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PROSPECÇÃO E ISOLAMENTO DE MICRORGANISMOS DO CERRADO BRASILEIRO PARA O DESENVOLVIMENTO DE BIOPROCESSO DE PRODUÇÃO DE PROTEASE

PROSPECTING AND ISOLATION OF MICROORGANISMS FROM THE BRAZILIAN CERRADO FOR THE DEVELOPMENT OF A PROTEASE-PRODUCTION BIOPROCESS

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“O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo fará coisas admiráveis.”

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Resumo

A prospecção e isolamento de novas espécies de microrganismos tem sido incentivada em habitats naturais, a fim de descobrir biomoléculas com aplicações de interesse industrial. A rica biodiversidade brasileira ainda é pouco explorada e relatos acerca das enzimas proteolíticas provenientes do Cerrado Brasileiro são escassos, especialmente na região do Triângulo Mineiro, Minas Gerais. Assim, o objetivo do presente trabalho foi prospectar e isolar microrganismos do Cerrado Brasileiro para o densenvolvimento de bioprocesso de produção de proteases. Inicialmente, amostras de solo, casca de frutos, folhas e flor foram coletadas na região do Cerrado Mineiro e 65 microrganismos foram isolados, formando um banco de microrganismos. As cepas foram testadas frente à produção de proteases extracelulares em fermentação submersa (glicose 10 $g.L^{-1}$; peptona $5g.L^{-1}$ e extrato de levedura $3g.L^{-1}$) a 30 °C por 120 horas, destacando-se a bactéria SRM-2AA, isolada do solo, a qual foi selecionada para o otimização do meio de cultivo. As análises das fontes de nitrogênio (nitrato de amônio, citrato de amônio, sulfato de amônio, peptona e extrato de levedura) e carbono (glicose, peptona, permeado de soro de leite, melaço de cana e melaço de soja) identificaram o extrato de levedura (16,46 $U.mL^{-1}$) e permeado de soro de leite (23,32 $U.mL^{-1}$) como melhores precursores para produção de proteases, sendo o primeiro relato de uso do permeado de soro de leite, um resíduo agroindustrial, utilizado para este propósito. Em seguida, a composição do meio de cultura foi otimizado utilizando otimização estatística por meio da metodologia da superfície de resposta, ANOVA e teste-F. Logo, o meio de culivo composto por permeado de soro de leite a 12,83 $g.L^{-1}$ e extrato de levedura a 4,41 $g.L^{-1}$ preparados com tampão fosfato salino, pH 7.0, incubado a 25 °C por 24 horas foi validado em 100 mL com as condições otimizadas (30,97 $U.mL^{-1}$), além do aumento de escala em biorreator (1500 mL) que, satisfatoriamente, atingiu 34,13 $U.mL^{-1}$. A protease apresentou atividade máxima em pH 8,2 e 45 °C, além de preservar sua atividade entre temperaturas de 1 a 40 °C e pH entre 5,8 a 7,4. Enzimas proteolíticas provenientes da bactéria SRM-2AA mostraram-se ser adequadas para aplicações biotecnológicas em indústrias alimentícias, sendo favorável à fabricação de queijos e como amaciante de carne.

Palavras-chave: peptidase; bioprospecção; Cerrado Brasileiro; fermentação submersa; biorreator.

Abstract

The bioprospection and isolation of new microorganism species have been encouraged in natural habitats, in order to found biomolecules with industrial applications. The rich Brazilian biodiversity is poorly investigated and there are few researches describing proteolytic enzymes from Brazilian Cerrado, especially in Triangulo Mineiro region, Minas Gerais State. For this reason, the aim of the present work was prospect and isolate microorganisms from Brazilian Cerrado to development of protease production bioprocess. Initially, samples were collected from soil, peel fruits, flower and leaves located in Cerrado Mineiro region, where 65 microorganisms were isolated obtaining a microorganism dataset. The strains were tested as protease producing under submerged fermentation (glucose 10 $g.L^{-1}$, peptone 5 $g.L^{-1}$ and yeast extract 3 $g.L^{-1}$) at 30 °C during 120 hours. The bacterium coded as SRM-2AA was the best extracellular protease-producer, which it was selected to medium culture optimization. Analysis of different nitrogen (ammonium nitrate, ammonium citrate, ammonium sulfate, peptone and yeast extract) and carbon (glucose, peptone, whey permeate, sugarcane molasses and soy molasses) sources allowed identifying the yeast extract (16.46 $U.mL^{-1}$) and whey permeate (23.32 $U.mL^{-1}$) as the best precursors for protease production. This is the first report that use whey permeate, an agro-industrial residue, for this purpose. Statistical optimization of medium composition was performed by response surface methodology, ANOVA and Fisher-based test. Culture media composed by whey permeate at 12.83 $g.L^{-1}$ and yeast extract at 4.41 $g.L^{-1}$ prepared with phosphate-buffered saline, pH 7.0, incubated at 25 °C during 24 hours was validated as optimized conditions in 100 mL (30.97 $U.mL^{-1}$) and satisfactory scaled up in 1500 mL-bioreactor attaining 34.13 $U.mL^{-1}$ of proteolytic activity. The protease displayed optimum activity at pH 8.2 and 45 °C and had activity preserved from 1 to 40 °C and a pH range 5.8 to 7.4. Food preliminary analyses suggest that proteolytic enzymes from bacterium SRM-2AA its suitability in industries; it could be a good candidate for cheese making as well as a meat tenderizer.

Keywords: peptidase; bioprospection; Brazilian Cerrado; submerged fermentation; bioreactor.

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AM	Arbuscular Mycorrhizal
ANOVA	Analysis of Variance
$CaCl_2$	Calcium Chloride
CAZymes	Carbohydrate-active Enzymes
CCD	Central Composite Design
CENARGEN	Centro Nacional de Pesquisa de Recursos Genéticos e Biotecnologia
CHNS	Carbon, Hydrogen, Nitrogen and Sulfur
C/N	Carbon and Nitrogen Proportion
C_{YE}	Yeast Extract Concentration
C_{WP}	Whey Permeate Concentration
DNA	Deoxyribonucleic Acid
EMBRAPA	Empresa Brasileira de Pesquisa Agropecuária
$F_{calculated}$	Fisher's Calculated Value
$F_{tabulated}$	Fisher's Tabulated Value
GOS	Galactooligosaccharide
IBGE	Brazilian Institute of Geography and Statistics
KCl	Potassium Chloride
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
MCA	Milk Clotting Activity
MCU	Milk Clotting Unit
PE	Polyethylene
PGPB	Plant Growth-Promoting Bacteria

PNSC	Sete Cidades National Park
R^2	Coefficient of Determination
R^2_{adj}	Adjusted Coefficient of Determination
RNA	Ribonucleic Acid
RSM	Response Surface Methodology
SSF	Simultaneous Saccharification and Fermentation
Tris	Tris(hydroxymethyl)aminomethane
YPD	Yeast Extract, Peptone and Dextrose
WBSF	Warner-Bratzler Shear Force

Sumário

Capítulo 1 – Introdução

1.1. Organização estrutural da tese

A disposição dos conteúdos abordados ao longo deste traabalho é evidenciada nesta seção. A tese está organizada em capítulos, os quais apresentam, inicialmente, uma introdução geral e os objetivos. Em seguida, a revisão bibliográfica aborda fundamentos teóricos em formato de artigo de revisão. A parte experimental da tese é mostrada em dois capítulos, que contemplam o delineamento inicial dos experimentos com a prospecção e isolamento dos microrganismos e; o desenvolvimento de bioprocesso de produção de proteases pela bactéria SRM-2AA. Por fim, as conclusões finais e sugestões para trabalhos futuros são expostos.

O **Capítulo 1** expõe uma visão geral sobre o tema proposto da tese, no qual evidencia-se a importância da prospecção de novas biomoléculas provenientes de habitats naturais, como o Cerrado Brasileiro que é rico em biodiversidade. Além disso, os microrganismos recebem destaque como fonte mais utilizada para obtenção de novos biocompostos, como as enzimas. O capítulo também apresenta dados do mercado mundial de enzimas com destaque ao uso das enzimas proteolíticas.

O **Capítulo 2** contempla o objetivo geral e os objetivos específicos do trabalho.

O **Capítulo 3** descreve a revisão bibliográfica em forma de artigo de revisão, o qual é intitulado "*Biotechnological products from Brazilian Cerrado microbiota*". O artigo aborda características do bioma Cerrado Brasileiro e atuais ações governamentais de preservação. A microbiota compreendendo bactérias, leveduras e fungos provenientes de solos ou superfícies de frutos típicos do Cerrado são descritos, evidenciando os principais filos e/ou gêneros. Por fim, o artigo trata das principais biomoléculas isoladas de microrganismos do Cerrado Brasileiro com potencial aplicação biotecnológica para as indústrias, com ênfase às enzimas como proteases, xilanases, catalases, lipases, celulases e, biocompostos com atividade antimicrobiana e anticancerígena.

O **Capítulo 4** retrata todo o procedimento inicial realizado na construção do banco de microrganismos provenientes do Cerrado Mineiro, desde a coleta de dados, prospecção e isolamento de cada colônia. Além disso, avalia o potencial enzimático dos microrganismos isolados frente à enzimas proteolíticas.

O **Capítulo 5** contempla o artigo experimental intitulado "*Screening and optimization of protease production by bacterium SRM-2AA isolated from Brazilian Cerrado*", no qual a investigação acerca da microbiota presente no solo do Cerrado Mineiro foi realizada a fim de encontrar potenciais candidatos produtores de enzimas proteolíticas. A bactéria com maior produção de proteases foi selecionada e seu meio de cultura foi otimizado por meio de planejamentos experimentais, em que um resíduo agroindustrial foi utilizado, permeado de soro de leite. Além disso, o aumento de escala do bioprocesso na produção de protease, a parcial caracterização bioquímica e testes de aplicação frente a atividade de coagulação do leite e análise de textura, por meio do teste de amaciamento da carne, foram analisados.

O **Capítulo 6** apresenta as principais conclusões e considerações finais, o **Capítulo 7** descreve algumas propostas para trabalhos futuros e, o **Capítulo 8** evidencia as referências bibliográficas.

1.2. Prospecção de novas biomoléculas no Cerrado Brasileiro

O Brasil é um dos países mais ricos em biodiversidade, apresentando cerca de 9,5% de todas as espécias conhecidas no mundo (1). Sua grande extensão, 8,5 milhões km^2, exibe diversas zonas climáticas que levam a variações ecológicas e resultam na formação de diferentes zonas biogeográficas ou biomas, representadas pela Amazônia, Mata Atlântica, Cerrado, Caatinga, Pantanal e Pampa (2). O Cerrado é o segundo maior bioma brasileiro, o qual representa 23,92% do território nacional (3) e ocupa aproximadamente 2 milhões km^2 em extensão (4). A savana brasileira, típica da zona tropical, está localizada principalmente na região central do país, sendo a vegetação caracterizada por camadas praticamente contínuas de gramíneas, interrrompidas apenas por arbustos e árvores em proporções variáveis. O clima destaca-se por verões chuvosos e invernos secos, os quais estão intimamente associados aos principais padrões de crescimento dos organismos entre as estações seca e úmida. (5).

Dentre a megadiversidade biológica em animais, plantas e microrganismos, os microrganismos do solo destacam-se por suas diferentes funções, desde a degradação de compostos orgânicos, ciclagem de nutrientes, fixação biológica de nitrogênio e auxílio na absorção de nutrientes para as plantas (6). Além disso, a flora do Cerrado inclui diversas espécies frutíferas, as quais microrganismos epifíticos estão presentes em folhas e frutos e, são responsáveis pela produção de diversas biomoléculas como enzimas com atividade amilolítica, celulósica, proteolítica e, também, antimicrobiana (4). Os microorganismos são uma abundante reserva de diversidade genética e funcional acumulada durante os milhões de anos de evolução

e adaptação por inúmeras pressões seletivas (7). Estes são as principais fontes de novas biomoléculas comerciais, uma vez que seus produtos apresentam diversidade química e estrutural (8).

A fonte mais utilizada para obtenção de enzimas é a microbiana, uma vez que apresenta menores custos para produção e recuperação, e possui maior facilidade no aumento de escala de produção (9). As enzimas, proteínas que catalizam reações químicas, tem sido amplamente utilizadas por facilitarem processos industriais. A indústria catalítica tem desenvolvido, significativamente, devido ao aumento da demanda global por tecnologias limpas e economicamente viáveis, em substituição aos agentes químicos por catalisadores biológicos e biodegradáveis. O mercado global destes catalisadores biológicos atingiu, aproximadamente, $ 4,6 bilhões em 2014, $ 4,9 bilhões em 2015 e $ 6,4 bilhões em 2021 (10). Atualmente, estima-se uma taxa de crescimento de 6,3% ao ano para 2021-2026, o qual atingirá $ 8,7 billhões em 2026 (11). As aplicações incluem diversos produtos e processos em indústrias químicas, detergentes, têxteis, alimentícias, de rações, couro e, polpa e papel (12).

A utilização de enzimas oferece vantagens para os setores industriais por ser um produto natural, o qual apresenta alto grau de especificidade nas reações, contribuindo para eficiência do processo. A atividade reacional permite ser controlada, atuando em baixas concentrações e condições brandas de pH e temperatura (13). Assim, o uso comercial atual de enzimas, juntamente com novas aplicações, resultam em economias significativas de recursos, como matérias-primas e consumo de água. Além da melhoria na eficácia energética em benefício tanto da indústria quanto do meio ambiente, contribuindo com a sustentabilidade de gerações futuras. Dentre as enzimas industriais mais utilizadas encontram-se as hidrolases, incluindo proteases e lipases, que são amplamente aplicadas em laticínios, indústrias químicas e detergentes (12).

As proteases são enzimas proteolíticas, as quais compreendem mais de 65% do mercado total de enzimas (14). Estas tem a capacidade de agir sobre ligações peptídicas (15). As peptidases, como também são designadas, tem sido investigadas não apenas no campo científico, como nas áreas de química de proteínas e engenharia de enzimas, mas também para aplicações práticas como potentes agentes de limpezas e aditivos alimentares (16). Além disso, podem ser aplicadas em diversos setores, principalmente na indústria alimentícia como em laticínios, queijos, cervejaria e panificadoras, além de aplicações detergentes, como lavagens e tingimento em indústrias têxteis, de couro e seda (17; 18).

A prospecção da microbiota em ambientes naturais tem sido incentivada para obtenção de novos biocatalisadores. Até 1980, apenas 2% dos microrganismos do mundo tinham sido testados como fontes enzimáticas (7). A rica biodiversidade da flora brasileira é pouco estudada, tanto para espécies frutíferas, quanto microrganismos e fauna. As propriedades biotecnológicas ou medicinais são popularmente conhecidas fazendo parte do conhecimento tradicional, mas por outro lado, ainda são desconhecidas no meio científico. Assim, a bioprospecção segura da microbiota do Cerrado contribui para o descobrimento de novos compostos e moléculas bioativas com aplicações biotecnológicas, como já tem sido demonstrado em alguns estudos (19; 20). A busca por bactérias, leveduras e/ou fungos em nichos ecológicos novos e/ou menos investigados é a chave para coleta de diferentes cepas (19) e, logo, novas biomoléculas como as enzimas proteolíticas.

No Brasil, diversos programas do governo têm incentivado o estudo da biodiversidade, levando os pesquisadores brasileiros a investigarem sistemas enzimáticos de microrganismos nativos com potencial aplicação biotecnológica (2). Enzimas provenientes destes microrganismos são muito utilizadas na indústria e apresentam vantagens em sua produção devido ao curto tempo de fermentação, alta estabilidade e diversidade bioquímica (21). Além disso, a maioria das proteases são diretamente secretadas para o meio de cultivo, facilitando as posteriores etapas de recuperação e purificação. Portanto, o isolamento, identificação, conservação e exploração de enzimas proteolíticas provenientes de microrganismos se tornam importante para o desenvolvimento científico do país (2), além da grande capacidade de aplicação de proteases nas indústrias.

Capítulo 2 – Objetivos

2.1. Objetivo geral

Prospectar e isolar potenciais microrganismos do Cerrado Brasileiro para o desenvolvimento de bioprocesso de produção de protease.

2.2. Objetivos específicos

- Isolar microrganismos do Cerrado, na região do Triângulo Mineiro, por meio do solo, casca de frutos, folhas e flor;
- Selecionar o melhor microrganismo produtor de enzimas proteolíticas extracelulares;
- Otimizar o meio de cultivo do microrganismo selecionado utilizando resíduos agroindustrais, como permeado de soro de leite, melaço de soja e melaço de cana-de-açúcar;
- Caracterizar a protease produzida pelo microrganismo selecionado, por meio de testes de pH e temperatura ótimos, estabilidade e influência de íons;
- Aumentar a escala de produção da enzima proteolítica utilizando biorreator;
- Realizar estudo da cinética microbiana e de produção de proteases em escalas diferentes de fermentação (100 mL e 1500 mL);
- Averiguar a aplicação das proteases produzidas pelo microrganismo selecionado, frente a atividade de coagulação do leite e análise de textura, por meio do teste de amaciamento da carne.

Capítulo 3 – Artigo de revisão a publicar:

Biotechnological products from Brazilian Cerrado microbiota

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Abstract

Isolation and screening of microorganism in natural habitats has become a strategy to obtain new biomolecules with potential applications in industry. This review provides the characteristics of Brazilian Cerrado biome and current government preservation actions, further to the focus on biotechnological products from microorganisms. Biodiversity, water and soils characterize the importance and heterogeneity of the Cerrado. Microbial communities are influenced by seasonal patterns with rainy summers and dry winters and, the different gradient of vegetation cover (*Campo Limpo*; *Campo Sujo*; *Cerrado sensu stricto*; *Cerrado Denso* and *Mata de Galeria*). The native vegetation is richness in biodiversity when compared to perennial and annual crops and pastures. Proteobacteria, Actinobacteria and Acidobacteria phyla are highlighted on Brazilian savannah soil, whereas the genus *Glomus* is the most abundant on arbuscular mycorrhizal diversity. Biotechnological products as enzymes as well as proteases, lipases, catalases, xylanases, cellulases and chitinase, further to bio-compounds with antimicrobial and anticancer activities have been described from bacteria, yeasts and fungi species. Microbial community from Brazilian Cerrado is poorly known and efforts for new investigations have to be encouraged, owing to attend the growing global market for biological catalysts in industrial applications.

Keywords: Brazilian savannah; biomolecules; bio-compounds; microorganisms.

3.1. Introduction

Enzymes are mostly proteins that catalyze chemical reactions. Industrial enzymes have been widely used to accelerate industrial processes and the production of products. They were used in baking, brewing and cheese making dated to the ancient times, contributing to microorganisms growths or added to preparations, such as chymosin and papain from calves rumen and papaya fruit, respectively (1). The development in advanced bioprocess enabled to produce enzymes on large-scale, purified and well-characterized, resulting in diverse biotechnological applications such as detergent, chemical, textile, food, animal-feed, leather, and pulp and paper industries (2; 1).

Further enzymes, diverse biotechnological products as secondary metabolites of microorganisms and plants; organic acids, among others, such as citric, gluconic, acetic and lactic acids as well as pharmaceuticals products such as antibiotics, amino acids, monoclonal antibodies and hormones have also been investigated and developed, resulting in novel products and technologies in food, environmental, medicine, energy and bulk commodities applications (3).

The demand for industrial enzymes and biomolecules is emerging as a continuous stream of innovative products in the market, in order to meet the new requirements of industries, and soon, the maintenance and enhancement of the quality life of humanity. Also considering the sustainability due to biodegradability of bio-compounds (1). The bioprospecting in natural habitats has been encouraged currently in order to obtain new biomolecules and biocatalysts. Brazil is an abundant country in biodiversity and efforts have been made recently to investigate the diversity of biomes (4).

The Cerrado biome is considered the largest savannah region in South America, in which vegetation covering and temporal variations in soil moisture influence microbial communities (4). The Brazilian richness biodiversity is sparsely explored to microorganisms, as well as fauna and fruit species. On the other hand, biotechnological and/or medicinal properties are popularly known as part of traditional knowledge; however, they are still unknown in scientific aspects (5). The safe bioprospecting of the Cerrado contributes to the discovery of different strains and, consequently, new bioactive compounds with biotechnological applications.

In this context, the present review manuscript aimed to describe the biotechnological products from Cerrado biome reported in the literature, focusing on microorganisms bio-

compounds. Furthermore, the research includes the overview of Brazilian savannah characteristics, government preservation actions as well as the perspectives on global market of enzymes.

3.2. Cerrado biome

The Brazilian savannah is one of the five biggest biomes in Brazil occupying 23.92% of national territory (2,036,448 Km^2) (6). Cerrado biome comprises, predominantly, the states of Goiás, Tocantins, Mato Grosso do Sul, southern Mato Grosso, western Minas Gerais, Distrito Federal, western Bahia, southern Maranhão, western Piauí and portions of São Paulo State (Figure 3-1). Furthermore, there are portions of Cerrado in other states, as in Paraná, or in areas within other biomes, like in Amazonian Forest (7; 8).

The Brazilian Savannah biodiversity represents around 5% of world's biodiversity (7). The biome is considered one of the 25 most vital terrestrial biodiversity hotspots, i.e., one of the richest and most threatened biomes in the world (10). It is estimated that this region have more than 6 thousand species of trees and 800 species of birds. Over than 40% of woody plant and 50% of bees are considered endemic. The biome is crucial for maintaining the hydrological balance in the country and South American continent. It captures rainwater that supply springs and form rivers in the basins of the Amazonas, Tocantins, Parnaíba, São Francisco, Paraná and Paraguay. Moreover, immense aquifers are found, as Guarani Aquifer (7). The Cerrado biome rainfall is well-defined by rainy and dry seasons comprising water deficit to 5-6 months from April/May to September, which implies on precipitation and distribution variation patterns with a yearly average of 750-2000 mm on rainy season and 4-791 mm on dry season (6).

Biodiversity, water and also soils characterize the heterogeneity and importance of the Cerrado (7). The diversity of environments forms a complex mosaic of habitats and plant physiognomies showing a gradient of vegetation cover, which range from grasslands (*Campo Limpo* and *Campo Sujo*) to savannas (*Cerrado sensu stricto* and *Cerrado Denso*), even as riverine forests (*Mata de Galeria*). The soils characterized as weathered, acidic and with high clay content demonstrate low natural fertility, as well as limitations of native-nutrient availability (P, K, Ca, Mg, S, Zn, B, Cu) and high aluminum content. However, the relatively smooth reliefs are suitable for agricultural mechanization. These conditions provide physical and geochemical extreme characteristics of temperature, pH, water availability, radiation, salt content and redox potential for the soil and whole environment (6) contributing to the non-

uniform distribution of species, which increase the need for prospection and conservation programs (7; 11).

Figure 3-1: Political division of Brazil map. Cerrado biome area is highlighted in yellow. The map was defined by the Brazilian Institute of Geography and Statistic (7; 9).

In addition to the environmental aspects, the Cerrado is distinguished by its social importance, represented by 1,500 Brazilian municipalities. Countless human populations survive and know the available variety from biome, such as agroextractivists from southern Maranhão, northern Minas Gerais and western Bahia, and the few remaining indigenous populations from Goiás (7).

3.2.1. Government preservation actions

The Cerrado should stand out on environmental conservation. Until 2006, only 4% of the area was defined as a protected area, distantly to the ecological conservation world goal (10%) (7). The central problem of the territorial and economic occupation of the Cerrado is the predatory character of the predominant agricultural model. The existing Conservation Units suffer constantly from the lack of effective implementation and problems in their surroundings, such as burning, predatory hunting and fishing, contamination by pesticides and other threats of disorderly occupation registered in the region (7). In the 1950s, the construction of the new Brazil capital in central of Cerrado increased the population in this region 50-fold. As a consequence, the biome suffers degradation to human activity and land use changes (4).

In 2003, the Brazilian Ministry of Environment established the Working Group on the Cerrado Biome in order to prepare a program proposal at conservation and sustainable use of the biome. After a year, they presented the proposal for the National Program for Conservation and Sustainable Use of the Cerrado Biome – Sustainable Cerrado Program (12). The Sustainable Cerrado Program has as its main objective the promotion of conservation, restoration, recovery and sustainable management of natural ecosystems, as well as the valorization and recognition of its traditional populations (7).

According to the Brazilian Conservation Units panel, 8.61% of the Cerrado biome, i.e., 178,456.62 km^2, is protected by Conservation Units. This percentage includes 5.79% for integral protection areas and 12.30% for sustainable use areas. The biome has a total of 481 Conservation Units, distributed in many management categories such as private reserves of natural patrimony (182), environmental protection areas (105), parks (98), ecological stations (22), relevant ecological interest areas (20), natural monuments (18), forests (11), wildlife refuges (10), extractive reserves (7), biological reserves (6), sustainable development reserves (2) (13). Parks, ecological station, natural monument, biological reserves and wildlife refuge are considered integral protection areas, and the others are categorized as sustainable use areas (14).

Other projects have been developed in addition to action plans for the conservation of species. Such efforts are noticeable in preventing and controlling deforestation and burning in the Cerrado, which has the main goal to reduce the greenhouse gas emissions by at least 40% until 2020 (12) (results not published until now). On the other hand, a constitutional proposal to turn Cerrado and Caatinga as a national patrimony has been in the Brazilian National Congress for many years. The recognition of these biomes as national patrimony could provide a basis for sustainable development policies for these regions, which was not specified and determined by the 1988 Federal Constitution (12).

3.3. Microbiota from Brazilian savannah

The microbes in natural habitats are the reservoir of biodiversity and are involved in the ecosystem maintenance of the forests (15). Microbial communities are variable taking into account spatial scales, soil physicochemical properties and plant diversity, which influence the microbiota disposal in the environment (16). Researches in Brazilian Cerrado have revealed that microbial community structure are significant influenced by vegetation and soil physicochemical properties, i.e., plant diversity influence directly in microorganisms community through carbon sources and, indirectly with soil characteristics (17; 18).

Sequencing techniques have been widely used jointly with bioinformatics and statistical analyses in order to describe microorganism diversity in diverse ecosystems (Table 3-1). Such researches on Cerrado microbiota have been utilized ribosomal gene sequencing to explore microbial diversity and interactions of microbial community functioning (17; 4), as well as investigate new alternative microbial resources in biotechnology applications (19). Ohara et al. (19) surveyed the diversity of the flora and fauna of State of São Paulo, describing an extensive project that resulted on more than 12 thousand species and databases with contents 35 biological collections.

Environmental variations on Cerrado vegetation reflects differences in microbiota community composition. The first molecular survey of the bacterial communities associated with Cerrado *sensu stricto* soil and an area converted to pasture were reported by Quirino et al. (21). The authors identified the α-Proteobacteria as the most abundantly bacterial group in Cerrado *sensu stricto* soil while in Cerrado converted to pasture the most abundant group was Actinobacteria. In addition, results showed that the expected richness of species in Cerrado *sensu stricto* is approximately 10 times greater than that of Cerrado converted to pasture. The

survey highlighted the importance of Cerrado biome and evidenced the consequences of the expansion of human activity on bacterial community richness (21).

Table 3-1: Techniques employed in microorganisms diversity researches from Brazilian Cerrado.

Local investigated	Technique	Reference
Quadrilatero Ferrifero region, Minas Gerais state, Brazil.	Metabarcoding	(20)
Sete Cidades National Park (PNSC), located in the northeastern state of Piauí, Brazil.	Illumina sequencing	(17)
Ecological Reserve of Brazilian Institute of Geography and Statistics (IBGE), Federal District, Brazil.	High-throughput DNA sequencing	(4)
Ecological Reserve of Brazilian Institute of Geography and Statistics (IBGE), Federal District, Brazil.	Pyrosequencing	(18)
Ecological Reserve of Brazilian Institute of Geography and Statistics (IBGE) area and a pasture area in Federal District, Brazil.	16S rDNA-based approach	(21)

Other researches have been analyzed metagenomics data from microbial community in different types of native vegetation in Cerrado. Castro et al. (4) investigated four types of native vegetation (*cerrado denso*, cerrado *sensu stricto*, campo sujo and gallery forest) during dry and rainy seasons. The results revealed that bacterial, archaeal and fungal community structures are strongly correlated with seasonal patterns of soil water uptake. The genes investigated were associated with an adaptation to water stress. During the dry season was described an increase in genes related to iron acquisition and metabolism, dormancy and sporulation, whereas during the rainy season was noticed a significant increase in genes associated with respiration and DNA and protein metabolism. Furthermore, the relative abundance of phyla AD3, WPS-2, Planctomycetes, Thermoprotei, and Glomeromycota diminished in the rainy season, while the relative abundance of Proteobacteria and Ascomycota increased (4).

Araujo et al. (18) compared the bacterial communities in Cerrado soil of central of Brazilian Savannah in Federal District, Brazil. Samples of four different vegetation physiognomies were investigated: ‘Cerrado denso’, Cerrado *sensu stricto*, Campo sujo and

Mata de Galeria. The bacterial communities from 'Cerrado Denso' and 'Cerrado *sensu stricto*' were characterized as similar, differently from 'Campo Sujo' that forms a separate group and 'Mata de Galeria' which is the most distinct with higher species richness. Acidobacteria (40-47%) were the dominant bacterial phylum followed by Proteobacteria (34-40%) (18).

Additionally, Araujo et al. (17) using the 16S ribosomal RNA gene sequencing (Illumina) evidenced that the bacterial community structure are different across the Cerrado in the Northeast of Brazil. 'Cerrado *stricto sensu*' and 'Cerradao' show more similarities between edaphic properties and vegetation, and the study found more similarity within bacterial communities. Whereas 'Floresta decidual' and 'Campo graminoide' represent the largest environmental variation and more distinct bacteria. Proteobacteria (26%), Acidobacteria (21%) and Actinobacteria (21%) were the most abundant phyla within the four areas (17).

Vieira and coworkers (20) investigated the soil microbiome in the Quadrilatero Ferrifero region, Minas Gerais State. Soil samples were collected in the dry and rainy seasons in an iron-mining site under revegetation, semi-decidual forest, *canga* and Cerrado *strictu sensu*. The community composition was not influenced by season within each site. Among the Prokaryotes, Acidobacteria and Proteobacteria were the most abundant phyla for semi-decidual forest, Cerrado *strictu sensu* and iron-mining site under revegetation, while Actinobacteria and Acidobacteria were dominant in *canga*. For Eukarya, Opisthokonta, Stramenopiles, Alveolates, Rhizaria and Archaeplastida were the most abundant groups and Fungi accounted for 51–71% of total sequences. The authors concluded that microbiota are dissimilar among sites at the family and genus level, however sites shared the same dominant phylum level of Eukaryota and Prokaryota, and the differentiation of the soil microbiome was driven mainly by plant community composition (20).

Plant growth-promoting bacteria (PGPB) provide an important role on plant productivity improvement, enhancing plant growth and protecting plants from biotic and abiotic stresses. Silva et al., (22) evaluated the diversity, structure, and composition of PGPB in soil from Brazilian Cerrado and concluded that there were no differences between the diversity and richness of the PGPB community among the three types of physiognomies (Campo Graminoide, Cerrado *stricto sensu*, and Cerradao). Nevertheless, each physiognomy demonstrated different composition and structure of the PGPB community due to environmental conditions, highlighting to soil temperature, macronutrients, pH and moisture. *Bacillus* (36.2%), *Alicyclobacillus* (22.1%), *Mycobacterium* (12.2%), *Paenibacillus* (9.1%),

Burkholderia (7%), *Streptomyces* (5.7%), *Saccharopolyspora* (1.7%), and *Brevibacillus* (1.4%) were the dominated PGPB community (22).

On the other hand, Araujo et al. (23) evaluated the arbuscular mycorrhizal (AM) on different Cerrado physiognomies Campo graminoide, Cerrado *stricto sensu*, and Cerradao based on the 18S rRNA. The genus *Glomus* was the most abundant on all physiognomies and arbuscular mycorrhizal diversity and richness did not change across Cerrado physiognomies. Authors findings indicate *Glomus* as a sensitive AM that can indicate environmental disturbances in Cerrado systems. Furthermore, Campo graminoide may promote a negative effect on AM community structure due to low natural fertility soil, resulted by lower C/N content and high soil temperature demonstrated in low plant density in this area (23).

3.4. Biotechnological products from Cerrado microbiota

Over million years of evolution and adaptation, environmental microorganisms have been accumulated genetic and functional diversity against many selective pressure. As a result, the isolation of microbes has become a strategy to obtain new sources of biomolecules with potential applications in food, pharmaceutical, agricultural and fuels industries (19; 24). Until 1980, only around 2% of the world's microbiota were tested as enzymes sources. Accordingly, bioprospecting in natural habitats has been recommended to obtain new sources of enzymes (19).

Biotechnological products from Cerrado microbiota are scarce in literature and, consequently, in industrial applications. The safe bioprospecting have been illustrating the biotechnological products from microorganisms isolated from Brazilian savannah as proteases, lipases, catalases, xylanases, cellulases and chitinase, further to biomolecules with antimicrobial and anticancer activities. Microorganisms from Cerrado also evidence functions as growth-promoting strains and bioagent controls (Table 3-2).

The bacterial genus *Streptomyces* as known to be a protease-producer microorganism widely distributed to Brazilian Cerrado soil (2527). Pereira et al. (28) isolated a new specie of *Streptomyces* under cerrado vegetation cover and described it to produce chitinolytic proteases. Souza et al. (29) isolated from the soil of Brazilian Savannah a fungus and evaluated kinetic and thermodynamic of an acid protease produced by *Aspergillus foetidus*. On the other hand, antimicrobial activities from Cerrado microbiota were found on fungi species (30; 33).

Table 3-2: Biotechnological products from microorganisms isolated from Brazilian savannah.

Biotechnological product	Potential applications	Source	Local	Reference
Protease	Enzymatic and/or microbiological hydrolysis at the industrial level	*Streptomyces* sp. 594 from soil	Central Plateau, Brasília, Federal District	(25; 38)
Protease	Peptide synthesis	*Streptomyces cyaneus*	Experimental Station, EMBRAPA, Brasília, Federal District	(27)
Proteases and xylanases	Biotechnologial potential	*Streptomyces malaysiensis AMT-3* from soil	-	(39; 40; 26)
Proteases	Biotechnologial potential	*Streptomyces odonnellii sp.* from soil	Ecological park and research station at CENARGEN/EMBRAPA, Brasília, Federal District	(28)
Proteases	Biotechnologial potential	*Aspergillus foetidus*	Culture bank of the Enzymology Laboratory of Cell Biology Department, University of Brasília (Brasília, Federal District)	(29)
Catalase and protease	Biotechnologial potential	Bacteria from rice roots cultivated in Cerrado soil	Goias State	(41)

Xylanase	Food, feed, ethanol, pulp and paper industries	A bacterium *Lysinibacillus* sp. and a fungus *Neosartorya Spinose* isolated from soil	Selviria city, Mato Grosso do Sul State	(42)
Chitinase	Biocontrol agent	*Streptomyces* sp. from soil	Central Plateau, Brasília, Federal District	(43; 44)
Cellulase	Biotechnologial potential	Genera of *Bacillus*, *Streptomyces*, *Paenibacillus*, *Enterobacter* and *Burkholderia* from fruits	Regions near the cities of Arcos, Luminárias and Passos, Minas Gerais State	(45)
Lipase, biotin and riboflavin	Biotechnologial potential	*Pichia caribbica* (lipase and riboflavin) and *Candida oleophila* (lipase and biotin)	Atlantic Rain Forest (Ilha Bela city), Savannah (Ribeirão Preto city) and the transition area between both biomes (Campinas city) in the State of São Paulo	(19)
Antimicrobial and anticancer activities	Source for novel drugs	Endophytic fungi associated with the medicinal plant *Stryphnodendron adstringens*	Serra do Cipó National Park and Serra de São José National Park, Minas Gerais State	(31)
Antimicrobial compounds	Biotechnologial potential	*Trichoderma* spp., *Fusarium* spp., *Acremonium* spp., *Penicillium* spp., and *Paecilomyces* spp.	Savannah and Atlantic Rainforest biomes in São Paulo State	(30)
Antimicrobial activity	Biotechnologial potential	*Penicillium* sp. isolated from soil	Serra do Cipó National Park, Minas Gerais State	(32)

Antibacterial activity	Anti-infective agents industry	Fungi isolated from soil	Serra do Cipó National Park, Minas Gerais State	(33)
Mushroom	Agroextractive product for food	*Lentinus crinitus*		(46)
Lipase and amylase	Biotechnologial potential	Yeasts	Yeasts from typical fruits from Tocantins State	(36)
Invertase, pectinase and β-glycosidase	Biotechnologial potential	*Meyerozyma* (*Pichia*) *caribbica*	Yeast from pinha fruit (*Annona squamosa*)	(37)
Antibacterial activity	Probiotic properties	*Lacticaseibacillus casei/paracasei*	Lactic acid bacteria from guapeva fruit residue (*Pouteria caimito*)	(47)
Antibacterial activity	Biocontrol agent of citrus green mould (*Penicillium digitatum*)	*Aureobasidium pullulans*	Leaves and fruit of *Byrsonima crassifolia* and *Eugenia dysenterica*	(48)

Fruits are essential microhabitats for a diverse yeasts species in nature due to a high concentration of simple sugars, low pH and insect vectors. Yeasts isolated from fruits are phylogenetically diverse and the study of yeasts from different fruit communities is useful for the isolation of new species (34; 35). Such efforts on yeast investigations from the peel of typical Cerrado fruits have been isolated a potential pool of enzymes. Oliveira (36) investigated the yeasts strains isolated from babassu (*Orbignya* sp.), buriti (*Mauritia flexuosa*), tucum (*Bactris inundata*), inajá (*Attalea maripa*) and macaúba (*Acrocomia aculeata*), typical fruits of the Cerrado from Tocantins State. The capacity production of lipase, amylase, protease and pectinase enzymes were tested and among 142 yeasts, 61 strains showed positive results for lipase production and only one yeast showed an amylase production halo. There were no positive results for pectinase and protease producers (36). Araujo (37) isolated and evaluated the biotechnological potential of yeasts associated with ingá (*Inga edulis*), guavira (*Campomanesia adamantium*) and pinha (*Annona squamosa*) fruits. Among nine isolates it were possible to obtain invertase, pectinase and β-glycosidase enzymes produced by *Meyerozyma* (*Pichia*) *caribbica* (37).

Trindade et al. (47) evaluated lactic acid bacteria from fruit processing residues from the Brazilian Cerrado with probiotic properties. The authors identified 14 isolates by sequencing 16S rRNA gene in *Lactiplantibacillus plantarum/pentosus*, *Lacticaseibacillus casei/paracasei*, *Pediococcus adicilactici* and *Weissella cibaria/confuse*. Eleven isolates inhibited the growth of *Esherichia coli*, *Staphylococcus aureus* and *Salmonella* sp., in which *L. casei/paracasei* from guapeva residue showed the greater ability to survive in gastrointestinal conditions. Authors aimed future investigations of probiotic properties for selected lactic acid bacteria. Sperandio et al. (48) isolated yeasts from leaves and fruit of *Byrsonima crassifolia* and *Eugenia dysenterica* from Cerrado conservation areas in order to explore their potential for inhibiting citrus green mould, a filamentous fungi *Penicillium digitatum* that frequently deteriorate postharvest oranges. Identification by 26S rDNA classified yeasts as *Aureobasidium*, *Meyerozyma*, *Candida*, and *Pichi*a genera. *Aureobasidium pullulans* was capable to reduce the incidence of disease *in vitro* and *in vivo* tests and increase the storage time of fruit, evidencing yeasts as important postharvest biocontrol agents.

Genetic engineering have made possible to access non-cultivable microorganisms that are difficulty to cultivate under laboratory conditions. Inserting DNA into substitute cultivable hosts allows the constructions of libraries of enzymes, antibiotics, and other biological

compounds with biotechnological potential (49). Lopes et al. (50) performed a whole-genome sequencing of a filamentous fungi *Acremonium strictum* AAJ6 isolated from plant debris in Cerrado biome. The fungus produce lignocellulase-degrading enzymes, which is interesting for sustainable biofuels production. Using metagenomic methods, authors expressed the carbohydrate-active enzymes (CAZymes) from plasmid or genome-integrated in order to evaluate the ethanol production from cellulosic substrates in Brazilian industrial *Saccharomyces cerevisiae* strains evolved for thermotolerance. Results indicated the improved performance of thermotolerant industrial strains with genome-integraded CAZymes in the simultaneous saccharification and fermentation (SSF) process for 2G ethanol.

Native soils and perennial and annual crops differentially affect biochemical activity in Brazilian Cerrado soils. The native vegetation replacement causes a decrease on biodiversity and reduces biochemical activity; however annual crops stimulate microbial activity and maintain nutrient cycling (51). Hydrolases and oxidoreductases are influenced by land-use change working as soil quality indicators. Zago et al. (52) investigated soil samples from crop-livestock-forest system, old pasture and a native ecosystem of the Brazilian Cerrado, and the activity of β-glucosidase, acid phosphatase and phenoloxidase indicating a competition of soil microorganisms in a low resource environment (old pasture). On the other hand, the higher the content of soil organic matter, the greater the specificity constant for β-glucosidase and arylsulfatase on enzyme kinetic parameters.

Studies have been investigated potential microorganisms in Cerrado biome that suffer human activity and/or extensive agriculture. Fernandes et al. (53) tested esterase and peroxidase from a bacilococcus of the genus *Klebsiella* isolated from Brazilian Cerrado soy crop and enzymes incubated with epoxiconazol and pyraclostrobin resulted in an absorbance reduction, suggesting chemical modification of fungicides. Peixoto et al. (54) revealed a microbial potential for unpretreated polyethylene (PE) biodegradation from plastic debris found in soil of the Brazilian Cerrado. Isolates of genera *Comamonas, Delftia,* and *Stenotrophomonas* showed metabolic activity and cellular viability after a 90-day incubation with PE as unique carbon source, further to PE chemical changes. Coworkers concluded that these microorganisms were able of degrading unpretreated PE of very high molecular weight, 19000 $g.mol^{-1}$, and survive prolonged intervals at this circumstance, propounding waste management approaches and environmental decontamination, as well as future understood of synthetic polymers metabolism.

3.5. Conclusion and perspectives

Flora and fauna from Cerrado biome are often the focus of studies and preservation efforts while microbial community is neglected (55; 21). Investigations in the Cerrado biome have described the influence of different vegetation and soil physicochemical properties, as well as the dry and rainy seasons on characteristics of microorganisms. The focus of this review were describe unexploited biotechnological products from Brazilian Cerrado biome from microbiota. Natural habitats has become the focus for isolation and screening of new microorganism species and, consequently, new sources of biomolecules with potential biotechnological purposes. The global market for biological catalysts in industrial applications reached $ 6.4 billion in 2021, and estimates show grow to $ 8.7 billion in 2026 at compound annual growth rate of 6.3% for the period of 2021-2026 (56). Explore native preservation and agroextractivism areas have to be encouraged to local population, researchers and government actions in order to find out biotechnological compounds with potential interest for application in industries. Our research group have been investigated microorganisms from Cerrado in order to found enzyme producers in a meaningful scale for biotechnological purposes. Samples were collected from soil, peel fruits, flower and leaves located in Triangulo Mineiro region, Minas Gerais State. We formed a microorganism dataset with 65 isolates, including bacteria and yeasts that have been tested for enzymes production, such as proteases and amylases.

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Capítulo 4 – Delineamento inicial dos experimentos: Análise do potencial enzimático de microrganismos isolados do Cerrado Mineiro frente à enzimas proteolíticas

4.1. Prospecção e isolamento de microrganismos

4.1.1. Coleta de dados

As amostras foram coletadas na estação quente e período chuvoso do mês de dezembro de 2018 no município de Uberlândia - Minas Gerais, no qual o bioma característico é o Cerrado. O clima predominante é o tropical com temperatura média anual em torno de 22 °C. O índice pluviométrico é de 1600 mm concentrando-se nos meses de verão (22). As amostras foram coletadas em uma fazenda com coordenadas 18°58'14.8"S 48°17'33.2"W. Quatorze amostras foram coletadas assepticamente e aleatoriamente, sendo proveniente do solo, superfície de folhas, frutos e flor (Tabela 4-1). O solo obtido foi coletado a uma profundidade máxima de 5 cm.

Tabela 4-1: Descrição das amostras coletadas no Cerrado Mineiro.

Dados	Quantidade de Amotras
Solo	2
Frutos	9
Folhas	2
Flor	1
Total	14

Obs.: Uma das amostras de frutos foi descartada no laboratório, uma vez que continha larvas.

4.1.2. Isolamento das colônias

No mesmo dia em que realizou-se a coleta das amostras, estas foram manuseadas em laboratório não ocorrendo o armazenamento. As amostras de solo foram diluídas em água salina esterilizada 0,9% NaCl, ou seja, uma porção de 1 grama de solo foi suspensa em 9 mL de água salina. As amostras de frutos, folhas e flor foram colocados em 100 mL de água peptonada esterilizada 0,1%, as quais foram colocadas sob agitação por 1 hora a 120 rpm, em temperatura ambiente. Os frutos não tiveram a casca retirada, com exceção do abacate. A tabela 4-2

evidencia os tipos de amostras que foram coletadas juntamente com as espécies e a quantidade utilizada para diluição, seja em água peptonada 0,1% ou salina 0,9%.

Tabela 4-2: Detalhamento das amostras utilizadas em cada recipiente para isolamento de microrganismos.

Tipo de Amostra	Amostras	Quantidade utilizada (g)	Quantidade utilizada em unidades
Fruto	Pitanga	2,5	1
	Manga	3,34	1
	Café	3,2	4
	Goiaba	7,49	1
	Acerola	4,09	1
	Abacate	3,6	2 pedaços da casca
	Parece Goiaba	-	5
	Fruto X	5,6	1
Flor	Flor X	1,0	6 pétalas
Folha	Folha da Mangueira*	1,0	1 folha grande
	Folha da Pintagueira	1,0	5 folhas pequenas
Solo	Barranco	1,0	
	Raiz da Mangueira	1,0	

Obs.: As quantidades não foram proporcionais, uma vez que uma unidade de um fruto era diferente em volume de uma unidade de outro fruto. Assim, foram considerados a área superficial de cada fruto e, também, a quantidade a qual foi possível coletar. * Presença de patógenos na folha.

Em seguida, foi realizado o plaqueamento de todas as amostras em triplicata, utilizando meio universal de levedura YPD, composto por glicose (10 $g.L^{-1}$), peptona (5 $g.L^{-1}$), extrato de levedura (3 $g.L^{-1}$) e ágar (20 $g.L^{-1}$). Assim, 100 uL foi disposto em cada placa e plaqueado com auxílio de um bastão de vidro. Não foram realizadas diluições em série, visto que era necessário

observar o crescimento direto das suspensões do solo em água salina e da água peptonada das cascas dos frutos, folhas e flor. Também não foi utilizado um meio seletivo cotendo antibiótico.

Logo, cada placa que continha inúmeros microrganismos foi utilizada para iniciar o isolamento das colônias. Devido ao grande número de microrganismos foi selecionado 5 colônias diferentes de cada amostra, ou seja, das placas em triplicata de uma amostra foram selecionados cinco microrganismos totais. Para isolamento foram considerados aspectos como coloração, tamanho da colônia e pureza. As placas foram incubadas a 30 °C e, após 48 horas de crescimento, cada colônia foi transferida para uma nova placa contendo o meio YPD. A codificação de cada microrganismo foi feita levando em consideração: a fonte em qual foi encontrada; o número da placa retirada a qual estava em triplicata (1, 2 ou 3); e a(s) colônia(s) retirada(s) de uma placa seguiam as letras do alfabeto (A, B, C ou D) (Tabela 4-3). Para as amostras PG, FX, CA e AB, as colônias destas foram transferidas com 72 horas de incubação. A amostra proveniente da pitanga foi descartada, pois não houve nenhum crescimento.

Assim que as colônias foram obtidas puras foi feito o armazenamento em microtubos contendo meio líquido YPD e glicerol esterilizados, na proporção 1:1. Em seguida, estes foram acondicionados no ultrafreezer a -70 °C. Dessa forma, foi construído um banco de microrganismos o qual totalizam em 65 cepas.

Tabela 4-3: Levantamento do banco de dados dos microrganismos isolados e a sua origem.

Local de armazenamento	Origem da coleta	Codificação do microrganismo
Caixa 1	Solo da raiz da mangueira (SRM)	SRM 1A
		SRM 1B
		SRM 2AA
		SRM 2B
		SRM 3AA
		SRM 3ABA
		SRM 3ABB

	Solo barranco (SB)	SB 1A
		SB 2A
		SB 2B
		SB 2C
		SB 3A
	Folha da pitangueira (FP)	FP 1A
		FP 1BA
		FP 1BB
		FP 2A
		FP 3A
		FP 3B
	Folha da mangueira (FM)	FM 3B
		FM 3D
		FM 3E
		FM 3F
		FM 3GA
		FM 3GB
	Flor X (FLX)	FLX 3A
		FLX 3BA
		FLX 3C
		FLX 3BBA
		FLX 3BBBB
Caixa 2	Goiaba (GO)	GO 1A
		GO 1B

		GO 2A
		GO 2B
		GO 3A
	Abacate (AB)	AB 1A
		AB 2A
		AB 2B
		AB 3A
		AB 3B
	Acerola (AC)	AC 1A
		AC 2A
		AC 2B
		AC 3AA
		AC 3B
	Café (CA)	CA 2A
		CA 2B
		CA 3A
		CA 2B
Caixa 3	Fruto X (FX)	FX 1A
		FX 1B
		FX 2A
		FX 3A
		FX 3B
	Manga (MA)	MA 1A
		MA 1BA

	MA 1BB
	MA 1C
	MA 1D
	MA 1E
Parece Goiaba (PG)	PG 1A
	PG 1B
	PG 1C
	PG 1DA
	PG 1DB
	PG 3A

Obs.: Há amostras em que foram armazenadas mais de 5 microrganismos, pois no processo de obtenção da colônia pura foram encontrados mais de 1 microrganismo em uma mesma placa, o qual também foi considerado para a formação do banco de microrganismos.

4.2. Seleção de microrganismos protudores de enzimas proteolíticas

Inicialmente, o intuito foi utilizar meio sólido contendo gelatina (23) para identificação de halos em torno da colônia. Estes halos corresponderiam à digestão da gelatina pelas respectivas proteases produzidas pelo microrganismo. Contudo, não foi possível tal visualização com os testes realizados. Outros meios também foram testados, como agar milk (24) e agar Sabouraud (25). A utilização de uma destas metodologias facilitaria todo o processo de identificação de culturas potencialmente produtoras de proteases levando em consideração tempo, além de uso de reagentes e equipamentos. Contudo, deu-se início às fermentações submersas em meio YPD líquido, um meio universal de leveduras, em que a avaliação da atividade proteolítica foi realizada por teste quantitativo utilizando o substrato azocaseína.

As fermentações submersas foram realizadas em triplicata para cada isolado presente no banco de microrganismos. Primeiramente, cada microrganismo era reativado dos microtubos armazenados a -70 °C em placas contendo meio YPD sólido. Em seguida, esta placa era utilizada para repique de 3 novas placas com mesmo meio de cultivo. Estas eram utilizadas para

dar início à fermentação, na qual o microrganismo era transferido para 100 mL de meio YPD líquido.

Os frascos erlenmeyers foram colocados sob agitação orbital a 120 rpm, em temperatura ambiente (30 °C) durante 120 horas. A cada 24 horas alíquotas eram retiradas de cada fermentação, centrifugadas (7000 g durante 20 minutos) e o sobrenadante submetido às análises de caracterização da biomassa por densidade óptica (600 nm) e avaliação da atividade proteolítica utilizando substrato azocaseína, em que a leitura final era feita em espectrofotômetro (440 nm) (26) . Os resultados de produção máxima das enzimas proteolíticas, em $U.mL^{-1}$, de cada isolado estão evidenciados na tabela 4-4, juntamente com o tempo de fermentação em que foi atingido.

Tabela 4-4: Produção máxima e tempo de fermentação das enzimas proteolíticas dos microrganismos isolados.

Microorganismo	Atividade Proteolítica ($U.mL^{-1}$)	Tempo de fermentação (h)
SRM 2AA	7,8044	48
SRM 2B	1,7636	48
FLX 3C	1,5929	120
AB 2A	1,2587	48
FX 3B	1,2196	120
GO 2A	1,1698	48
PG 1A	1,0524	120
AB 3A	0,9600	120
GO 2B	0,9173	24
FX 1B	0,8996	120
CA 3B	0,8178	24
FX 3A	0,8142	48
PG 1DB	0,8036	72

AC 3B	0,7893	48
SRM 1A	0,7573	96
SRM 3AA	0,7289	120
FLX 3BBA	0,7289	120
SB 3A	0,7218	96
FLX 3A	0,7076	120
SRM 1B	0,7004	48
CA 2A	0,6969	96
AC 3AA	0,6933	48
FX 1A	0,6809	48
FLX 3BBBB	0,6756	120
CA 2B	0,6684	96
SB 1A	0,6471	120
AC 1A	0,6436	120
GO 3A	0,6400	96
AC 2B	0,6329	48
SB 2A	0,6151	96
AB 1A	0,6116	120
SRM 3ABB	0,6080	96
FM 3D	0,6044	24
PG 1DA	0,6044	72
AB 3B	0,6009	120
FX 2A	0,5867	48
FM 3GB	0,5582	96

MA 1A	0,5493	96
MA 1D	0,5440	72
FM 3GA	0,5369	96
SB 2C	0,5262	48
SB 2B	0,5227	24
MA 1BA	0,5173	72
GO 1A	0,5120	72
MA 1C	0,5084	120
FP 3A	0,4942	120
FP 1BB	0,4942	48
MA 1BB	0,4800	120
PG 3A	0,4693	120
GO 1B	0,4658	72
SRM 3ABA	0,4587	96
FM 3F	0,4587	72
FP 2A	0,4409	48
MA 1E	0,4373	120
CA 3A	0,4267	120
AB 2B	0,4124	96
FP 3B	0,4089	96
AC 2A	0,4089	120
FM 3B	0,4018	24
FLX 3BA	0,3840	72
PG 1B	0,3556	120

PG 1C	0,3342	120
FM 3E	0,3307	96
FP 1BA	0,3271	48
FP 1A	0,3200	48

A maior produção de proteases foi pelo microrganismo SRM-2AA, o qual pode ser visualizado em microscópio (Figura 4-1). Pela análise microscópica e o método de Coloração de Gram (27) pode-se concluir que são bactérias do tipo cocobacilo Gram-negativo.

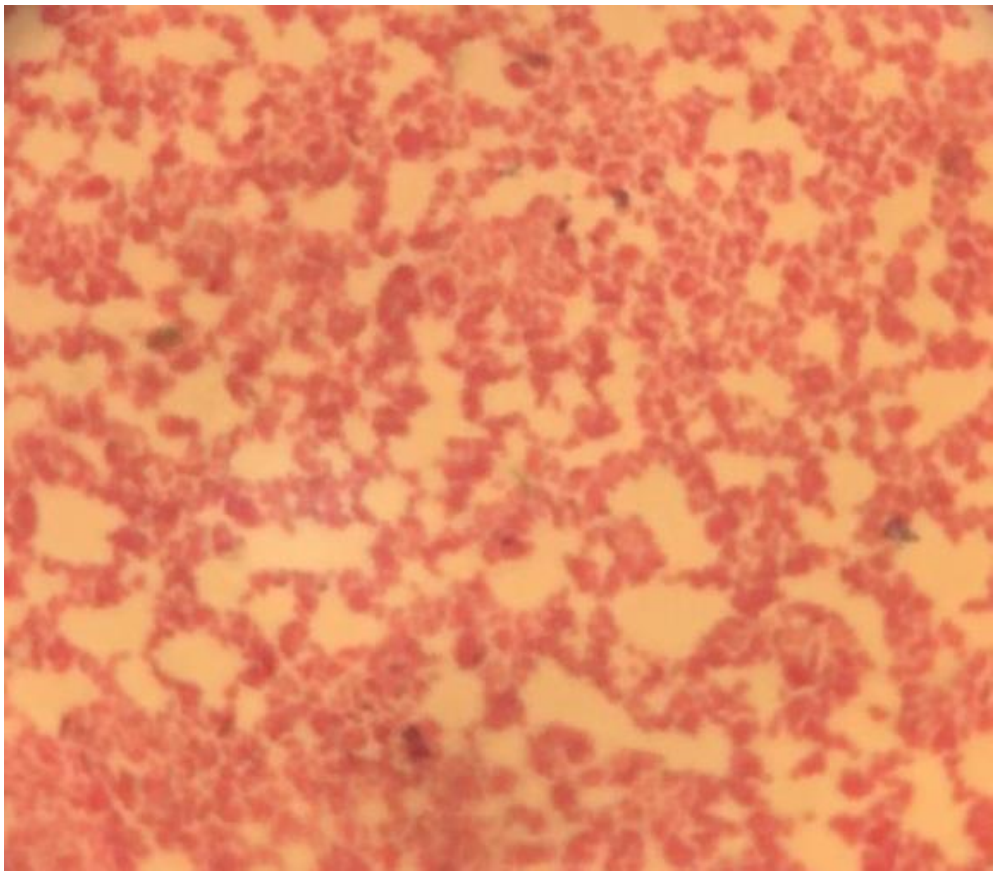

Figura 4-1: Bactéria SRM-2AA cocobacilo Gram-negativo.

Capítulo 5 - Artigo experimental a publicar

Screening and optimization of protease production by bacterium SRM-2AA isolated from Brazilian Cerrado

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Abstract

Cerrado biome is a natural habitat with potential for obtaining new biocatalysts. Screening of protease producing from Triangulo Mineiro region, Minas Gerais State (Brazil) was conducted under submerged fermentation (glucose 10 $g.L^{-1}$, peptone 5 $g.L^{-1}$ and yeast extract 3 $g.L^{-1}$) at 30 °C during 120 hours. The best extracellular protease-producer, a bacterium coded as SRM-2AA isolated from soil, had its medium composition optimized and scaled up. Analysis of different nitrogen (ammonium nitrate, ammonium citrate, ammonium sulfate, peptone and yeast extract) and carbon (glucose, peptone, whey permeate, sugarcane molasses and soy molasses) sources allowed identifying the yeast extract (16.46 $U.mL^{-1}$) and whey permeate (23.32 $U.mL^{-1}$) as the best precursors for protease production. Statistical optimization of media composition was performed by a full factorial design of four factors (whey permeate concentration, yeast extract concentration, temperature and initial medium pH), in which the first two variables was the most significant ones (positive effects), followed by temperature and pH that were fixed at 25 °C and 7.0. A central composite design was carried out next distinguishing optimal levels of whey permeate concentration and yeast extract concentration. Statistics indicated that proteolytic activity was maximized when factors were selected at their highest values. Culture media-100mL composed by whey permeate at 12.83 $g.L^{-1}$ and yeast extract at 4.41 $g.L^{-1}$

prepared with phosphate-buffered saline, pH 7.0, incubated at 25 °C during 24 hours was validated as optimized conditions (30.97 $U.mL^{-1}$) and satisfactory scaled up in 1500 mL-bioreactor attaining 34.13 $U.mL^{-1}$ of proteolytic activity. The protease displayed optimum activity at pH 8.2 and 45 °C and had activity preserved from 1 to 40 °C and a pH range 5.8 to 7.4. Preliminary applications suggest that proteolytic enzymes from bacterium SRM-2AA its suitability in food industries, being a good candidate for cheese making as well as a meat tenderizer.

Keywords: proteinase; bioprocess; bioreactor; fermentation; scale up.

5.1. Introduction

Brazil is one of the most abundant countries in biodiversity, reaching around 9.5% of all known species in the world (1). The Brazilian Cerrado, a savanna-like region of central Brazil, is the second largest biome after the Amazon rainforest, occupying 23.92% of the country's territory with 2,036,448 km^2 (2). The Cerrado biome offers habitations to a wide range of microorganisms, animals and plants, being considered one of the 25 most vital terrestrial biodiversity hotspots (3).

Environmental microorganisms are the reservoir of biodiversity in natural habitats. They are involved in nutrient transformations for ecosystem maintenance and microbial communities structure are influenced by carbon sources of plant roots systems and physicochemical properties of soil (4). Microbial community have been accumulated genetic and functional diversity over million years of evolution and adaptation to various selective pressure. Consequently, microbes are the main sources of new biomolecules for biotechnology industries, due to a broad chemical and structural diversity (5).

Proteases are proteolytic enzymes that act on peptide bonds and represent one of the most important catalysts classes, comprising 65% of total worldwide enzyme sale (6). Peptidases have diverse applications in industrial market, especially in food processing and detergent formulations as well as textiles, silk, tanning, leather, pharmaceuticals industries, biosynthesis, silver recovery and bioremediation. *Bacillus* and *Aspergillus* species are the most representative microbial strains used to obtain proteases (7).

Until 1980, only around 2% of the world's microorganisms were analyzed as enzymes sources. As consequence, prospection of microbiota has been recommended in natural habitats for obtaining new biocatalysts (5). Some studies have described the safe Cerrado biome

bioprospecting as Barbosa et al. (8) that isolated filamentous fungus with antimicrobial activities from soil of Savannah and Atlantic Rainsforest biomes in São Paulo State. Takahashi et al. (9) also isolated fungus species from “Serra do Cipó” National Park, in Sete Lagoas region, Minas Gerais State, that investigated antibacterial activity against pathogenic microorganisms in order to develop new antibiotics. On the other hand, Castro et al. (10) explored the bacterial, archaeal and fungal associations in Cerrado soils within four different vegetation physiognomies, at a protected area in the Federal District of Brazil, in order to elucidate taxonomic composition and potential functions of microorganisms.

However, there are few researches describing proteolytic enzymes producer from Cerrado soil, especially in Triangulo Mineiro region, Minas Gerais State. For this reason, we investigated microbiota present in Cerrado soil in order to select potential proteolytic enzymes producers. The best bacteria producer was chosen to optimize culture medium, using agro-industrial residues as carbon sources, and scale up its production. Further, a partial biochemical characterization and applications were performed with proteases.

5.2. Material and methods

5.2.1. Isolation and screening of microorganisms for extracellular protease production

5.2.1.1. Microorganisms isolation from Brazilian Cerrado

Microorganisms were collected on a farm located at Uberlandia, Minas Gerais State, with coordinate’s 18°58'14.8"S 48°17'33.2"W. The sampling was carried out on the warm and rainy season of December 2018. A total of 14 samples of soils (2), fruits peel (9), sheets (2) and flower (1) were collected aseptically and randomly. Soil samples were obtained up to a maximum depth of 5 cm. In laboratory, one gram of each soil sample were diluted in 10 mL of sterile saline water 0.9% NaCl. The fruits peel, sheets and flower samples were disposed in 100 mL of sterile solution containing 0.1% of peptone and submitted to orbital agitation (110 rpm) on Erlenmeyer flasks for 1 hour. Then, aliquots of each dilution (100 uL) was plated in triplicate on Petri dishes, containing solid YPD medium (glucose 10 $g.L^{-1}$, peptone 5 $g.L^{-1}$, yeast extract 3 $g.L^{-1}$ and agar 20 $g.L^{-1}$). Inoculated Petri dishes were incubated at room temperature (30 °C) for 48 to 72 hours and every 24 h growing colonies were observed. Thereafter, five colonies of each triplicate sample were selected to isolation. The pure colonies were obtained and stored in microtubes containing YPD medium and glycerol (1:1) in the ultra-freezer at - 70 °C. Consequently, we created a microorganism databank including 65 strains.

5.2.1.2. Screening for protease-producer microorganism

Each strain was investigated to produce proteases by submerged fermentations. From –70 °C storage, each microorganism was reactivated in Petri dishes containing solid YPD medium. Strains were spread in triplicate in solid YPD medium and then transferred to 100 mL of liquid YPD medium (glucose 10 $g.L^{-1}$, peptone 5 $g.L^{-1}$ and yeast extract 3 $g.L^{-1}$). Fermentations were submitted to agitation (110 rpm) at room temperature (30 °C) during 120 hours. Every 24 h, the cell-free supernatant obtained by centrifugation (7,000 g, 20 minutes) was obtained for proteolytic activity and cell growth analyses.

5.2.2. Elemental analysis

The bacteria cells were grown in 100 mL of YPD medium during 48 hours. Cells were harvested in their exponential phase, washed three times (by centrifugation and resuspension) using pure water and dried in a vacuum freeze dryer for 21 hours. Thereafter, bacteria biomass sample was submitted to elemental carbon, hydrogen, nitrogen and sulfur (CHNS) analyzer Elementar Vario MACRO (Hanau, Germany). The duplicate bacteria samples were packed in tin sheets capsules, weighed and taken to the equipment for analysis. Elemental analysis was carried out by combustion in a pure oxygen atmosphere (99.999%) and the gases formed from this combustion were quantified on a thermal conductivity detector. Each element was expressed in percentage of the total mass of the original sample.

5.2.3. Analysis of carbon and nitrogen sources

The microorganism growth and protease production were analyzed using different carbon and nitrogen sources. Carbon and nitrogen proportions were determined from CNHS analysis. Ammonium nitrate, ammonium citrate, ammonium sulfate, peptone and yeast extract were analyzed at 2 $g.L^{-1}$ for nitrogen sources investigation, using glucose (8 $g.L^{-1}$) as carbon source. For carbon sources evaluation, yeast extract (2 $g.L^{-1}$) was fixed as nitrogen source and sucrose, glucose, peptone, whey permeate (SOORO), sugarcane molasses (British Petroleum, Brazil) and soy molasses (Selecta) tested at 8 $g.L^{-1}$. Each submerged fermentation contained 100 mL of medium was submitted at 110 rpm during 72 hours. At every 12 hours, cell growth and proteolytic activity were measured using cell-free supernatant obtained by centrifugation (7,000 g, 20 minutes).

5.2.4. Bioprocess optimization of culture medium composition for protease production

5.2.4.1. Inoculum preparation of bacterium SRM-2AA

A bacterial colony was inoculated in 50 mL-Erlenmeyer flasks containing 20 mL of YPD medium and incubated for 24 h at 30 °C under agitation (110 rpm). A cell suspension with optical density of 1 at 600 nm was used to inoculate a 250 mL-Erlenmeyer flask containing 100 mL of each medium.

5.2.4.2. Full factorial design

A full factorial design 2^4 was chosen to screen the fermentation parameters for protease production. The independent variables evaluated were whey permeate concentration, yeast extract concentration, pH and temperature (Table 5-1). Fermentations were prepared in 250 mL-Erlenmeyer flasks using phosphate-buffered saline (sodium chloride 8.0 $g.L^{-1}$, potassium chloride 0.2 $g.L^{-1}$, disodium phosphate 1.44 $g.L^{-1}$ and monopotassium phosphate 0.24 $g.L^{-1}$) and the final pH was adjusted with NaOH and/or HCl 0.1 M. The mediums were inoculated and carried out during 24 h on an orbital shaker (110 rpm). The cell-free supernatant obtained by centrifugation (7,000 g, 20 minutes) was used for determining the proteolytic activity and cell growth. Statistica 12.0 software was used to handle statistical analyses and the significance of all coefficients was assessed from the error estimate at 95% confidence level. The experiments were conducted in random order.

Table 5-1: Actual and coded levels of factors used in the 2^4 full factorial design to investigate the protease production.

Factor	Low (-1)	Central (0)	High (+1)
Whey permeate ($g.L^{-1}$)	6	8	10
Yeast extract ($g.L^{-1}$)	1	2	3
PH	6	7	8
Temperature (°C)	25	30	35

5.2.4.3. Central composite design

In order to improve proteolytic production, pH and temperature was fixed at best values at 7.0 and 25 °C, respectively, while whey permeate and yeast extract concentrations were modified according to a 2^2 central composite design (CCD). Two consecutively CCDs were

performed due to the first design was not enough to identify optimum conditions (Table 5-2 and 5-3). The experiments were carried out in random order and Statistica 12.0 utilized to analysis. The experimental data was submitted to adjustments using coefficient of determination, analysis of variance (ANOVA) and a Fisher-based test.

Table 5-2: Actual and coded levels of factors used in the first 2^2 central composite design to investigate the protease production.

Factor	Low (-1.41)	Low (-1)	Central (0)	High (+1)	High (+1.41)
Whey permeate ($g.L^{-1}$) (x_1)	7.17	8.00	10.00	12.00	12.83
Yeast extract ($g.L^{-1}$) (x_2)	1.58	2.00	3.00	4.00	4.41

Table 5-3: Actual and coded levels of factors used in the second 2^2 central composite design to investigate the protease production.

Factor	Low (-1.41)	Low (-1)	Central (0)	High (+1)	High (+1.41)
Whey permeate ($g.L^{-1}$) (x_1)	7.17	8.00	10.00	12.00	12.83
Yeast extract ($g.L^{-1}$) (x_2)	3.00	3.41	4.41	5.51	5.82

5.2.4.4. Optimized medium validation

The optimized culture medium was performed in a quintuplicate to validate the system model. The medium containing whey permeate 12.83 $g.L^{-1}$ and yeast extract 4.41 $g.L^{-1}$ prepared with phosphate-buffered saline, pH 7.0 was incubated at 25 °C during 24 hours. Then, the cell-free supernatant obtained by centrifugation (7,000 g, 20 minutes) was utilized for proteolytic activity and cell growth analyses.

5.2.5. Proteolytic activity and cell growth determination

The enzyme activity assay was performed using azocasein as substrate (11; 12). The microtube, containing 125 µL of azocasein 1% (solubilized in 4% ethanol (v/v) and 100 mM phosphate buffer, pH 7.0) and 125 µL of enzyme extract, was incubated for 15 min at 37 °C. The reaction stopped by addition of 750 µL of trichloroacetic acid 5% (w/v) and samples centrifuged at 7,000 g for 5 min at room temperature. One unit of activity was defined as the

amount of enzyme required to produce an increase in optical density of 1.0 at 440 nm and 37 °C within 1 hour.

A cell suspension was measured in spectrophotometer at 600 nm after fermentation. One optical density correspond to 2.6 x 10^{13} cells per milliliter (cells.mL^{-1}).

5.2.6. Effects of pH and temperature on proteolytic activity

The pH of the reaction mixture containing 1.0% azocasein was analyzed over the range 5.0-13.0. The 0.1 M buffers used were citrate-phosphate (pH 5.0; 5.8), sodium phosphate (pH 5.8; 6.6; 7.4); Tris-HCl (pH 7.4; 8.2; 9.0), carbonate-bicarbonate (pH 9.8; 10.6), phosphate-NaOH (pH 11.0) and KCl-NaOH (pH 12.0; 13.0). The effect of temperature on protease activity was evaluated by incubating the reaction mixture, 1:1 (v/v), proteolytic enzymes and azocasein 1.0% prepared with 0.1 M Tris-HCl buffer, pH 8.2, at selected temperatures: 0, 5,10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80 °C. Relative activity was expressed as the percentage of activity detected in relation to maximum protease activity (13).

5.2.7. Influence of pH and temperature on protease stability

The thermal stability was measured after enzyme pre-incubation in 0.1 M Tris-HCl buffer, pH 8.2, 1:1 (v/v) at different temperatures – from 1 to 80 °C, ranging from 5 to 5 °C, during 360 minutes. To define pH stability, the protease was mixed with predetermined 0.1 M buffer solution 1:1 (v/v) - citrate-phosphate (pH 5.0; 5.8), sodium phosphate (pH 5.8; 6.6; 7.4); Tris-HCl (pH 7.4; 8.2; 9.0), carbonate-bicarbonate (pH 9.8; 10.6), phosphate-NaOH (pH 11.0) and KCl-NaOH (pH 12.0) - at 45 °C for 360 minutes. Aliquots were withdrawn at intervals of 0, 20, 40, 60, 120, 180, 240, 300 e 360 minutes. Residual proteolytic activity was determined under standard assay conditions (14).

5.2.8. Effect of metal ions on proteolytic activity

The 1.0% azocasein solutions were prepared with 0.1 M Tris-HCl buffer, pH 8.2, containing different metal ions: Mg^{+2}, Ca^{+2}, Cu^{+2}, Co^{+2} and Ni^{+2}, at 5 mM and 10 mM concentrations. The effect on enzyme activity was measured using standard assay protocol at 45 °C and expressed in percentage of relative activity. The proteolytic activity without metal ions was taken as the control and considered as 100% (15).

5.2.9. Growth kinetics and protease production by the bacterium SRM-2AA

Inoculum preparation of bacterium SRM-2AA was firstly prepared in an YPD medium. The proteolytic enzyme production and bacterial growth was carried out in optimized medium containing whey permeate 12.83 $g.L^{-1}$ and yeast extract 4.41 $g.L^{-1}$ prepared with phosphate-buffered saline, pH 7.0. A cell suspension with 1.0 optical density at 600 nm was added to each 250 mL-Erlenmeyer flasks containing 100 mL of optimized medium. The flasks were incubated at 25 °C during 24 h on an orbital shaker (110 rpm). The cell-free supernatant obtained by centrifugation (7,000 g, 20 minutes) was used for determining the cell growth and proteolytic activity at every 2 hours. Optimums pH and temperature were used on protease activity assay.

5.2.10. Scale up of protease production by the bacterium SRM-2AA in a 1500-mL bioreactor

Production was scaled up in Biostat B fermenter - B. Braun Biotech International, with 1.5-Lworking volumes. The bioreactor was equipped with monitors to measure and control variables such as temperature, pH, stirring rate and dissolved oxygen (16). Fermentation was carried out in optimized medium conditions (whey permeate 12.83 $g.L^{-1}$ and yeast extract 4.41 $g.L^{-1}$ prepared with phosphate-buffered saline, pH 7.0) during 24 hours and was maintained between 25 °C, 1 vvm and 110 rpm. At every 2 h, the sample was drawn and cell-free supernatant obtained for cell growth and proteolytic assay analyses, in which pH and temperature were used on optimums conditions for protease activity assay.

5.2.11. Applications

5.2.11.1. Milk clotting activity (MCA)

A 10% solution of skim milk powder (Itambé) was supplemented with $CaCl_2$ at different concentrations: 0.01; 0.02; 0.04; 0.06; 0.08 and 0.1 $mol.L^{-1}$. Test tubes containing five milliliters of each solution were pre-incubated in a thermostatic bath at 35 °C for 10 minutes. Then, 0.5 mL of proteolytic enzyme was added to the test tubes and they were placed on rotating supports to be shaken. The time when the first particles were formed was measured. One milk clotting unit (MCU) was defined as the amount of enzyme required to clot 1 mL of substrate in 40 min at 35 °C and was calculated as follows: MCU=2400/t x S/E, where t is the time required for clot formation, S is the milk volume, and E is the enzyme volume (17).

5.2.11.2. Mechanical texture measurement

In order to analyze tenderization effect of proteases produced by bacterium SRM-2AA, beef meat without fat was cut into 3 cm x 3 cm x 3 cm cubes and incubated with proteolytic enzymes at 4 °C during 20 hours. Meat cubes incubated in distilled water were used as a negative control. The bromelain extract obtained from the processing of pineapple fruit (12) was used as a positive control and samples had enzyme concentration standardized in 29.97 $U.mL^{-1}$. After incubation, the beef cubes were heated at 70 °C for 20 minutes in a thermostatic bath (18). The shear force required to cut the muscle fibers was measured using a Texture Analyser TA.XT.plus (Stable Micro Systems Ltd., Vienna Court, UK) with a Warner-Bratzler shear force blade and a heavy duty platform attached to equipment. A blade cut meat sample perpendicular to the longitudinal orientation of the muscle fibers. The texturometer calibration was performed at 2.00 $mm.sec^{-1}$ speed, 40.00 mm distance and 30 g for calibration weight. The maximum shear force was expressed in N.

5.3. Results

5.3.1. Screening of protease-producing bacteria

The selection of an ideal producer of extracellular enzymes is strongly influenced by the components of the medium. We selected a standard YPD medium for screening microorganisms isolates in order to find out protease-producer candidates. The bacterium coded SRM-2AA showed the maximum protease activity with significant discrepancy from the other strains (Figure 5-1). Followed by SRM-2B, FLX-3C, AB-2A and FX-3B strains that obtained 22.60, 20.41, 16.13 and 15.63% of relative proteolytic activity. The majority of microorganisms did not produce meaningful proteases by the methodology employed, not being shown in Figure 5-1 producers below 10% of relative activity.

5.3.2. Preliminary screening of carbon and nitrogen sources for protease production by bacterium SRM-2AA

The bacterial strain coded as SRM-2AA was selected as a potential protease-producer to be researched in this survey. The CHNS analysis was used to quantify the mass fraction and determine the ratio of main macro elements of bacterium: carbon, hydrogen, nitrogen and sulfur of the bacteria sample (Table 5-4). The highest elemental percentage recorded is carbon with 47.61% followed by nitrogen with 12.49%. The results of hydrogen and sulfur represent the lowest percentages recorded with 7.76% and 0.65%, respectively. Carbon and nitrogen ratio

resulted in 4:1, approximately. Therefore, we investigated and optimized medium composition based on elemental analysis, supported by C/N proportion focusing on protease production. Concentrations of carbon and nitrogen were defined as 8.0 and 2.0 $g.L^{-1}$, respectively, in order to test different carbon and nitrogen sources.

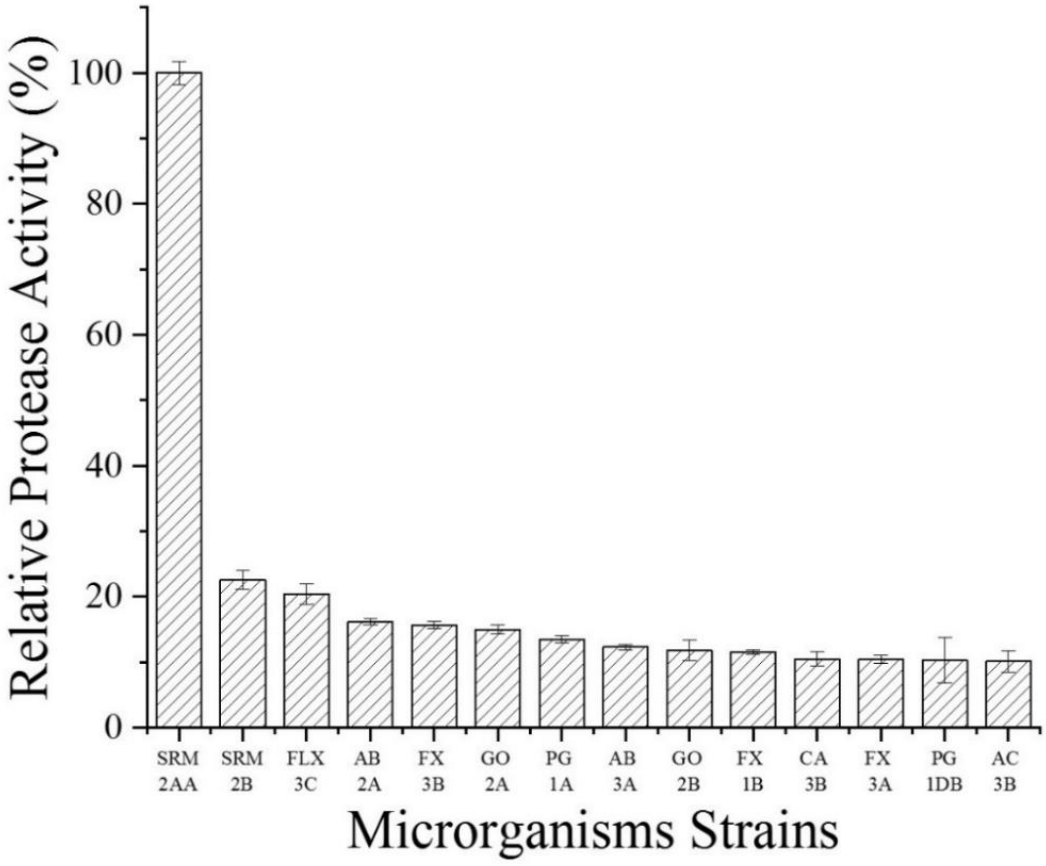


Figure 5-1: Top fourteen protease-producers from 65 strains of databank. Relative activity was expressed as the percentage of activity detected in relation to maximum protease activity. Only values up to 10% are shown.

Table 5-4: Percentages of carbon, hydrogen, nitrogen and sulfur in bacteria sample coded as SRM-2AA.

Sample	% C	% H	% N	% S
1	47.86	7.65	12.74	0.68
2	47.37	7.87	12.26	0.63
Average	47.61	7.76	12.49	0.65

Carbon and nitrogen sources have different influences on extracellular enzyme production. We tested, firstly, many nitrogen sources using glucose (8 $g.L^{-1}$) as carbon source standard, evaluating kinetics on growth and extracellular protease production. We analyzed ammonium nitrate, ammonium citrate, ammonium sulfate, peptone and yeast extract (2 $g.L^{-1}$) and the last two sources presented the highest results on protease activity with maximum activity of 15.42 $U.mL^{-1}$ after 48 h of fermentation using peptone and 16.46 $U.mL^{-1}$ after 36 h using yeast extract. The other nitrogen sources combined with glucose had inhibitory effect on protease production (Figure 5-2).

For carbon source investigation, we selected yeast extract (2 $g.L^{-1}$) as nitrogen substrate standard due to it showed the highest protease activity in a shorter fermentation time. Sucrose, glucose, peptone and agro-industrial wastes as whey permeate, sugarcane molasses and soy molasses were investigated as a potential inducer in protease production. All carbon substrates contributed to produce proteolytic enzymes (Figure 5-3). Agro-industrial wastes displayed similar values when compared to analytical substrates. Whey permeate stood out for producing the highest protease activity (23.32 $U.mL^{-1}$) in just 24 hours of fermentation. Thereafter, peptone as carbon source showed a pick of protease production (23.42 $U.mL^{-1}$) at the same period. Sucrose, sugarcane molasses and soy molasses displayed similar results at same period of fermentation (60 h), reaching picks of 20.61 $U.mL^{-1}$, 20.36 $U.mL^{-1}$ and 19.94 $U.mL^{-1}$ of proteolytic activity, respectively. Glucose was responsible to the lowest activity when compared to the others carbons sources tested, reaching 16.46 $U.mL^{-1}$ in 36 h of fermentation.

Kinetic growth of microorganism was also accomplished and different nitrogen and carbon sources influences on microorganism growth profile. When we tested nitrogen substrates jointly with glucose as carbon source, peptone and yeast extract were the main sources to support a biomass production, reaching the final exponential phase with 36 hours (with 1.09×10^{14} and 1.10×10^{14} $cells.mL^{-1}$, respectively). In carbon source investigation in which yeast extract was fixed as nitrogen source, sugarcane (1.81×10^{14} $cells.mL^{-1}$) and soy (1.51×10^{14} $cells.mL^{-1}$) molasses attained the highest biomass production with final exponential phase in 60 and 36 hours, respectively. Peptone, sucrose and glucose stood out next with 1.35×10^{14} $cells.mL^{-1}$, 1.23×10^{14} $cells.mL^{-1}$ and 1.10×10^{14} $cells.mL^{-1}$, respectively, with final exponential phase with 36 hours.

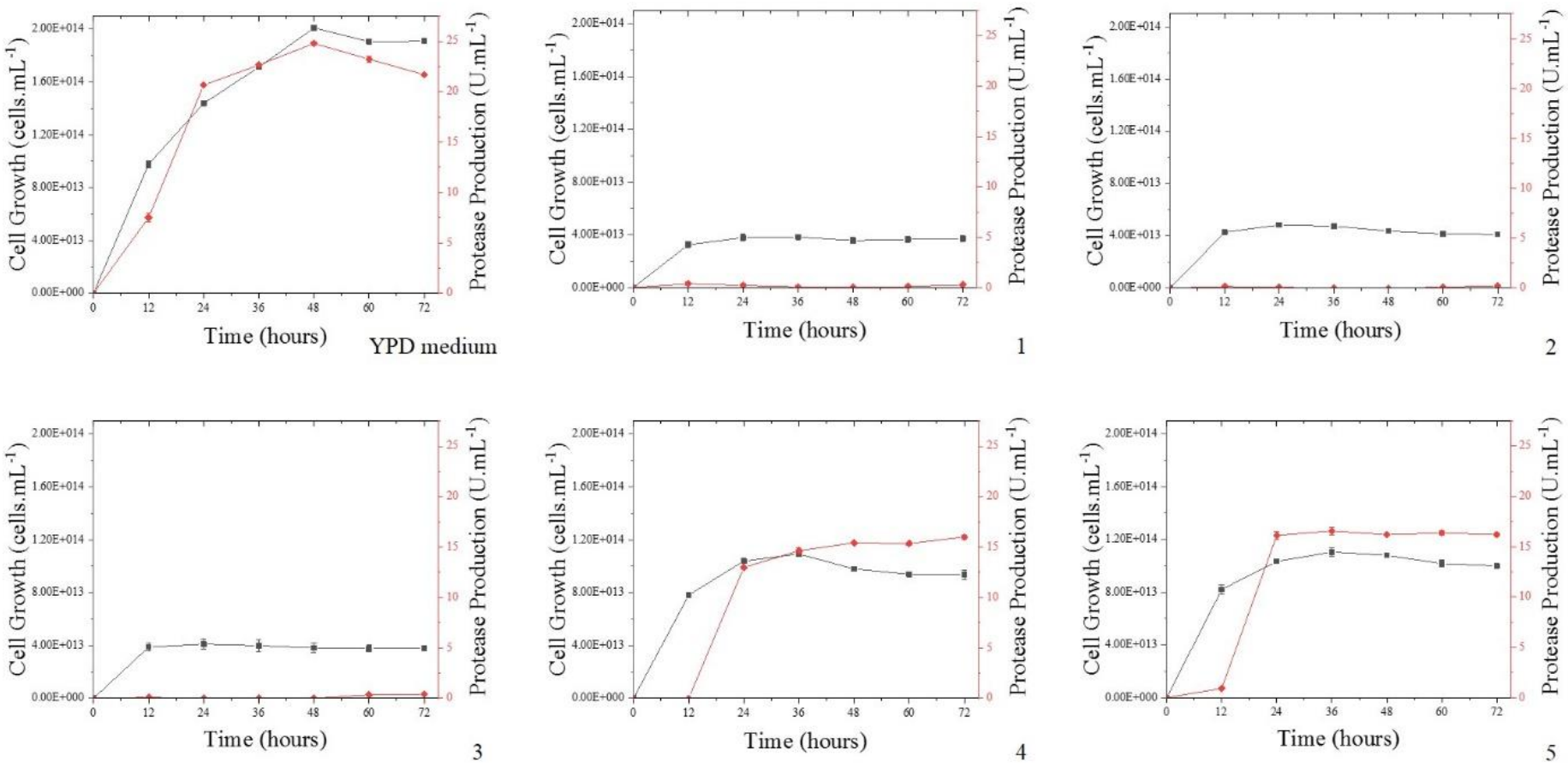


Figure 5-2: Cell growth and protease production by bacterium SRM-2AA in different nitrogen sources using glucose as standard carbon source. Nitrogen sources tested were (1) ammonium nitrate; (2) ammonium citrate; (3) ammonium sulfate; (4) peptone and (5) yeast extract. YPD medium was used as control fermentation. Red lines express proteolytic enzymes produced by bacterium and black lines show cell growth.

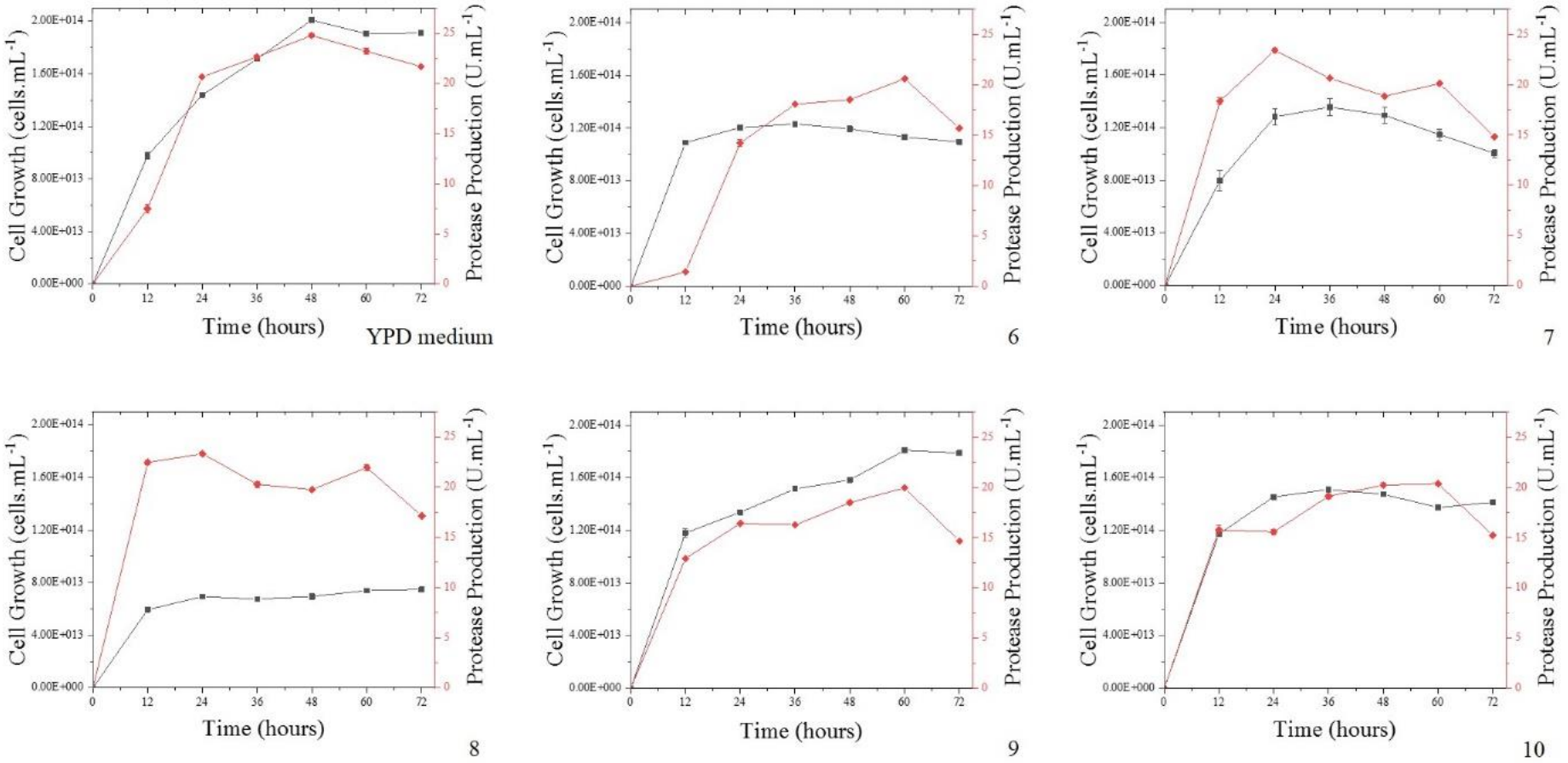


Figure 5-3: Cell growth and protease production by bacterium SRM-2AA in different carbon sources utilizing yeast extract as standard nitrogen source. Carbon sources tested were (6) sucrose; (7) peptone; (8) whey permeate; (9) sugarcane molasses; (10) soy molasses. YPD medium was used as control fermentation. Red lines express proteolytic enzymes produced by bacterium and black lines show cell growth.

A lower bacterial cell growth was observed when compared to microorganism growth of others carbon substrates tested (Figure 5-3:8). Ammonium nitrate, ammonium citrate, ammonium sulfate and even whey permeate produced low biomass production when compared to the others. However, the focus on carbon and nitrogen analysis was to investigate substrates capable to stimulate production of proteolytic enzymes. For this reason, we selected whey permeate as carbon source jointly with yeast extract for statistical optimization of the medium composition.

5.3.3. Statistical optimization of culture medium composition for protease production by bacterium SRM-2AA

The influence of whey permeate concentration, yeast extract concentration, temperature and initial medium pH on protease production from bacterium SRM-2AA was analyzed by a 2^4 full factorial design. Table 5-5 lists the experimental results of selected responses: cell growth and proteolytic activity. The best results on protease production evaluated by proteolytic activity were obtained in runs 4 with 26.58 $U.mL^{-1}$ (performed at 10 $g.L^{-1}$ whey permeate, 3 $g.L^{-1}$ yeast extract, pH 6 and 25 °C) and 8 with 25.87 $U.mL^{-1}$ (10 $g.L^{-1}$ whey permeate, 3 $g.L^{-1}$ yeast extract, pH 8 and 25 °C). Values obtained in center points represented an average of 25.27 $U.mL^{-1}$ next. Estimates of statistical effects (at 95% confidence level) revealed that yeast extract concentration and whey permeate concentration had positive effect, whereas temperature and interaction of yeast extract concentration and pH had negative effect. PH was not statistically significant at 95% of confidence level.

For cell growth the best values were detected in runs 8 (6.82×10^{13} $cells.mL^{-1}$) and 16 (6.74×10^{13} $cells.mL^{-1}$) performed at 10 $g.L^{-1}$ whey permeate, 3 $g.L^{-1}$ yeast extract, pH 8 and 25 °C and 10 $g.L^{-1}$ whey permeate, 3 $g.L^{-1}$ yeast extract, pH 8 and 35 °C, respectively. Estimates of statistical effects (at 95% confidence level) revealed that all the main effects and half of interactions were statistically significant for cell growth response, which had yeast extract concentration, whey permeate concentration, pH and temperature as positive effect.

Values of significant effects indicate that proteolytic activity and cell growth were the highest when yeast extract concentration and whey permeate concentration were selected at their highest values (positive effects).

Table 5-5: Results on 2^4 full design of culture medium optimization.

Run	Actual values				Results	
	Whey permeate ($g.L^{-1}$)	Yeast extract ($g.L^{-1}$)	pH	Temperature (°C)	Cell growth ($cells.mL^{-1}$)	Proteolytic Activity ($U.mL^{-1}$)
1	6	1	6	25	2.38×10^{13}	18.17
2	10	1	6	25	3.33×10^{13}	22.19
3	6	3	6	25	5.31×10^{13}	23.97
4	10	3	6	25	6.35×10^{13}	26.58
5	6	1	8	25	2.73×10^{13}	20.51
6	10	1	8	25	4.19×10^{13}	21.60
7	6	3	8	25	5.92×10^{13}	24.62
8	10	3	8	25	6.82×10^{13}	25.87
9	6	1	6	35	2.55×10^{13}	17.97
10	10	1	6	35	3.31×10^{13}	20.96
11	6	3	6	35	5.41×10^{13}	22.29
12	10	3	6	35	6.59×10^{13}	23.40
13	6	1	8	35	3.21×10^{13}	18.79
14	10	1	8	35	4.32×10^{13}	20.30
15	6	3	8	35	5.59×10^{13}	25.06
16	10	3	8	35	6.74×10^{13}	24.79
17	8	2	7	30	4.96×10^{13}	25.49
18	8	2	7	30	5.10×10^{13}	25.07
19	8	2	7	30	5.08×10^{13}	25.43
20	8	2	7	30	5.03×10^{13}	25.10

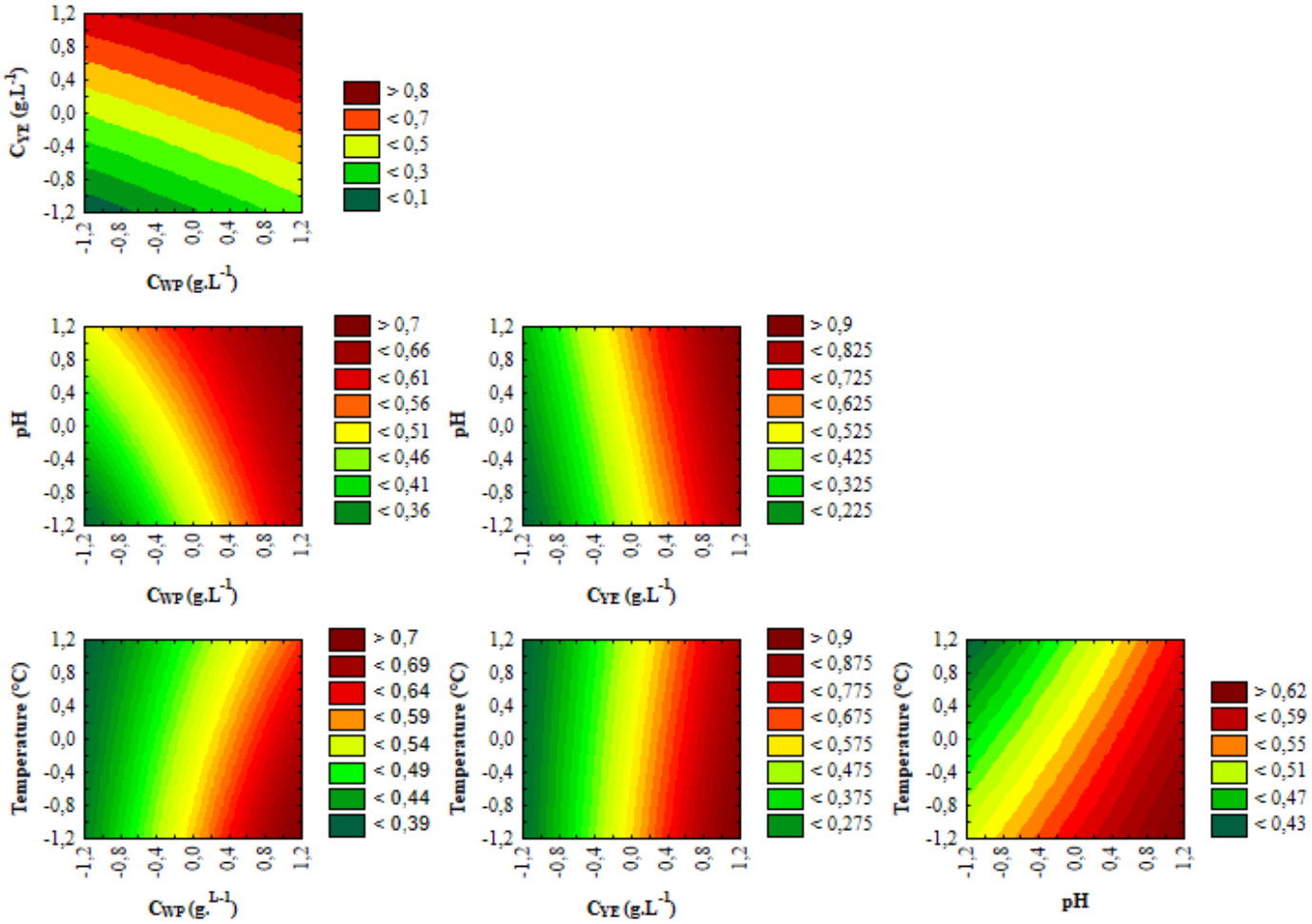


Figure 5-4: Desirability optimization methodology to culture medium composition applying 2^4 full factorial design for proteases from bacterium SRM-2AA. Each graph shows the most desirable response as the color intensity increases (highest values in red). C_{YE} and C_{WP} mean yeast extract concentration and whey permeate concentration, respectively.

Desirability surface (Figure 5-4) evidences that the minimum value for temperature had better results for all interactions of others independent variables, highlighting to whey permeate concentration and pH. PH influences good results in it central point to maximum value. Desirability tool combines all variables into one response assisting the direction to optimum response conditions. Therefore, we fixed temperature and pH at 25°C and pH 7, respectively, to next investigation on central composite design.

Table 5-6: Results on first 2^2 central composite design of culture medium optimization.

Run	Actual values		Results	
	Whey permeate ($g.L^{-1}$)	Yeast extract ($g.L^{-1}$)	Cell growth ($cells.mL^{-1}$)	Proteolytic Activity ($U.mL^{-1}$)
1	8.00	2.00	4.62×10^{13}	22.22
2	8.00	4.00	7.29×10^{13}	26.40
3	12.00	2.00	5.50×10^{13}	23.55
4	12.00	4.00	8.25×10^{13}	27.19
5	7.17	3.00	5.47×10^{13}	24.26
6	12.83	3.00	7.21×10^{13}	25.96
7	10.00	1.59	4.41×10^{13}	21.90
8	10.00	4.41	5.53×10^{13}	28.18
9	10.00	3.00	5.94×10^{13}	24.18
10	10.00	3.00	5.88×10^{13}	24.66
11	10.00	3.00	5.98×10^{13}	24.79
12	10.00	3.00	5.89×10^{13}	25.32

A central composite design was used for a detailed investigation of the effect of whey permeate concentration and yeast extract concentration. The first 2^2-CCD attained the maximum protease production at 28.18 $U.mL^{-1}$ and 27.19 $U.mL^{-1}$ in runs 8 (10 $g.L^{-1}$ whey permeate, 4.41 $g.L^{-1}$ yeast extract) and 4 (12 $g.L^{-1}$ whey permeate, 4 $g.L^{-1}$ yeast extract), respectively (Table 5-6). For cell growth response, the best values were obtained in runs 4 (12

$g.L^{-1}$ whey permeate, 4 $g.L^{-1}$ yeast extract) and 2 (8 $g.L^{-1}$ whey permeate, 4 $g.L^{-1}$ yeast extract) with 8.25 x 10^{13} and 7.29 x 10^{13} $cell.mL^{-1}$, respectively. Figure 5-5-A evidences the response surface methodology (RSM) showing the tendency of the optimum result when yeast extract concentration increase and whey permeate concentration is in central point to maximum values. The optimum curvature on the graph was not attained which evidences that experimental design had to be reevaluated in new conditions. Therefore, we decided to adopt an additional 2^2-CCD shifting yeast extract concentration at higher levels (Table 5-3).

Table 5-7: Results on second 2^2 central composite design of culture medium optimization.

Run	Actual values		Results	
	Whey permeate ($g.L^{-1}$)	Yeast extract ($g.L^{-1}$)	Cell growth ($cells.mL^{-1}$)	Proteolytic Activity ($U.mL^{-1}$)
1	8.00	3.41	6.64 x 10^{13}	24.13
2	8.00	5.41	8.71 x 10^{13}	26.69
3	12.00	3.41	7.59 x 10^{13}	26.59
4	12.00	5.41	9.68 x 10^{13}	27.58
5	7.17	4.41	7.79 x 10^{13}	25.69
6	12.83	4.41	8.89 x 10^{13}	27.67
7	10.00	3.00	6.50 x 10^{13}	25.74
8	10.00	5.83	9.37 x 10^{13}	27.13
9	10.00	4.41	8.33 x 10^{13}	26.82
10	10.00	4.41	8.43 x 10^{13}	27.27
11	10.00	4.41	8.41 x 10^{13}	26.94
12	10.00	4.41	8.46 x 10^{13}	26.38

The second composite design enhanced cell growth to 9.68 x 10^{13} $cells.mL^{-1}$, what it is observed in run 4 (12 $g.L^{-1}$ whey permeate, 5.41 $g.L^{-1}$ yeast extract), followed by run 8 with 9.37 x 10^{13} $cells.mL^{-1}$ (10 $g.L^{-1}$ whey permeate, 5.83 $g.L^{-1}$ yeast extract) (Table 5-7). Is possible to note the influence on the bacterial growth by increase on yeast extract concentration at culture

medium. The results obtained for proteolytic activity response do not exceed protease production on the first central composite design. However, similar values were obtained what can be seen on runs 6 (12.83 $g.L^{-1}$ whey permeate, 4.41 $g.L^{-1}$ yeast extract) and 4 (12 $g.L^{-1}$ whey permeate, 5.41 $g.L^{-1}$ yeast extract) with 27.67 and 27.58 $U.mL^{-1}$. The response surface methodology for proteolytic activity is shown on Figure 5-5-B, which evidences the high curvature point on the graph describing the improvement of the model in the second central composite design when compared to the first-CCD (Figure 5-5-A).

The statistical significance of second-order polynomial model was confirmed by the F-test analysis of variance (Table 5-8). The calculated F-value ($F_{5,6} = 12.274$) was higher than the tabulated one ($F_{5,6} = 4.387$), while the lack of fit between the predicted model and experimental values are in agreement ($F_{calculated} < F_{tabulated}$). In addition, the high value of the determination coefficient (R^2=0.9109) indicates that model explained 91.09% of the variability data, and the regression was statistically significant at 95% confidence level. Thereafter, the full quadratic model fitted the data following the equation 1 where all terms were maintained for proteolytic activity response:

$$Y = 26.8533 + 0.7707.(x_1) + 0.6891.(x_2) – 0.1647.(x_1)^2 - 0.287.(x_2)^2 – 0.3920 .x_1.x_2 \quad [Eq. 1]$$

Table 5-8: Analysis of variance (ANOVA) of proteolytic activity response in second central composite design.

Factor	SS[a]	df[b]	MS[c]	F-value	p-value
C_{WP} (L)	4.7521	1	4.7521	34.5656	0.0098
C_{WP} (Q)	0.1735	1	0.1735	1.26225	0.3430
C_{YE} (L)	3.7992	1	3.7992	27.6343	0.0134
C_{YE} (Q)	0.5284	1	0.5284	3.84333	0.1448
1L by 2L	0.6146	1	0.6146	4.47083	0.1248
Lack of Fit	0.5429	3	0.1809	1.31624	0.4133
Pure Error	0.4124	3	0.1374		
Total SS	10.7263	11			

[a] sum of square; [b] degree of freedom; [c] mean of square.

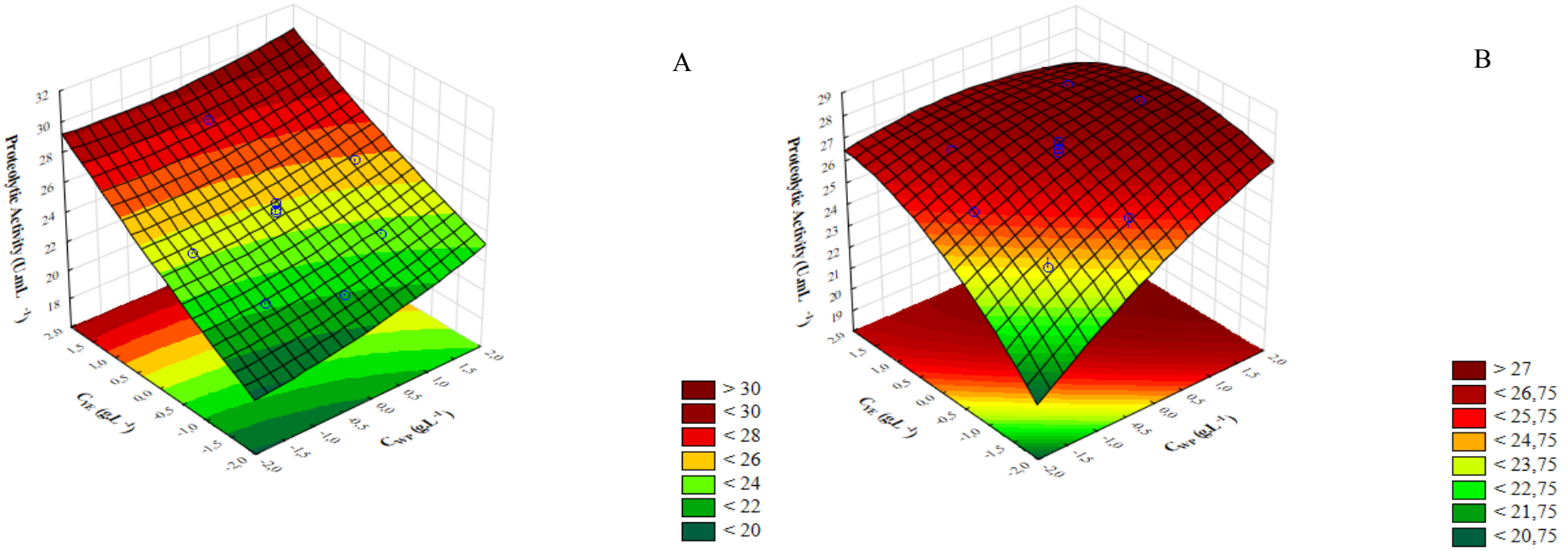


Figure 5-5: Contour plot showing the interactive effects of whey permeate concentration and yeast extract concentration on proteolytic activity from proteases produced by bacterium SRM-2AA. The blue dots show the conditions tested in the study. (A) First central composite design. (B) Second central composite design in the adjusted model. C_{YE} is the yeast extract concentration and C_{WP} is the whey permeate concentration.

Only linear factors were significant for proteolytic activity at 95% confidence level. Nevertheless, we used the complete model, because we did not obtain greater adjusted coefficient of determination (R_{adj}). Non-significant quadratic factors, it does not mean that the quadratic model is not appropriate. In this situation, the quadratic model described the model well as we can see from ANOVA, Fisher-based test and response surface methodology.

Considering the cell growth and proteolytic activity obtained on second CCD and our focus on improve protease production by bacterium SRM-2AA, we selected the optimized culture medium at 12.83 $g.L^{-1}$ whey permeate, 4.41 $g.L^{-1}$ yeast extract, pH 7 and 25°C. The optimized culture medium was validated in a quintuplicate experiments that confirmed the similarity between the predicted and experimental data. The observed values on validation experiments for proteolytic activity and cell growth response were 30.97 ± 0.3311 $U.mL^{-1}$ and 9.09 x 10^{13} ± 1.4 x 10^{12} $cells.mL^{-1}$, respectively, whereas predicted values were 27.99 $U.mL^{-1}$ and 11.227 x 10^{13} $cells.mL^{-1}$. Protease production exceed the predicted values.

5.3.4. Effects of pH and temperature on proteolytic activity

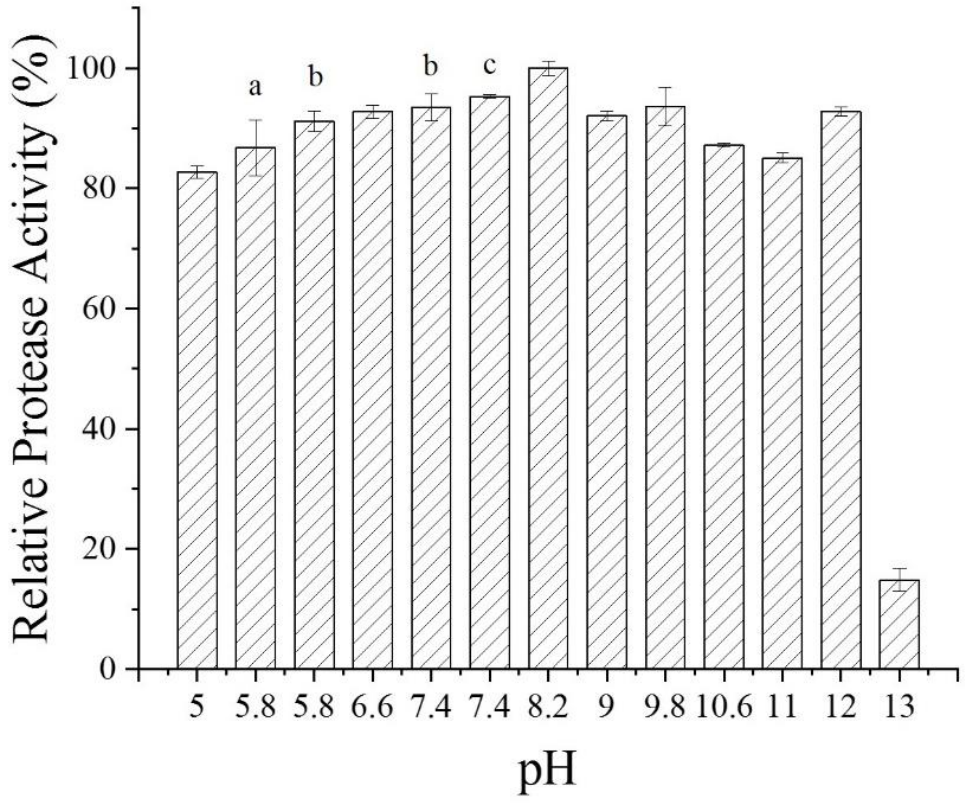


Figure 5-6: PH effect on proteolytic enzymes from bacterium SRM-2AA expressed in percentage of relative protease activity; ‘a’, ‘b’ and ‘c’ evidences citrate-phosphate, sodium

phosphate and Tris-HCl buffers, respectively. Relative activity was expressed as the percentage of activity detected in relation to maximum protease activity.

The optimum pH (5.0-13.0) of the enzyme extract was determined at 37 °C. The proteases exhibited activity over a broad pH range, which maximum activity at pH 8.2 (26.62 ± 0.010 U. mL^{-1}) (Figure 5-6). Enzymes showed activity above 80% in the majority when incubated from acidic to alkaline solution, in exception in pH 13.0. The effect of different temperatures on enzyme extract exhibited maximum activity at 45 °C and pH 8.2 (32.32 ± 0.007 U. mL^{-1}) (Figure 5-7). The proteases were active in a broad temperature range from 35 to 50 °C, with 84, 92 and 87% activity preserved. In other temperatures, relative activity exhibited activities less than 75% accentuating the losses at more extreme temperatures.

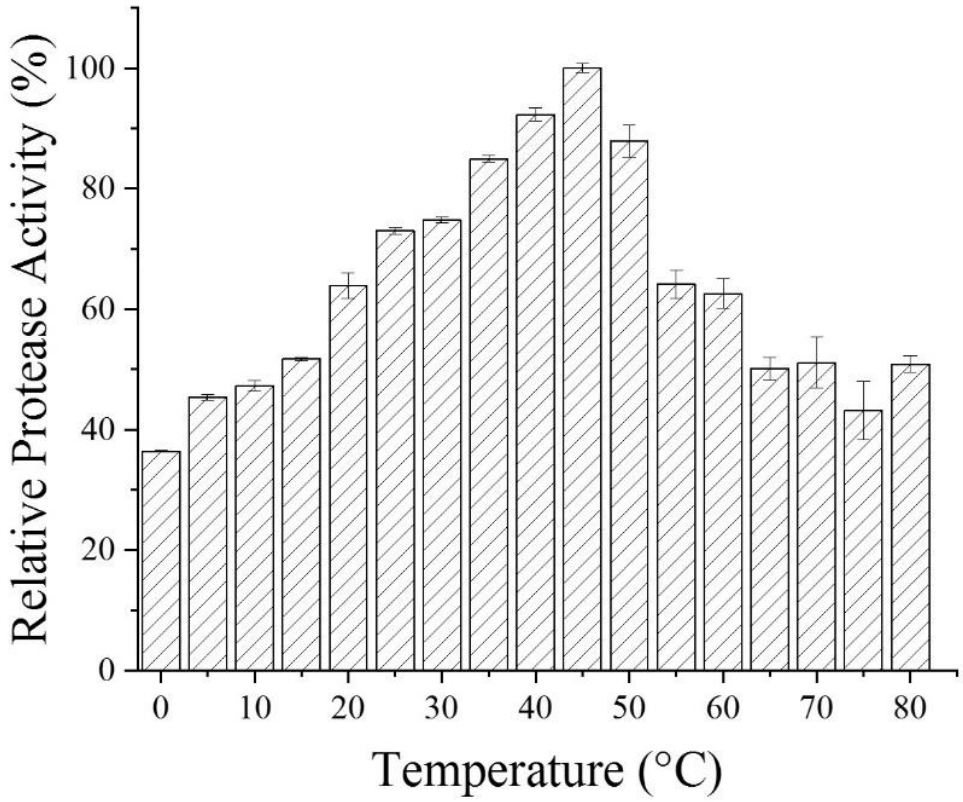


Figure 5-7: Temperature effect on proteolytic enzymes from bacterium SRM-2AA expressed in percentage of relative protease activity. Relative activity was expressed as the percentage of activity detected in relation to maximum protease activity.

5.3.5. Temperature and pH stability of proteolytic enzymes

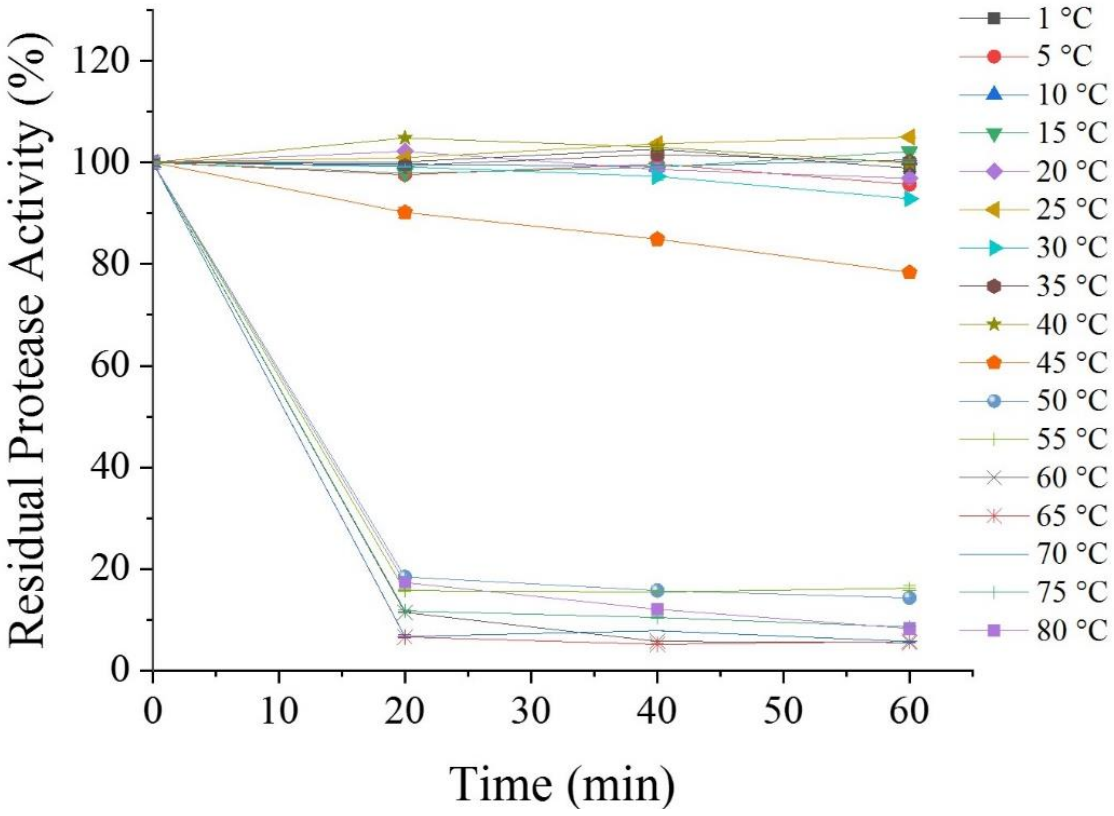


Figure 5-8: Thermostability of proteases from bacterium SRM-2AA. Residual activity was defined as the percentage of activity compared to the standard assay of proteolytic activity.

Thermal stability was investigated by performing an activity measurement at 1 to 80 °C temperature range at 20, 40, 60, 120, 180, 240, 300, and 360 minutes. The protease retained up to 100% at 1-35°C (107.31; 108.39; 109.23; 110.92; 111.31; 109.75; 108.30 and 109.24%) after 360 min (Figure 5-8). At 40 °C, the protease preserved 91.27% after 360 min of incubation. Following 60 min at 45 °C, enzymes diminished its activity to 78.32%, and then 29.39% from 360 min. Thereby, the proteolytic enzymes retained down of 16.50% on its activity at 50-80 °C.

When the pH stability profile for the protease enzyme from bacterium SRM-2AA was examined; it was determined that enzyme had preserved its activity by more than 65% at the range of pH 4.2-8.2 (70.44; 77.60; 104.00; 103.73; 95.43; 96.85; 65.32%, respectively) after 60 min-incubation at 45 °C (Figure 5-9). Proteolytic enzymes lost its activity at the rate 8.35; 26.23; 18.40; 17.10; 18.33 and 33.95% at pH 3.4; 9.0; 9.8; 10.6; 11.0 and 12.0, respectively. The pH stability results showed that the protease retained up 95% of its activity at pH 5.8b-7.4c after 60 min-incubation and, after 360 minutes proteases maintained up to 50% of its activity

at same pHs, highlighting pH 5.8b and 6.6 with 100.17 and 99.46% of residual activity, respectively.

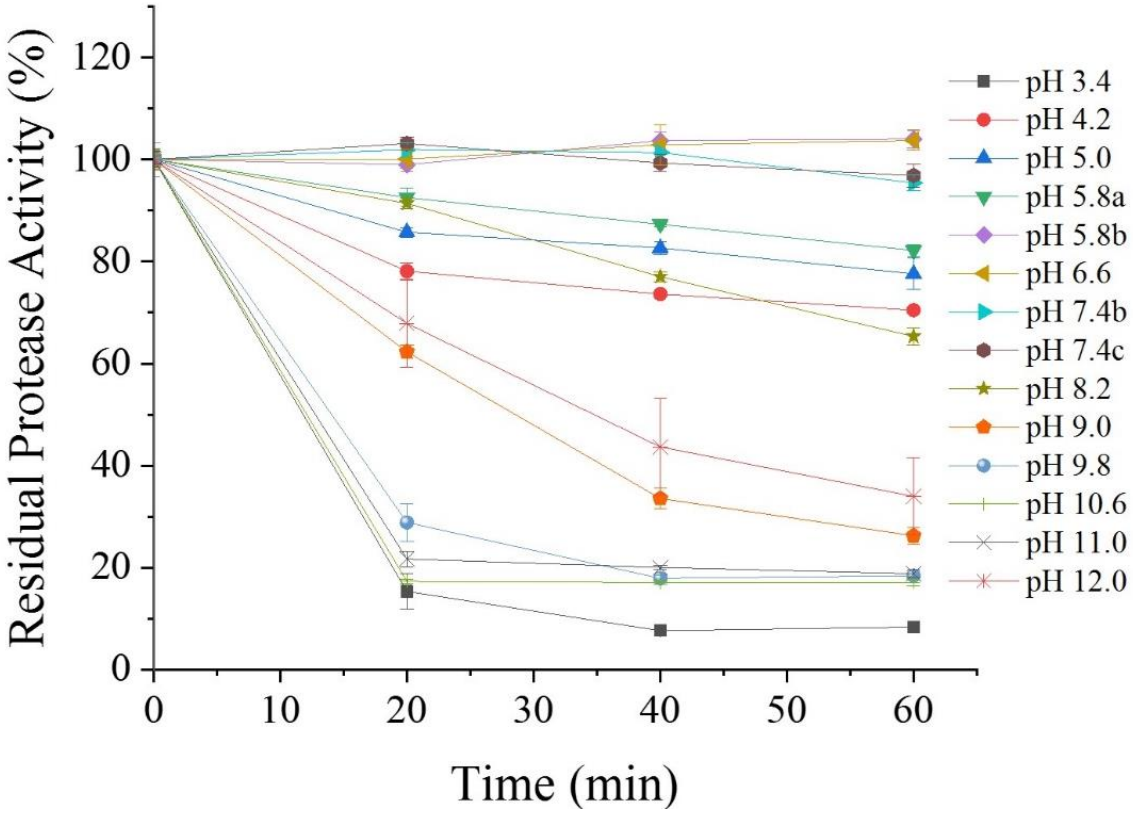


Figure 5-9: PH stability of proteases from bacterium SRM-2AA. 5.8a and 5.8b define activity of citrate-phosphate and sodium phosphate buffers, respectively. 7.4b and 7.4c are the respective activities of sodium phosphate and Tris-HCl buffers.

5.3.6. The effect of metal ions on protease activity

Proteases enhanced its activity in the presence of Mg^{+2} at 5 and 10 mM, and Ca^{+2} 5 mM; whereas in the presence of Ca^{+2} 10 mM 97% of the enzyme activity have remained (Table 5-9). However, at 5 mM, Ni^{+2} and Co^{+2} had some negative impact; 29.22 and 38.40% activity was lost in the presence of ions Ni^{+2} and Co^{+2}, respectively. A highly inhibitory effect on protease activity was observed with Cu^{+2} 5 and 10 mM, Co^{+2} 10 mM and Ni^{+2} 10 mM, in which more than 50% activity was inactivated.

5.3.7. Kinetic growth and protease production in optimized medium fermentation at 100 mL and 1500 mL-bioreactor

Samples in 100 mL (Erlenmeyer- flasks) and 1500 mL (bioreactor) of optimized culture medium were collected every 2 hours to establish the growth behavior and protease production

of bacterium SRM-2AA. Fermentation on Erlenmeyer-flasks containing 100 mL of optimized culture medium outcomes a maximum exponential biomass concentration at 24 hours with 5.43 x 10^{13} ± 1.02x 10^{12} cells.mL^{-1} (Figure 5-10-A). In contrast, protease activity attained its maximum production at 22 hours with 34.19 ± 0.2058 U.mL^{-1}. Scaling up the culture medium to 15-fold, bioreactor fermentation showed similar behavior on bacterial growth when compared to 100 mL-fermentation, with maximum cells at 24 hours with 5.07 x 10^{13} ± 4.36 x 10^{12} cells.mL^{-1} (Figure 5-10-B). Proteases reached its maximum production with 34.13 ± 0.1390 U.mL^{-1} at 24 hours. Kinetic growths revealed that maximum cells were attained at 24 hours and scaling up fermentation brings slight reduction on growth (6.65%). However, proteases had similar production on 100 and 1500 mL, with a slightly decrease of 0.17% in 1500 mL bioreactor. In both fermentations scale, 100 and 1500 mL, is possible to note that biomass exponential phase started at 2 hours and had a slightly reduction when attained 8 to 10 hours of fermentation, whereas exponential producing protease initiated at 4 hours. It is noticed that initial exponential protease production phase affects cell growth log phase.

Table 5-9: Relative protease activity of proteases from bacterium SRM-2AA submitted to metal ions effect.

Metal Ion	Concentration	
	5.0 mM	10.0 mM
Control	100.00 ± 0.32	100.00 ± 0.32
Mg^{+2}	113.38 ± 2.84	107.68 ± 1.43
Ca^{+2}	109.64 ± 0.14	97.97 ± 0.38
Cu^{+2}	43.22 ± 0.50	19.82 ± 7.64
Co^{+2}	61.60 ± 4.16	46.40 ± 6.92
Ni^{+2}	70.78 ± 3.41	45.04 ± 7.22

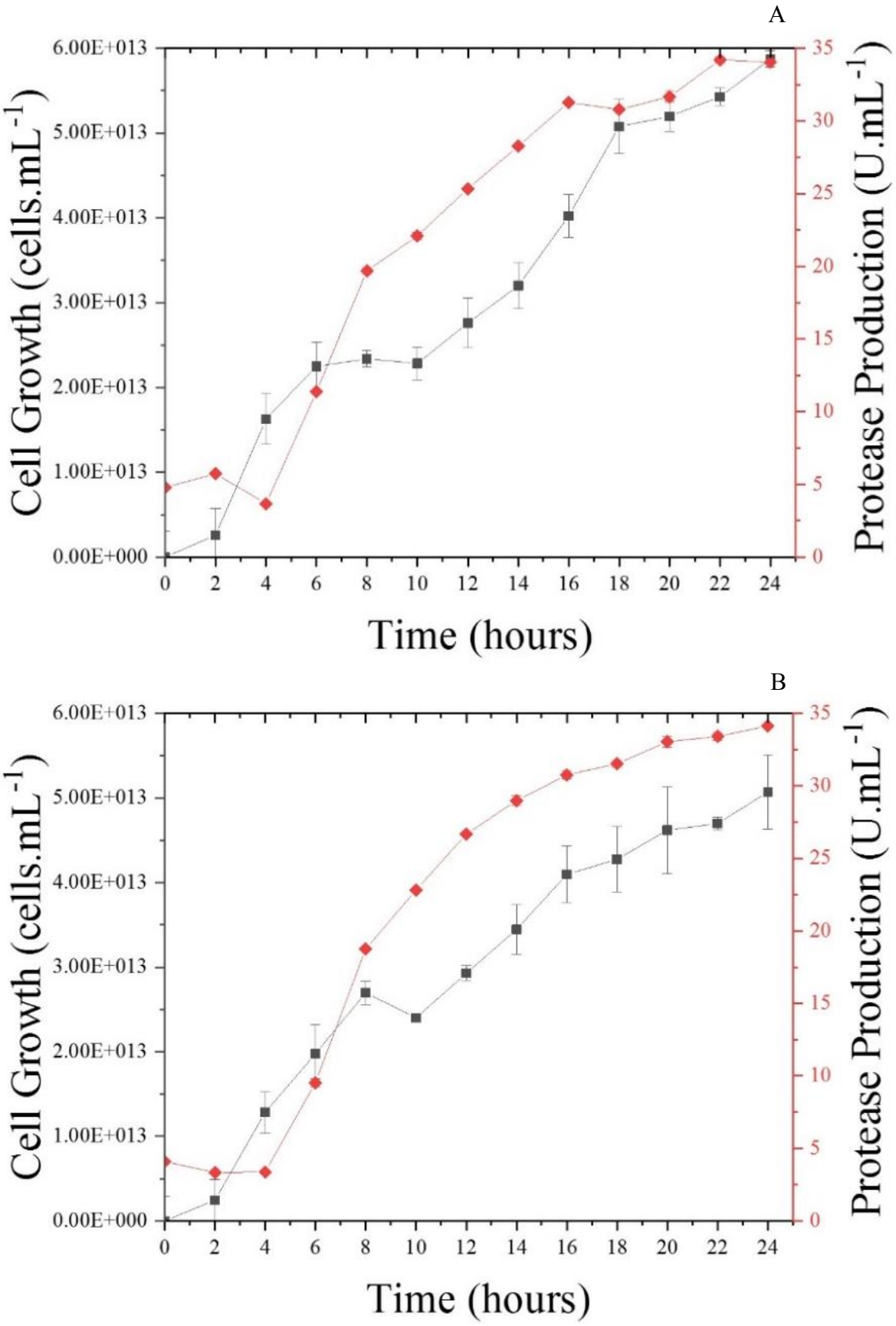


Figure 5-10: Protease production and cell growth by bacterium SRM-2AA during 24 hours.

Red lines express proteolytic enzymes produced by bacterium and black lines show cell growth. (A) Submerged fermentation in 100 mL-Erlenmeyer flasks and (B) 1500 mL-bioreactor.

5.3.8. Applications

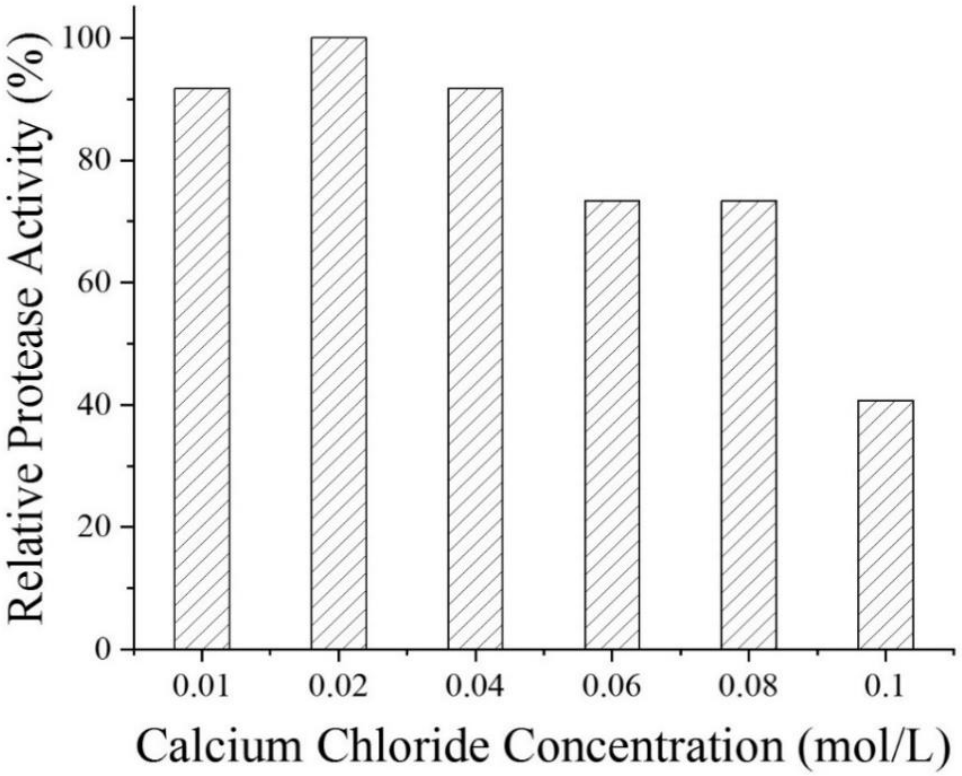
Proteolytic enzymes produced by bacterium SRM-2AA were tested as milk clotting formation to dairy industry and mechanical texture measurement to tenderization effect. Milk clotting activity revealed a maximum result with 2 181.82 $U.mL^{-1}$ at 0.02 $mol.L^{-1}$ $CaCl_2$. Calcium chloride concentration effect on milk clotting activity was investigated, which evidences a peak of activity at 0.02 $mol.L^{-1}$ of $CaCl_2$ (Figure 5-11). The decrease on MCA was observed at 0.01 concentration and superior concentrations (0.04, 0.06, 0.08 and 0.1 $mol.L^{-1}$).

Shear force is one of the physical parameters that most available meat tenderness. Untreated meat cubes (negative control) had a shear force of 19.77 ± 7.43 N, and bromelain used as positive control showed 12.38 ± 2.37 N (Figure 5-12). Meat treated by proteolytic enzymes from bacterium SRM-2AA resulted in 11.73 ± 2.20 N shear force, signing a reduction of 40.67% when compared to untreated meat and 5.25% to bromelain. Protease from bacterium SRM-2AA showed inferior shear force, meaning a better performance when compared to bromelain.

5.4. Discussion

Isolation and screening of microorganisms have become a strategic to succeed new sources of biomolecules with potential applications in industries, focusing on natural habitats as Brazilian Cerrado biome. Our efforts in investigate proteolytic enzymes from soil, fruit peel, sheet and flower microbiota resulted in a databank with 65 strains. This particularly survey reports the bioprospection of extracellular protease-producer from our databank collection and the results obtained showed that a bacterium isolated from soil was able to produce the target bioproduct in more expressive quantities from the other strains. For this reason, the bacterium strain coded as SRM-2AA was selected to study. Investigations on protease-producer from Brazilian Cerrado soil has been carried out as Petinate et al. (19) and Nascimento et al. (20) that partially characterized proteinases isolated from Brazilian Cerrado soil from *Streptomyces cyaneus* and *Streptomyces malaysiensis*, respectively. A new specie of *Streptomyces* was isolated under cerrado vegetation cover and described to produce chitinolytic proteases (21). Souza et al. (22) could kindly use the Culture bank of the Enzymology Laboratory of Cell Biology Department, University of Brasilia (Brasilia, DF, Brazil) in which *Aspergillus foetidus*,

also isolated from the soil of Brazilian Savannah, had the kinetic and thermodynamic of an acid protease explored.



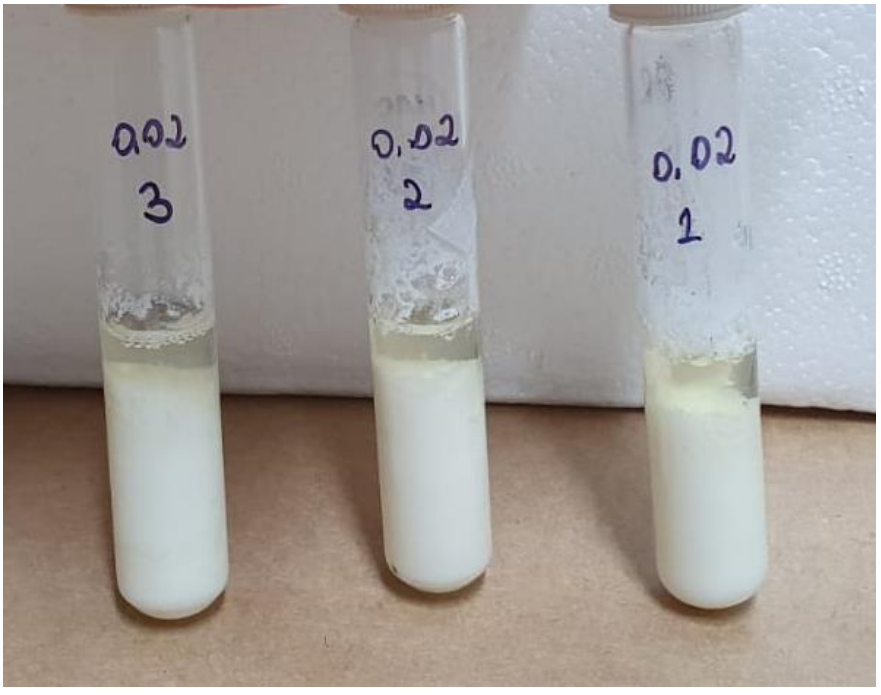


Figure 5-11: Calcium chloride effect on proteases from bacterium SRM-2AA to milk clotting formation. (A) Relative activity was expressed as the percentage of activity detected in relation to maximum protease activity. (B) Milk clotting triplicate at 0.02 $mol.L^{-1}$ $CaCl_2$.

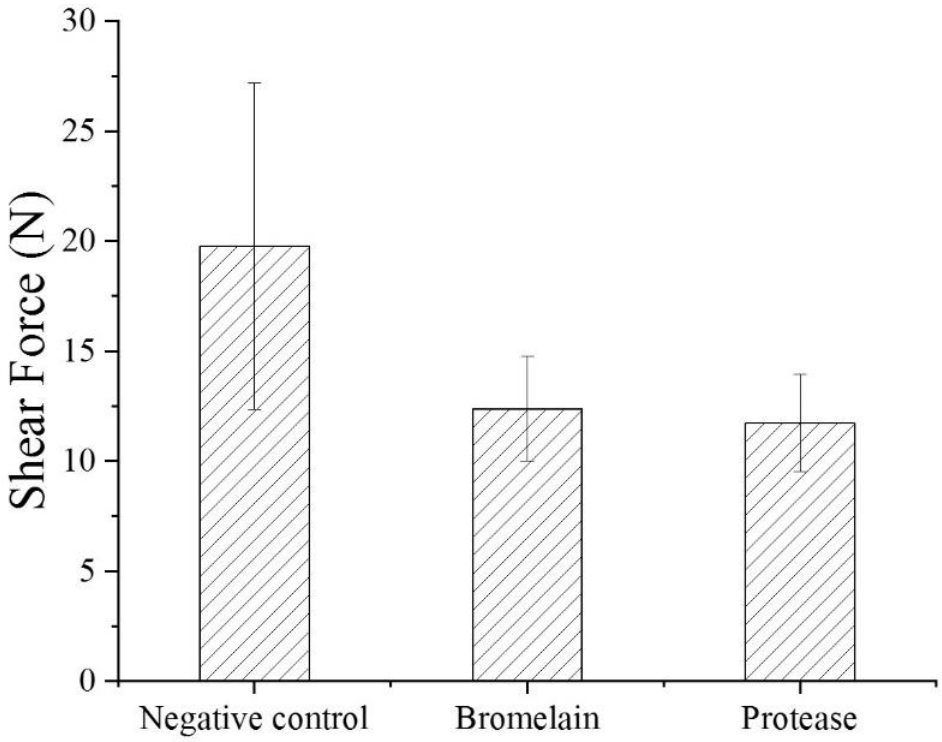


Figure 5-12: Texture measurement by the shear force of cubes meats treated with bromelain from pineapple and proteases produced by bacterium SRM-2AA.

The optimized culture medium of bacterium SRM-2AA was investigated next. The selection of an appropriate protease production model depends on several factors established between the physiology of microorganism and physicochemical factors as temperature, pH, substrates sources, aeration and bed properties (7). The carbon, hydrogen, nitrogen and sulfur analysis was carried out firstly to estimate the minimum quantities needed for optimal biomass production and minimize excess or insufficiency of the culture medium components (23). Inorganic and organic nitrogen sources affect differently the growth and protease production of each strain of microorganism. In this study, the bacteria preferred organic nitrogen source from yeast extract and peptone, in which yeast extract had highest proteolytic production. These results are in accordance with Gibb et al. (24) and El-Sayed et al. (25) that also related yeast extract as the best inducer for protease production from *Streptomyces* C5-A13 and *Streptomyces pseudogrisiolus* NRC-15, respectively. Joshi et al. (26) combined these two forms of nitrogen to achieve maximum protease production of *Bacillus cereus* MTCC 6840.

Our effort in analyzing the different carbon sources, detected peptone (23.42 $U.mL^{-1}$) with a slightly higher proteolytic activity when compared to whey permeate (23.32 $U.mL^{-1}$). Due to the similarity of results and the desire for whey permeate exploitation for optimization

of the culture medium to proteolytic enzyme production we considered this difference irrelevant (0.43% of increase). The use of agro-industrial waste is relevant in reutilize a worthless residue and cheapen the cost of the culture medium (27; 28). The whey permeate was defined as the carbon source for the medium composition. A by-product of cheese-making, the whey permeate is a portion of a liquid obtained through a membrane when whey or milk is pumped through ultrafiltration system. It is abundant in lactose, proteins and minerals that contributes for the growth of microorganisms and synthesis of microbial enzymes (28; 29). Therefore, we realize that bacteria has capacity in use lactose as energy source and convert to proteolytic enzyme production as well as reported by Mabrouk et al. (30) and Al-Dhali et al. (31).

It seems to be the first report that utilize whey permeate as an inducer for protease production. However, whey permeate had been investigated for other purposes as production of β-galactosidase (32), galactooligosaccharide (GOS) (33), a primary step of d-tagatose (34) and co-fermentation investigation with cattle slurry using an anaerobic digestion tank (35).

Culture medium optimization was evaluated using desirability function and response surface methodology (36; 37). The bioprocess optimization gave a final protease activity of 27.67 $U.mL^{-1}$ by setting whey permeate and yeast extract concentrations to the highest levels which was validated in a quintuplicate shake flask experiment, resulting in 30.97 ± 0.3311 $U.mL^{-1}$. Protease activity on validated experiments was superior and the optimum factor settings were employed to formulate medium for further investigations of protease production by SRM-2AA (12.83 $g.L^{-1}$ whey permeate, 4.41 $g.L^{-1}$ yeast extract, pH 7 and 25°C).

Our initial scaling up survey brought similar behavior on optimized culture medium in 100 mL-Erlenmeyer flasks and 1500 mL-bioreactor, highlighting outcomes obtained to protease production at 34.19 and 34.13 $U.mL^{-1}$, respectively. It was evidenced that cell growth and protease production is not correlated, i.e., the conditions which favors maximum microorganism growth not necessarily result in highest protease production (27). Large-scale production is challenging due to the difficulty in assessing the factors that influence cultivation. Parameters as aeration, transference rate of nutrients, agitation, pH, temperature and medium composition influence microbial metabolite production and cell growth (38). Thereafter, enzymes are susceptible to mechanical force, which may disturb molecule structure leading to denaturation and stress condition might contribute negatively toward cell growth and enzyme stability (16).

It is relevant to produce enzymes in inexpensive and optimized media on large-scale being the bioprocess commercially viable (16). Asitok et al. (39) optimized medium conditions of protease produced by *Stenotrophomonas acidaminiphila* mutant strain using response surface methodology and artificial neural network, in which proteolytic production was significantly improved to 411,713.87 U activity and had successfully scaled up in a 5L-bioreactor by 22-fold. Prasad and coworkers (16) improved protease production by a moderately halophilic *Bacillus* sp. utilizing wheat bran as cost effective substrate on medium composition and then was scaled up to 1500 mL in fermentor that reached a maximum activity of 4.32 ± 0.15 $U.mL^{-1}$. Espoui et al. (40) investigated protease enzyme production by *Bacillus licheniformis* bacteria also using wheat bran on culture medium and maximum protease activity in the Erlenmeyer flask and batch bioreactor was attained at 596 and 683.93 $U.mL^{-1}$.

The partial biochemical characterization of protease from SRM-2AA allowed to investigate effect of pH, in which proteolytic activity displayed it maximum at pH 8.2. Hence, proteases from SRM-2AA can be classified as an alkaline protease. These results are in accordance with literature, in which Espersen et al. (41) reported T-like proteases from *Amycolatopsis keratinophila* subsp. *keratinophila* $D2^T$ with optimum pH around 8-9. Nascimento et al. (42) characterized fibrinolytic proteases from *Mucor subtilissimus* UCP 1262 with maximum activity at pH 8.0 and Sarkar and K (43) encountered alkaline protease from *Streptomyces* sp. with optimum pH at 8.5. Optimum temperature on enzyme extract determined to be 45 °C, which implies that proteolytic enzyme is a thermo-tolerant enzyme. Other researches described maximum activity at 45 °C, as proteases from *Streptomyces* sp. (43), proteolytic enzymes from *Euphorbia resinifera* latex (44) and aspartic proteases from sábalo (*Prochilodus lineatus*) (45). Thermo- and alkaline properties suggest potential proteases application in detergent, leather and food industries (7; 43).

The thermal stability results of the protease from bacterium SRM-2AA demonstrated stability at 1-40 °C after 360 minutes. Additional surface charges, extensive ionic networks or random genetic drift might be responsible for extend thermal stability of proteolytic enzymes (46), i.e., the values up to 100% of residual activity. On the other hand, the enzyme destabilizes due to abrupt loss in its structure when weak interactions like hydrogen bonds are affected (37); that can be observed by activity losses above 40 °C. The thermostable proteases in this study suggest promise efficiency in terms of industry, because most of the thermostable proteases lose their stability at low temperatures even though proteolytic enzymes show catalytic efficiency and stability at higher temperature (13). Proteolytic enzymes showed up to 95% of

pH stability at 5.8 to 7.4 behind 60 min-incubation, indicating stability near at neutral pH conditions. At low and high pH, the increase or decrease in the hydrogen ion concentration in the reaction mixture changes the ionization states of amino acid that maintain the three-dimensional structure of the protein or the enzyme, in which can result on enzyme denaturation (37). Our findings are in accordance with Yi et al. (47) that had investigated extracellular protease from *Shewanella putrefaciens* isolated from bigeye tuna, in which protease demonstrated stability at pH 7.0-10.0 and low temperature (< 40 °C). It is seen that protease from bacterium SRM-2AA is a powerful candidate for industry application as it is capable to preserve more than 90% of its activity at a broad temperature range, from 1 to 40 °C, and a pH range 5.8 to 7.4.

In the presence of divalent metal ions protease activity slightly enhanced in the presence of Mg^{+2} and Ca^{+2}, by stabilizing its ternary structure and protecting it against thermal denaturation (48; 49). Proteases from different bacteria species in the literature showed similar positive effects on proteolytic activity in the presence of Mg^{+2} and Ca^{+2} (50; 39; 41). A protease activity inhibition was observed with Co^{+2}, Cu^{+2} and Ni^{+2} ions, that was also reported by Asitok et al. (39) and Bouacem et al. (48). These results indicates that the activity of protease from bacterium SRM-2AA is not metal-dependent.

For applications purpose, the chloride calcium was investigated in order to help milk coagulation. Calcium acts creating isoeletric conditions and connecting casein micelles as a bridge. $CaCl_2$ addition to pasteurized milk is interesting because ensure proper clot formation, reduction in clotting time, increased curd firmness and gel strength. In controversial point, the excess could produce a bitter taste (51; 17). Milk clotting activity diminished at concentration higher than 0.02 $mol.L^{-1}$ probably due to the saturation of micelles negative residues when Ca^{+2} concentration had raised up or to the increase on ionic force (51). Outcomes from literature reports highest milk clotting activity at 0.04 $mol.L^{-1}$ $CaCl_2$ concentration from *Thermomucor indicae-seudaticae* N31 (51; 17). Milk-clotting enzymes from *Solanum dubium* seeds showed a maximum activity when 0.2 and 0.4 $mol.L^{-1}$ $CaCl_2$ were applied (52) and ovine pepsin had better performance at 0.05 M of $CaCl_2$ (53).

Another food approach is the tenderization effect on meat. Several intrinsic and extrinsic characteristics affect meat quality features, including tenderness. This parameter is frequently available by Warner-Bratzler Shear Force (WBSF) that measures the maximum force (N) as a function of knife movement and the compression to cut off a meat sample (54). Texture is

relevant to consumers for food quality identification, Destefanis et el. (55) available consumer ability to discern three levels of beef tenderness established by WBSF: tough, tender and intermediate. Beef with superior values of 52.68 N is considered “tough” and inferior to 42.87 N is perceived by consumers as ‘‘tender’’. Considering these reliable thresholds, our results are in agreement with literature. Zhao et al. (18) realized a study with purified proteases from *Pseudoalteromonas* sp. SM9913 that demonstrated 23% of reduction on meat shear force when compared to untreated beef and bromelain effect brought similar results (22%). Proteases from bacterium SRM-2AA in this survey showed better performance on shear force reduction for both untreated meat (40.67%) and bromelain activity (37.38%).

5.5. Conclusion

Extracellular protease produced by bacterium SRM-2AA demonstrated superior protease activity from others collection strains. Preliminary screening for carbon and nitrogen sources for culture medium composition evidenced whey permeate and yeast extract as best protease inducer and CHNS analysis conducted the initial C/N proportions to study (4:1). It seems to be the first report that utilize whey permeate, an agro-industrial waste, as an inducer for protease production. Culture medium composition was improved by statistical optimization and validated by response surface methodology, ANOVA and Fisher-based test that attained a media composed by whey permeate at 12.83 $g.L^{-1}$ and yeast extract at 4.41 $g.L^{-1}$ prepared with phosphate-buffered saline, pH 7.0 at 25 °C. A final protease activity validation resulted in 30.97 $U.mL^{-1}$. Scaling up the fermentation 15-fold, the proteolytic production profile showed good performance reaching 34.13 $U.mL^{-1}$ of activity. Enzyme production gave promises results encouraging future detailed investigation in bioreactors with larger volumes. The partial characterization displayed optimum activity at pH 8.2 and 45 °C, indicating an alkaline and thermostable enzyme. Proteases stability against temperature and pH showed more than 90% of activity preserved from 1 to 40 °C and a pH range 5.8 to 7.4. Food approach analyses suggest that proteolytic enzyme from bacterium SRM-2AA its suitability in industries; it could be a good candidate for cheese making as well as a meat tenderizer.

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5.8. Supplementary file

Table 5-10: Effect estimates of proteolytic activity response in 2^4 full factorial design.

Factor	Effect	Sdt. Err. Pure Err	t(3)	P	-95, % Cnf. Limt	+95, % Cnf. Limt	Coeff	Std. Err. Coeff	-95, % Cnf. Limt	+95, % Cnf. Limt
Mean/Interc.	22.71947	0.114652	198.1599	0.000000	22.35459	23.08434	22.71947	0.114652	22.35459	23.08434
(1)CWP (g/L)	1.78800	0.256370	6.9743	0.006050	0.97212	2.60388	0.89400	0.128185	0.48606	1.30194
(2)CYE (g/L)	4.51067	0.256370	17.5944	0.000400	3.69478	5.32655	2.25533	0.128185	1.84739	2.66328
(3)Ph	0.75067	0.256370	2.9281	0.061096	-0.06522	1.56655	0.37533	0.128185	-0.03261	0.78328
(4)Temperature (°C)	-1.24133	0.256370	-4.8420	0.016805	-2.05722	-0.42545	-0.62067	0.128185	-1.02861	-0.21272
1 by 2	-0.61200	0.256370	-2.3872	0.096988	-1.42788	0.20388	-0.30600	0.128185	-0.71394	0.10194
1 by 3	-0.89467	0.256370	-3.4897	0.039774	-1.71055	-0.07878	-0.44733	0.128185	-0.85528	-0.03939
1 by 4	-0.45467	0.256370	-1.7735	0.174256	-1.27055	0.36122	-0.22733	0.128185	-0.63528	0.18061
2 by 3	0.27067	0.256370	1.0558	0.368580	-0.54522	1.08655	0.13533	0.128185	-0.27261	0.54328
2 by 4	-0.13200	0.256370	-0.5149	0.642170	-0.94788	0.68388	-0.06600	0.128185	-0.47394	0.34194
3 by 4	0.32667	0.256370	1.2742	0.292340	-0.48922	1.14255	0.16333	0.128185	-0.24461	0.57128

Table 5-11: Effect estimates of cell growth response in 2^4 full factorial design.

Factor	Effect	Sdt. Err. Pure Err	t(3)	P	-95, % Cnf. Limt	+95, % Cnf. Limt	Coeff	Std. Err. Coeff	-95, % Cnf. Limt	+95, % Cnf. Limt
Mean/Interc.	4.7006E+13	1.0592E+11	443.7689	0.000000	4.6669E+13	4.7343E+13	4.7006E+13	1.0592E+11	4.6669E+13	4.734310E+13
(1)CWP (g/L)	1.0680E+13	2.3685E+11	45.0910	0.000024	9.9262E+12	1.1434E+13	5.3400E+12	1.1843E+11	4.9631E+12	5.716888E+12
(2)CYE (g/L)	2.8395E+13	2.3685E+11	119.8838	0.000001	2.7641E+13	2.9149E+13	1.4197E+13	1.1843E+11	1.3820E+13	1.457439E+13
(3)pH	5.3475E+12	2.3685E+11	22.5772	0.000190	4.5937E+12	6.1013E+12	2.6737E+12	1.1843E+11	2.2969E+12	3.050638E+12
(4)Temperature (°C)	8.5500E+11	2.3685E+11	3.6098	0.036508	1.0122E+11	1.6088E+12	4.2750E+11	1.1843E+11	5.0612E+10	8.043882E+11
1 by 2	-1.5000E+10	2.3685E+11	-0.0633	0.953487	-7.6878E+11	7.3878E+11	-7.5000E+09	1.1843E+11	-3.8439E+11	3.693882E+11
1 by 3	8.4750E+11	2.3685E+11	3.5781	0.037335	9.3724E+10	1.6013E+12	4.2375E+11	1.1843E+11	4.6862E+10	8.006382E+11
1 by 4	-1.6500E+11	2.3685E+11	-0.6966	0.536160	-9.1878E+11	5.8878E+11	-8.2500E+10	1.1843E+11	-4.5939E+11	2.943882E+11
2 by 3	-1.8375E+12	2.3685E+11	-7.7579	0.004455	-2.5913E+12	-1.0837E+12	-9.1875E+11	1.1843E+11	-1.2956E+12	-5.418618E+11
2 by 4	-1.0200E+12	2.3685E+11	-4.3064	0.023048	-1.7737E+12	-2.6622E+11	-5.1000E+11	1.1843E+11	-8.8689E+11	-1.331118E+11
3 by 4	-3.6750E+11	2.3685E+11	-1.5516	0.218560	-1.1213E+12	3.8628E+11	-1.8375E+11	1.1843E+11	-5.6064E+11	1.931382E+11

Table 5-12: Effect estimates of proteolytic activity response on first 2^2 central composite design.

Factor	Effect	Sdt. Err. Pure Err	t(3)	p	-95, % Cnf. Limt	+95, % Cnf. Limt	Coeff	Std. Err. Coeff	-95, % Cnf. Limt	+95, % Cnf. Limt
Mean/Interc.	24.73867	0.234561	105.4681	0.000002	23.99219	25.48514	24.73867	0.234561	23.99219	25.48514
(1)C_{WP} ($g.L^{-1}$) (L)	1.13406	0.331719	3.4188	0.041881	0.07839	2.18974	0.56703	0.165859	0.03919	1.09487
C_{WP} ($g.L^{-1}$) (Q)	0.25333	0.370873	0.6831	0.543584	-0.92695	1.43362	0.12667	0.185436	-0.46347	0.71681
(2) C_{YE} ($g.L^{-1}$)	4.17592	0.331719	12.5888	0.001081	3.12025	5.23160	2.08796	0.165859	1.56012	2.61580
C_{YE} ($g.L^{-1}$) (Q)	0.18400	0.370873	0.4961	0.653877	-0.99628	1.36428	0.09200	0.185436	-0.49814	0.68214
1L by 2L	-0.27200	0.469121	-0.5798	0.602719	-1.76495	1.22095	-0.13600	0.234561	-0.88248	0.61048

Table 5-13: Effect estimates of cell growth response on first 2^2 central composite design.

Factor	Effect	Sdt. Err. Pure Err	t(3)	P	-95, % Cnf. Limt	+95, % Cnf. Limt	Coeff	Std. Err. Coeff	-95, % Cnf. Limt	+95, % Cnf. Limt
Mean/Interc.	5.9225E+13	2.2798E+11	259.7813	0.000000	5.8499E+13	5.9950E+13	5.9225E+13	2.2798E+11	5.8499E+13	5.9950E+13
(1)C_{WP} ($g.L^{-1}$) (L)	1.0778E+13	3.2241E+11	33.4293	0.000059	9.7520E+12	1.1804E+13	5.3890E+12	1.6120E+11	4.8759E+12	5.9020E+12
C_{WP} ($g.L^{-1}$) (Q)	7.9575E+12	3.6047E+11	22.0754	0.000203	6.8103E+12	9.1047E+12	3.9787E+12	1.8023E+11	3.4052E+12	4.5523E+12
(2) C_{YE} ($g.L^{-1}$)	1.7527E+13	3.2241E+11	54.3616	0.000014	1.6501E+13	1.8553E+13	8.7634E+12	1.6121E+11	8.2504E+12	9.2764E+12
C_{YE} ($g.L^{-1}$) (Q)	-5.7225E+12	3.6047E+11	-15.8752	0.000543	-6.8697E+12	-4.5753E+12	-2.8612E+12	1.8023E+11	-3.4348E+12	-2.2876E+12
1L by 2L	4.2000E+11	4.5596E+11	0.9211	0.424916	-1.0310E+12	1.8711E+12	2.1000E+11	2.2798E+11	-5.1553E+11	9.3553E+11

Table 5-14: Effect estimates of proteolytic activity response on second 2^2 central composite design.

Factor	Effect	Sdt. Err. Pure Err	t(3)	p	-95, % Cnf. Limt	+95, % Cnf. Limt	Coeff	Std. Err. Coeff	-95, % Cnf. Limt	+95, % Cnf. Limt
Mean/Interc.	26.85333	0.185392	144.8459	0.000001	26.26333	27.44333	26.85333	0.185392	26.26333	27.44333
(1)C_{WP} ($g.L^{-1}$) (L)	1.54145	0.262185	5.8793	0.009818	0.70706	2.37584	0.77072	0.131092	0.35353	1.18792
C_{WP} ($g.L^{-1}$) (Q)	-0.32933	0.293131	-1.1235	0.343018	-1.26221	0.60354	-0.16467	0.146566	-0.63110	0.30177
(2) C_{YE} ($g.L^{-1}$)	1.37826	0.262185	5.2568	0.013410	0.54387	2.21265	0.68913	0.131092	0.27194	1.10632
C_{YE} ($g.L^{-1}$) (Q)	-0.57467	0.293131	-1.9604	0.144790	-1.50754	0.35821	-0.28733	0.146566	-0.75377	0.17910
1L by 2L	-0.78400	0.370785	-2.1144	0.124840	-1.96400	0.39600	-0.39200	0.185392	-0.98200	0.19800

Table 5-15: Effect estimates of cell growth response on second 2^2 central composite design.

Factor	Effect	Sdt. Err. Pure Err	t(3)	P	-95, % Cnf. Limt	+95, % Cnf. Limt	Coeff	Std. Err. Coeff	-95, % Cnf. Limt	+95, % Cnf. Limt
Mean/Interc.	8.4065E+13	2.7125E+11	309.9203	0.000000	8.3202E+13	8.4928E+13	8.4065E+13	2.7124E+11	8.3201E+13	8.4928E+13
(1)C_{WP} ($g.L^{-1}$) (L)	8.6970E+12	3.8360E+11	22.6720	0.000188	7.4762E+12	9.9178E+12	4.3485E+12	1.9180E+11	3.7381E+12	4.9589E+12
C_{WP} ($g.L^{-1}$) (Q)	-5.8875E+11	4.2888E+11	-1.3728	0.263445	-1.9536E+12	7.7613E+11	-2.9437E+11	2.1444E+11	-9.7682E+11	3.8807E+11
(2) C_{YE} ($g.L^{-1}$)	2.0524E+13	3.8360E+11	53.5043	0.000014	1.9303E+13	2.1745E+13	1.0262E+13	1.9180E+11	9.6517E+12	1.0872E+13
C_{YE} ($g.L^{-1}$) (Q)	-4.6237E+12	4.2888E+11	-10.7810	0.001707	-5.9886E+12	-3.2589E+12	-2.3119E+12	2.1444E+11	-2.9943E+12	-1.6294E+12
1L by 2L	9.0000E+10	5.4249E+11	0.1659	0.878786	-1.6364E+12	1.8164E+12	4.5000E+10	2.7125E+11	-8.1823E+11	9.0823E+11

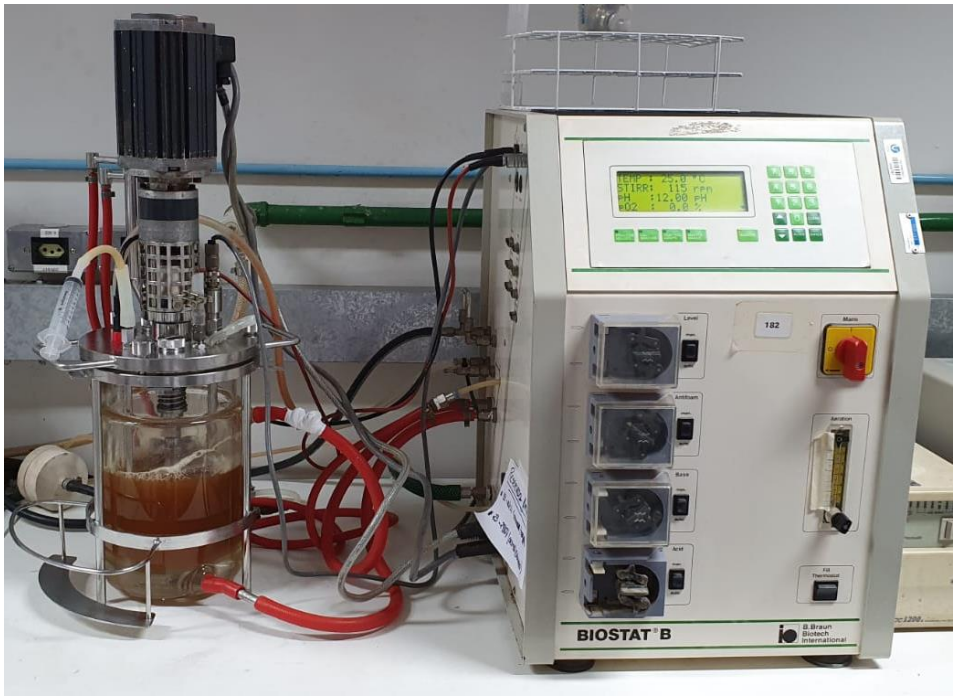

Figure 5-13: Biostat B fermenter utilized to protease production by bacterium SRM-2AA.

Capítulo 6 – Conclusão

Proteases extracelulares produzidas pela bactéria SRM-2AA demonstraram atividade proteolítica superior às demais cepas do banco de microrganismos. Os testes preliminares da escolha da fonte de carbono e nitrogênio para composição do meio de cultivo, iniciaram-se com a análise CHNS (carbono, hidrogênio, nitrogênio e enxofre) realizada na amostra da bactéria, a qual possibilitou a obtenção das proporções iniciais de C/N (4:1). Logo, a fonte de nitrogênio que resultou em maior produção de enzimas proteolíticas foi o extrato de levedura (16,46 $U.mL^{-1}$) e, para fonte de carbono, permeado de soro de leite (23,32 $U.mL^{-1}$). Este subproduto da fabrição de queijos é rico em lactose, proteínas e minerais que contribuem para o crescimento de microrganismos e síntese de enzimas microbianas. O presente trabalho é o primeiro a utilizar permeado de soro de leite para produção de proteases.

A composição do meio de cultivo foi determinada pela otimização estatística e validada pela metodologia de superfície de resposta, ANOVA e teste-F, os quais possibilitaram obter o meio com permeado de soro de leite a 12,83 $g.L^{-1}$ e extrato de levedura a 4,41 $g.L^{-1}$, preparado com tampão fosfato salino, pH 7, a 25 °C. A validação do meio de cultivo foi realizada em fermentação submersa (100 mL), a qual resultou em uma atividade proteolítica de 30,97 $U.mL^{-1}$. O aumento de escala em 15 vezes foi submetido em biorreator (1500 mL) que demonstrou boa performance no perfil de produção de proteases, atingindo 34,13 $U.mL^{-1}$. Estes resultados encorajam futuras investigações em biorreatores de maior volume.

A caracterização parcial apresentou atividade ótima em pH 8,2 e temperatura de 45 °C, indicando enzimas proteolíticas alcalinas e termoestáveis. A estabilidade frente a temperatura e pH evidenciaram mais de 90% de atividade preservada entre 1 a 40 °C e pH entre 5,8 a 7,4. Os testes de aplicação indicaram que as proteases apresentaram atividade de coagulação do leite de 2 181,82 $U.mL^{-1}$ a 0,02 $mol.L^{-1}$ de $CaCl_2$ e, também foram capazes de reduzir a força de cisalhamento da carne em 40,67% em comparação a carne não tratada com enzimas, contribuindo com uma maior maciez da carne. Assim, as aplicações preliminares da protease proveniente da bactéria SRM-2AA sugerem aplicabilidade destas nas indústrias alimentícias, tanto para fabricação de queijo quanto amaciante de carne.

Capítulo 7 – Sugestões para trabalhos futuros

- Identificação da bactéria SRM-2AA por meio de técnicas de biologia molecular;
- Otimização do estudo cinético de crescimento da bactéria SRM-2AA em reator estilo batelada (1500 mL);
- Aumentar a escala de produção da enzima proteolítica pela bactéria SRM-2AA em reatores estilo batelada.

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