, what is different from the results with crude protease in the same conditions (~5%).

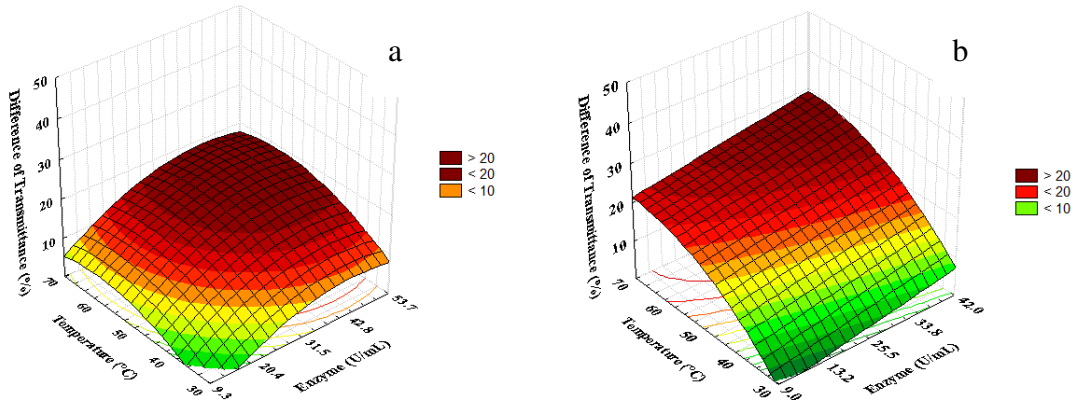

Figure IV.4. Response surfaces of the effect of the amount of protease from *Bacillus* sp. LBA 46 and temperature on the lysis of *X. campestris* cells: a- crude protease extract; b- partially purified protease

Although there are few reports on the use of protease extracts from *Bacillus* sp. strains on xanthan gum clarification, some patents can be found. Colegrove (1977) reported the clarification of xanthan gum in fermentation broths and other aqueous suspensions containing a dissolved xanthan gum and suspended solids resulting from the fermentation producing the xanthan gum. In the patent a minor amount of an alkaline protease from *B. subtilis, B. licheniformis, B. amyloliquefaciens* and *Bacillus pumilus* were used. In another patent, Pollock (1994) reported the enzymatic clarification of polysaccharides, using acid and/or neutral proteolytic enzymes for the degradation of the cells of the genus *Xanthomonas campestris*. Aliquots of 20 ppm of proteases from *B. subtilis* and *Aspergillus niger*, were used and transparency for all of the enzyme-treated samples increased. In the patent of Dimasi et al. (2003) the clarification of an aqueous xanthan gum solution was investigated using neutral proteases from *B. amyloliquefaciens* and alkaline

proteases from *B. subtilis*, *B. licheniformis*, and *B. pumilus*, among other proteases. After clarification of the xanthan gum solution the high purity xanthan gum provides substantially clear viscosified compositions, having most preferably, a transmittance of at least 93% for solutions containing 1% by weight of the high purity xanthan gum.

Taking into consideration the high cost of purification processes of enzymes the crude protease was selected for studying the influence of time in the *X. campestris* lysis. As it can be seen in Figure IV.5 when the reaction was carried out using 42 U of protease/mL of cell suspension of *X. campestris* at 65°C, an increase of 18% of Trans $_{620\ nm}$ was observed after 30 min of reaction, which is extremely close to the predicted value in the same conditions for crude protease. After 2h of reaction an increase in Trans $_{620\ nm}$ of almost 40% was obtained.

Besides *Bacillus* sp. Strains, other microorganisms are also reported for *X. campestris* lysis. In the work of Pollock and Yamazaki (1993) protease extracts from *Lysobacter* sp strains were applied in xanthan gum clarification and it was observed a considerable decrease in Absorbance values of the fermented broth indicating the lysis of *X. campestris* cells. *Trichoderma koningii* has been studied for production of protease extract and its application in *X. campestris* cell lysis. The authors observed that the culture filtrate containing protease activity found to lyse the cells from fermented broth optimally at pH 7 and 50°C, resulting in an increase of 44% after 24h of reaction. After 4 hours of reaction the increase observed was only 4% using 10% (v/v) of enzymatic extract (Triveni and Shamala, 1999).

Shastry and Prasad (2002) purified a protease from *Pseudomonas* sp. (CL 1457) and studied the lysis of *X. campestris*. After transmission electron microscopy analysis it was possible to observe that the lytic action of the enzyme against the cells involves initial lysis of the outer cell membrane and this effects cell disintegration.

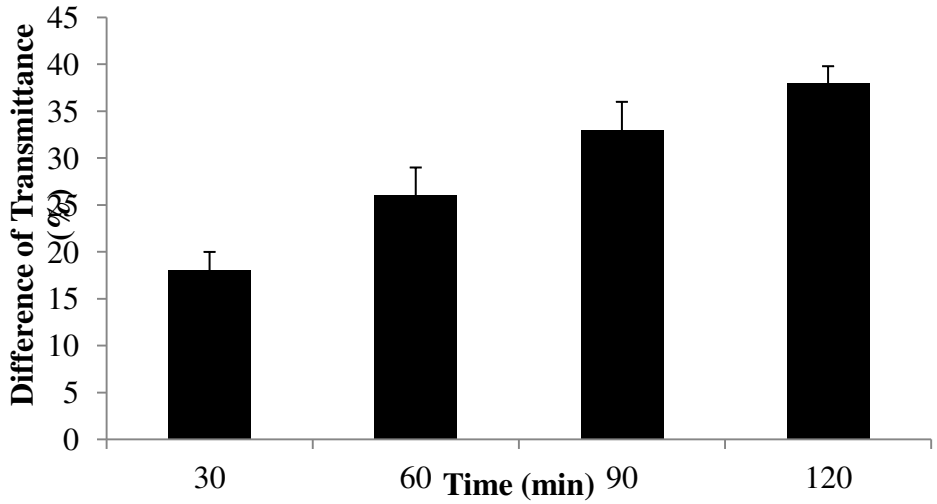

Figure IV.5. Influence of time on the lysis of *X. campestris* cells for xanthan gum clarification, using crude protease of *Bacillus* sp. LBA 46 (42U/mL) at 65°C.

Each data point represents the mean of three independent assays with the standard deviation.

IV.4. Conclusions

The proteases from *Bacillus* sp. LBA 46 strain were purified 3.69 times using ammonium sulfate fractionation and gel filtration using Sephadex G-100.

The crude and partially purified proteases preparations presented optimal activity at 55°C and pH 7.5.

The crude and partially purified proteases preparations were considerably stable in the pH range 5.7-9.3 after 1 hour incubation at 30-36°C, retaining above 95% of residual activity. Both enzyme preparations retained above 78.8% of residual activity after 1 hour incubation at pH 7.5 and 50°C. The enzyme was inactivated after 1 hour incubation at pH 7.5 and 70°C.

Crude and partially purified proteases from *Bacillus* sp. LBA 46 presented good results when applied in the lysis of *X. campestris* cells from the fermented broth for clarifying xanthan gum. The process was optimized using RSM for both protease preparations, however an increase of $Trans_{620\ nm}$

of the fermented broth is higher using higher amounts of partially purified protease, reaching more than 30%, whilst an improve of Trans $_{620\ nm}$ of 20% is obtained using the same quantity of crude protease (42 U/ mL of cell suspension of *X. campestris*). In addition, temperature showed to be highly significant in the *X. campestris* lysis indicating that better results are obtained when the temperature are above 60°C. When the crude protease was applied to the lysis of *X. campestris* cells almost 40% of increase of Trans $_{620\ nm}$ after 2h of reaction at 65°C was obtained, using 42 U of protease/mL of cell suspension.

IV.5. Acknowledgements

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V. CONCLUSÕES GERAIS

As linhagens de *Bacillus* sp. LBA 7, 8, 19, 39, 44, 46, 48, 50 and 60 foram selecionadas dentre cinquenta e nove linhagens devido a maior produção de proteases. Após cultivo em três diferentes meios, as linhagens de *Bacillus* sp. LBA 7, 8, 46, 48 e 50 apresentaram a maior produção de proteases, sendo que a linhagem de *Bacillus* sp. LBA 7, 48 e 8 produziram maior atividade protease no meio n° 1 composto de 10 g/L de caseina, 1 g/L de extrato de levedura, 7g/L de $(NH_4)_2HPO_4$, 1.5 g/L de K_2HPO_4, 0.5g/L de $MgSO_4.7H_2O$, 0.3 g/L de $CaCl_2.2H_2O$, 1 g/L de $FeSO_4.7H_2O$, 1 g/L de $MnCl_2.4H_2O$, 1 g/L de $ZnSO_4.7H_2O$ (222 U/mL), no meio n° 2 composto de 35g/L de melaço de cana de açúcar, 20 g/L de água de maceração de milho, 3 g/L de extrato de levedura Prodex-Lac SD® e 20 g/L de soro de queijo (548U/mL) e no meio n° 3 composto de farelo de trigo e água (1:1, m/m) (13480 U/gss), respectivamente.

As preparações de proteases de *Bacillus* sp. LBA 7, 8, 19, 39, 44, 46, 48, 50, 60 produzidas nos três meios apresentaram atividade ótima a 60°C e na faixa de pH 7-9, estabilidade na faixa de pH 5-9 por 24h a 4°C. Os extratos enzimáticos brutos de protease das nove linhagens de *Bacillus* sp. LBA obtidos de fermentação nos meios n° 2 e n° 3 foram estáveis a 50°C por uma hora em pH 7.0. As proteases de *Bacillus* sp. LBA 19, 44, 46, 48, e 50 obtidas da fermentação no meio n° 1 composto de caseína, extrato de levedura e sais manteve aproximadamente 60-80% da atividade inicial após incubação com detergente Ariel® líquido e sólido a 25°C por 2h. Os extratos de protease de *Bacillus* sp. LBA 44 e 46 obtidos do cultivo no meio n° 2 composto de melaço de cana de açúcar, água de maceração de milho, extrato de levedura Prodex-Lac SD® e soro de queijo apresentaram cerca de 60% de atividade residual após incubação com o mesmo detergente. Quando incubadas em solventes orgânicos alguns extratos enzimáticos brutos de protease mantiveram mais de 60% de atividade residual após 24h em acetona (*Bacillus* sp. LBA 8 e 44),

hexano (*Bacillus* sp. LBA 19, 29, 44, 46 e 60), clorofórmio (*Bacillus* sp. LBA 44 and 60) e etanol (*Bacillus* sp. LBA 60). Os extratos enzimáticos brutos de protease de *Bacillus* sp. LBA 46 and 48 apresentaram maior capacidade de clarificar o meio fermentado de *X. campestris* contendo goma xantana.

A linhagem de *Bacillus* sp. LBA 46 foi selecionada e as condições ótimas para a produção de proteases utilizando metodologia de superfície de resposta foi obtida em meio de cultura n° 4 composto de 40g/L de melaço de cana de açúcar, 6 g/L de água de maceração de milho, 2 g/L de extrato de levedura Prodex-Lac SD® e 20 g/L de soro de queijo, após 96h de fermentação a 30°C e 200 rpm, resultando em atividade de protease de aproximadamente 3000 U/mL. O extrato bruto de protease obtido do meio otimizado em conjunto com detergente Omo® (tratado termicamente para a inativação das enzimas comerciais) foi mais eficiente na remoção de manchas de sangue e de molho de tomate com carne moída do que apenas o detergente.

O extrato enzimático bruto foi purificado por fracionamento com sulfato de amonio e cromatografia de filtração em gel G100 em 3.69 vezes. Os extratos enzimáticos brutos e parcialmente purificados de protease foram caracterizados com MSR e observou-se que ambas as preparações apresentaram atividade ótima em pH 7,5 e 55°C. As preparações de proteases retiveram mais que 95% de atividade residual após serem incubadas por 1h na faixa de pH de 5,7-9,3 a 30-36°C, mantendo mais que 78.9% de atividade residual após incubação em pH 7.5 a 50°C. Foi utilizado MSR para otimizar a lise de células de *X. campestris* e observou-se que as condições ótimas para ambas as preparações de proteases foram 42 U de protease /mL de suspensão celulase de *X. campestris* e temperatura igual ou superior a 60°C. Um aumento de quase 40% na Trans $_{620\ nm}$ foi observado após 2h de lise de células de *X. campestris* utilizando 42 U de protease bruta/mL de suspensão celular de *X. campestris* a 65°C.

V. ANEXO I. PRODUCTION OF PROTEASES FROM *Bacillus* sp. LBA 46 UNDER SOLID STATE FERMENTATION

O objetivo da inclusão do anexo ao corpo desta tese é de registrar parte do trabalho experimental desenvolvido. Neste anexo estão informações sobre como foram definidos os caminhos seguidos durante o desenvolvimento do trabalho de pesquisa, e resultados que não foram apresentados nos capítulos, mas que podem ter importância para definição de futuros projetos de pesquisa nessa área.

V.1. Introduction

Proteases are applied in several industrial areas including detergents, protein recovery, organic synthesis and production of bioactive peptides. *Bacillus* sp. strains are studied for protease production due to secreting large amounts of alkaline proteases, and presenting proteases with remarkable proteolytic activity and stability (Shah et al., 2010, Chauhan and Gupta, 2004).

Although the great number of works focused on submerged fermentation (SmF) for production of proteases from *Bacillus* sp., solid state fermentation (SSF) can present some advantages in many cases. SmF is the classic technique and is responsible for the majority of the enzymes industrially produced and shows some advantages including reduction of contamination due to relatively short growth period, lower capital investment compared to continuous processes for same bioreactor volume, among other aspects (Gupta et al., 2002b). On the other hand, SSF requires solid substrates in complete or near absence of free water with enough moisture to support

growth and metabolism of microorganisms (Pandey, 1992) and therefore this technique has been receiving much attention due to some advantages compared to SmF, for instance the simplicity of the media, economy of space for fermentation and less energy demand (Lonsane et al., 1985).

Media compositions represent approximately 30–40 % of the production costs (Kirk et al., 2002), showing the necessity of low cost substrates, such as agroindustrial wastes and byproducts. They include wheat bran (Renganathan et al., 2011) as one of the most studied substrate for protease production due to its nutritional profile. In addition, the optimization of fermentation conditions and medium components is important and can reduce considerably the cost of the process. One interesting technique for this purpose is the use of response surface methodology (RSM), leading to the identification of the relationships between the variables and the response, which can result in the optimization of the process and simultaneously limiting the number of experiments. Therefore, the aim of this work was to optimize the production of proteases by *Bacillus* sp., 46 under SSF using RSM, considering this *Bacillus* sp. strain showed interesting results in the previous study of lysis of *X. campestris* cells from the fermented broth of xanthan.

V.2. Material and methods

I.2.1. Microorganism

The strains of *Bacillus* sp. LBA 46 from the culture collection of Food Biochemistry Laboratory, College of Food Engineering – UNICAMP was maintained in nutrient agar at 4°C, with sterile vaseline addition and transfers in every 2 months.

V.2.1. Production of proteases by *Bacillus* sp.

V.2.1.1. Optimization of production of proteases by Bacillus sp. LBA 46 under solid state fermentation

The production of protease from *Bacillus* sp. LBA 46 under SSF has been optimized using CCD and RSM. A 2^3 central composite design (2^3-CCD) with 2 axial points (+\–) at a distance of α=1.68 was used, with three replicates at the center point, resulting in a total of 17 runs. Aliquots of 2 mL of cell suspension of *Bacillus* sp. LBA 46 in saline solution (0.85% NaCl, m/v) and optical density at 620 nm between 0.49 and 0.51 were inoculated into 250 mL Erlenmeyer flasks containing 5 g of wheat bran (Natu's, BR) and different quantities of distilled water and saline solution (0.85% NaCl, m/v) and incubated at 30°C for 96 h. After the fermentation, 50 mL of 0.1 M sodium phosphate buffer pH 7.0 was added in each the flasks and they were stirred at 150 rpm for 1 h. The mixtures were filtered against cotton and centrifuged at 9600 x *g* for 15 min at 5°C. The supernatants were used as crude protease extracts and the dependent variable was the protease activity. The independent variables were ratio of wheat bran and water (m:m), inoculum size and temperature (Table VI.1). The statistical analyses were carried out using software Statistica 8.0®.

V.2.2. Determination of protease activity

The protease activity was determined as described by Charney & Tomarelli (1947) with modifications. The reaction mixture containing 0.5 mL of 0.5% (m/v) azocasein (Sigma, US) in 0.05 M phosphate buffer (pH 7.0) and 0.5 mL the enzyme solution was incubated at 50°C for 40 min. The reaction was stopped by adding 0.5 mL of 10.0% (m/v) trichloroacetic acid and the test tubes were centrifuged at 17000 x *g* for 15 min at 25°C. A 1.0 mL aliquot of the supernatant was neutralized with 1.0 mL 5 M KOH. One unit of protease activity was defined as the amount of enzyme which caused an

increase of 0.01 of absorbance at 428 nm per minute. The enzyme activity was expressed U/gds (gram of dried substrate).

V.3. Results and Discussion

V.3.1. Optimization of production of proteases by *Bacillus* sp. LBA 46 under solid state fermentation

The protease production by *Bacillus* sp LBA 46 under 96h of SSF was studied using CCD as described in the Table VI.1. The highest protease activity observed (6725 U/gds) was in the run 2 corresponding to the ratio of wheat bran and water of 6.2:3.8 (m:m), 8.5% (v/m) of inoculum size and 26.4°C. On the other hand, the lowest protease activity was observed when the ratio of wheat bran and water was 3.8:6.2 (m:m), 8.5% (v/m) of inoculum and 36.6°C (run 5). Therefore, it can be mentioned that using high proportions of wheat bran, high protease production is obtained. However, in the run 10 corresponding to the axial point, the ratio of wheat bran and water was 7:3 (m:m) and the protease activity was not as high as runs 2 and 4. In addition, according to the Table VI.1, high protease activity amounts were observed when the temperature was low (26.4°C) (runs 1, 2, 3 and 4) compared to the runs 5, 6, 7 and 8 in which the temperature was higher (36.6°C) and the protease activity amounts were lower.

The statistical analysis was carried out to evaluate the results observed and as can be seen in the Table VI.2, according to the regression coefficients the variable ratio of wheat bran and water and the variable temperature linear and quadratic were statistically significant with a 90% confidence level ($p < 0.1$), whilst the variable inoculum was not. The mathematical model (equation 1) can be obtained according to the analysis of variance analysis (ANOVA) and F test (Table VI.3). The R^2 was equal to 0.71 which is acceptable for biological process, mainly SSF that can present several interferences, including the extraction of the enzyme.

Table VI.1. Central composite design 2^3 coded for the study of the influence of ration of wheat bran and water, inoculum size and temperature on protease production by *Bacillus* sp. LBA 46, for 96h of fermentation time.

Runs	Variables			U/gds
	Wheat bran:water (m:m)	Inoculum (%, v/m)	Temperature (°C)	
1	-1 (3.8:6.2)	-1 (8.5)	-1 (26.4)	2907
2	+1 (6.2:3.8)	-1 (8.5)	-1 (26.4)	6725
3	-1 (3.8:6.2)	+1 (24.5)	-1 (26.4)	3532
4	+1 (6.2:3.8)	+1 (24.5)	-1 (26.4)	5314
5	-1 (3.8:6.2)	-1 (8.5)	+1 (36.6)	1519
6	+1 (6.2:3.8)	-1 (8.5)	+1 (36.6)	3620
7	-1 (3.8:6.2)	+1 (24.5)	+1 (36.6)	1657
8	+1 (6.2:3.8)	+1 (24.5)	+1 (36.6)	3483
9	-1.68 (3:7)	0 (16.5)	0 (31.5)	1958
10	+1.68 (7:3)	0 (16.5)	0 (31.5)	3335
11	0 (5:5)	-1.68 (3)	0 (31.5)	5700
12	0 (5:5)	+1.68 (30)	0 (31.5)	3830
13	0 (5:5)	0 (16.5)	-1.68 (23)	1680
14	0 (5:5)	0 (16.5)	+1.68 (40)	1525
15	0 (5:5)	0 (16.5)	0 (31.5)	4540
16	0 (5:5)	0 (16.5)	0 (31.5)	4380
17	0 (5:5)	0 (16.5)	0 (31.5)	4120

Table VI.2. Regression coefficients of the analysis of the 2^3 CCD for protease production by *Bacillus* sp. LBA 46 under 96h of solid state fermentation

	Regression coefficients.	Standard error	t(7)	P	-90,%	+90,%
Mean/Interc.	4682.582	415.0368	11.28233	0.000000	3942.87	5422.297
(1) Ratio (L)	867.756	259.6143	3.34248	0.005861	405.05	1330.464
Ratio (Q)	-539.829	273.3405	-1.97493	0.071737	-1027.00	-52.657
(3) T (°C) (L)	-619.972	259.6143	-2.38805	0.034257	-1082.68	-157.265
T (°C) (Q)	-909.727	273.3405	-3.32818	0.006018	-1396.90	-422.555

Table VI.3. Analysis of variance of the effect of wheat bran:water and temperature on protease production under 96h of solid state fermentation.

Source of variation	Sum of squares	Degrees of fredom	Mean square	F – Value
Regression	27144849	4	6786212.36	7.37
Residues	11035844	12	919653.69	
Lack of fit	10945978	10	1094597.76	
Pure error	89867	2	44933.33	
Total	38180694	16		

$R^2 = 0.71$; $F_{0.90;4;12} = 2.51$

Equation 1: Protease Activity = 4682.582 + 867.756.Ratio – 539.829.$Ratio^2$ – 619.972.T – 909.727. T^2

The response surface (Figure VI.1) shows that the protease production was optimized and protease activity amounts higher than 5000 U/mL can be obtained when the fermentation is carried out at around 30°C and wheat bran:water (m:m) ratio is approximately 6:4. Interestingly, according to the surface if the ratio of the substrate is superior for instance 7:3 the protease activity decreases considerably, probably due to the association with reduced availability of water for microbial growth. Furthermore, when the fermentation was carried out at 23°C and 40°C the protease activity is not considerable, resulting in less than 1000 U/gds.

Wheat bran is considered an efficient substrate for protease production due to the fact that it provides adequate source of protein, carbohydrates, and minerals necessary for the *Bacillus* sp. strains for growth and biosynthesis of enzymes. In addition, this substrate has also large surface area per unit volume for a good bacterial growth on the solid/gas interfaces. Natural substrates can also possess growth promoters in enough amounts covering the requirements of the bacterial growth and enzyme production.

In addition to the importance of agro-industrial substrate for protease production, another important factor is the moisture content of the culture medium. The optimum moisture content for growth and substrate use depends on the micro-organism and substrate used and in this case the initial moisture content is a critical factor for SSF, taking into account this variable influences growth, biosynthesis and secretion of enzymes. It must be considered that the higher the moisture level is, the higher the reduction in enzyme yield is. This is due to steric hindrance of the growth of strain by reduction in porosity of the solid substrate this interfering with oxygen transfer (Ramakrishna et al.,

2012). Considering, SSF processes are different from SmF, microbial growth and product formation occur at or near the surface of the solid substrate particle that have low moisture contents (Pandey et al., 2000). Therefore, it is extremely necessary providing optimized moisture level capable of controlling the water activity of fermenting substrate for achieving maximum enzyme production. On the other hand, the observed reduction in enzyme production at reduced moisture level might be associated with reduced availability of water for microbial growth.

In the study of Madhuri et al. (2012) maximum protease production (128.357 U/gds) using castor husk as solid substrate was observed with 80% moisture content and further increase in moisture level resulted in separation of liquid medium from solid substrate. On the other side, Renganathan et al. (2011) isolated the *Bacillus* RRM1 for protease production and reported that wheat bran was the best substrate the enzyme production under SSF. The microorganism showed maximum enzyme production in the medium composed of wheat bran with 60% of moisture and 15% of inoculum, after 60h of fermentation, at 37°C.

In spite of the fact that the inoculum size in the range studied has not affected significantly the protease production under SSF, some works report the influence of this variable. Ramakrishna et al. (2012) observed that the optimum protease production (7096 U/mL) was obtained at 0.5 mL inoculum size (1x 10^8 cells/mL). When the inoculum size was increased beyond the optimum level a decrease in protease production was observed.

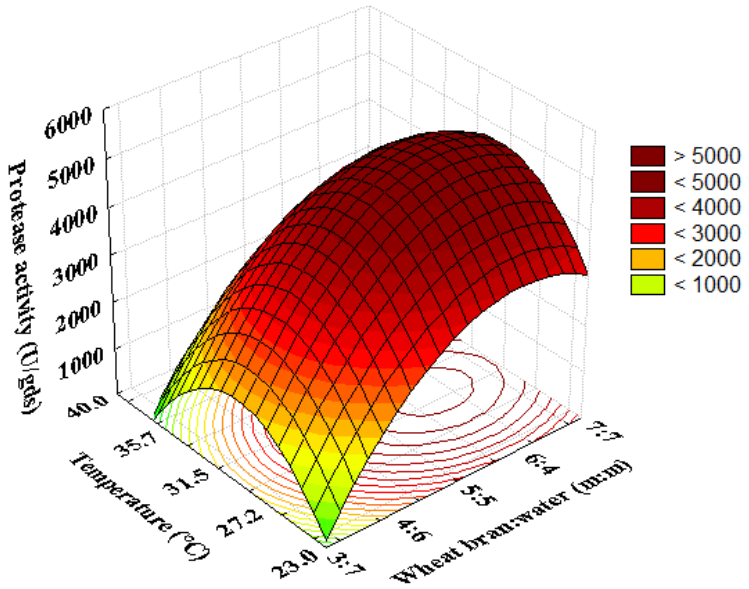

Figure VI.1. Response surface of the effect of ratio of wheat bran and water, and temperature on the protease production by *Bacillus* sp. LBA 46 under 96h of solid state fermentation.

V.4. Conclusions

In the study of optimization of proteases from *Bacillus* sp. LBA 46 under SSF using RSM, the highest protease production was obtained using medium no. 5, containing wheat bran: water (60%:40%) after 96 h of fermentation at 30°C.

V.5. References

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SUGESTÕES PARA TRABALHOS FUTUROS

1. Realizar estudos para produzir proteases utilizando escala piloto e industrial.

2. Realizar estudos de produção da enzima por fermentação em estado sólido

3. Avaliar diferentes técnicas de purificação das proteases, visando obter maior fator de purificação e maior rendimento.

4. Testar outras aplicações das proteases como reações enantioseletivas utilizando a enzima em meio orgânico, visando à obtenção de compostos com alto valor agregado; e na obtenção de peptídeos com atividade biológica.