Revista GEAMA – Ciências Ambientais e Biotecnologia
Scientific Journal of Environmental Sciences and Biotechnology
ISNN: 2447-0740

11
1

Cheese whey as a sustainable substrate for protease production by Aspergillus sp. UCP 1290

Soro de queijo como substrato sustentável para produção de protease por Aspergillus sp. UCP 1290

Jaqueline dos Santos Marinho[1], Galba Maria de Campos Takaki[1], Luciana de Oliveira Franco[2], Marcos Antonio Barbosa de Lima[2], Carlos Alberto Alves da Silva[1]

[1] Catholic University of Pernambuco, Pernambuco, Brazil
[2] Federal Rural University of Pernambuco, Pernambuco, Brazil

Contato: marcos.barbosalima@ufrpe.br

Keyword
co-products
alternative materials
waste
appreciation
environment

ABSTRACT
The objective of this study was to evaluate protease production by submerged fermentation of Aspergillus spp. by using cheese whey as the sole substrate. Initially, six Aspergillus spp. isolates were screened for protease production in a conventional medium containing gelatin and an alternative whey-based medium. A 23 factorial design was used to evaluate the main effects and interactions of the variables (i.e., whey concentration, temperature, and medium pH) on protease production. Aspergillus sp. UCP 1290 was selected for its higher proteolytic activity, which reached 28.75 U/mL in the conventional medium and 37.33 U/mL in the whey medium. The highest protease production, 129.80 U/mL, by Aspergillus sp. UCP 1290 was obtained at 20% whey concentration, pH 8.0, and temperature 32 °C under submerged fermentation at 150 rpm for 96 hours. Temperature and whey concentration were the most significant independent variables for enzyme production. Proteolytic activity was enhanced by the interaction between a low concentration of cheese whey and a higher temperature. The enzyme exhibited maximum catalytic activity at 60 °C and pH 7.0, classifying it as a neutral protease. Results have shown Aspergillus sp. UCP 1290 is an effective producer of protease using cheese whey as the sole substrate as well as the produced enzyme has potential applications in industrial processes.

Palavras-Chave
produtividade
macronutrientes
agricultura sustentável

RESUMO
O objetivo deste estudo foi avaliar a produção de protease por fermentação submersa de Aspergillus spp. utilizando soro de queijo como substrato único. Inicialmente, seis isolados de Aspergillus spp. foram selecionados para produção de protease em meio convencional contendo gelatina e em meio alternativo à base de soro de leite. Um planejamento fatorial 23 foi usado para avaliar os principais efeitos e interações das variáveis (i.e., concentração de soro de leite, temperatura e pH do meio) na produção de proteases. A amostra UCP 1290 foi selecionada por sua maior atividade proteolítica, que atingiu 28,75 U/mL no meio convencional e 37,33 U/mL no meio de soro de leite. A maior produção de protease, 129,80 U/mL, por Aspergillus sp. UCP 1290 foi obtida a 20% de concentração de soro de leite, pH 8,0 e temperatura de 32 °C sob fermentação submersa à 150 rpm por 96 horas. A temperatura e a concentração de soro de leite foram as variáveis independentes mais significativas para a produção da enzima. A atividade proteolítica foi aumentada pela interação entre uma baixa concentração de soro de queijo e uma temperatura mais alta. A enzima exibiu atividade catalítica máxima a 60 °C e pH 7,0, classificando-a como uma protease neutra. Os resultados mostraram que Aspergillus sp. UCP 1290 é um produtor eficaz de protease usando soro de queijo como único substrato, bem como a enzima produzida tem aplicações potenciais em processos industriais.

Informações do artigo
Recebido: 07 de agosto, 2024
Aceito: 13 de abril, 2025
Publicado: 30 de abril, 2025

Introduction

Proteases are an important group of multifunctional enzymes that are widely used in various industries and account for nearly 60% of all marketed enzymes worldwide. The global protease market is expected to reach $3 billion by 2024, with a compound annual growth rate of 6.1% (Naveed et al., 2021).

Microbial proteases are preferred in industrial applications due to their technical and economic advantages. Fungal proteases are in high industrial demand as they offer a good cost-benefit ratio. The separation of mycelium is easier compared to bacterial cells, and fungal proteases exhibit stability, high diversity, and substrate specificity (Razzaq et al., 2019).

In addition, fungi are generally recognized as safe (GRAS) to be added to food (Naveed et al. 2021 and Benerjee and Ray, 2017 and Souza et al., 2015). Among fungi, the genus *Aspergillus* is widely used in biotechnological processes and is an excellent producer of various enzymes, including proteases (Menezes et al., 2021 and Naveed et al., 2021). However, a major challenge in industrial enzyme production is to reduce production costs, particularly by replacing synthetic media, which account for about 40% of the total cost (Markawala et al., 2021).

Various low-cost substrates (e.g., wheat bran, oilseed cakes, soybean bran, and rice bran) have been reported to yield significant amounts of protease (Subhash et al., 2023). Cheese whey, a byproduct of cheese manufacturing, is highly polluting due to its high biochemical oxygen demand and chemical oxygen demand. However, cheese whey is rich in proteins, fermentable sugars and nutrients, making it suitable for reuse in co-processing (Ryan and Walsh 2016 and Pacuma et al., 2015). In Brazil, 13% of the dairy industry partially uses whey to produce other dairy products, while 27% discards all the whey produced, either to wastewater treatment or as animal feed (Trindade et al., 2019).

An alternative to increase the value of cheese whey is its bioconversion by fungi and bacteria into value-added products such as biofuels and bioplastics, offering both economic and environmental benefits (Ryan and Walsh 2016).

Therefore, the global demand for proteases requires the search for new species and strains of microorganisms with high protease production capacity as well as the exploration of sustainable and low-cost substrates (Ravindram et al., 2019). Therefore, the aim of this study was to evaluate the production of proteases by *Aspergillus* spp. in an alternative medium based on cheese whey.

Material and Methods

Proteases are an i

Microorganisms

Six strains of *Aspergillus* spp. (UCP 0076, UCP 1064, UCP 1132, UCP 1177, UCP 1290, UCP 1461) used in this study were kindly provided by the UCP Culture Collection of the Multi-user Center for Analysis and Characterization of Biomolecules and Surface of Materials, Catholic University of Pernambuco, registered with the World Federation for Culture Collection (WFCC). Stock cultures were maintained on Sabouraud agar at 4 °C.

Agrifood waste

Cheese whey was kindly provided by a small rural cheese producer from the city of Cachoeirinha, State of Pernambuco, Brazil.

Preparation of the inoculum

Strains of *Aspergillus* spp. were grown on Sabouraud agar at 28 °C for 72 hours. Twenty mycelial discs, each 10 mm in diameter, were then collected and used as inoculum in the selection and factorial design experiments.

Selection of protease producing fungi

Selection was performed in both conventional and alternative media under submerged fermentation. Erlenmeyer flasks (250 mL) containing 100 mL of standard medium with 1% gelatin according to Manachini et al. (1987) and an alternative medium prepared with 25% cheese whey and a saline base (0.23% KH_2PO_4, 0.25% K_2HPO_4, 0.1% $MgSO_4$, 0.1% $FeSO_4$, pH 6.0) were inoculated with 20 mycelial discs of *Aspergillus* spp. strains. These were then incubated at 28 °C, 150 rpm for 96 hours in triplicate. Culture supernatants were centrifuged at 10,000 rpm for 5 minutes at 4 °C, and the cell-free metabolic fluid was used to determine proteolytic activity.

Determination of proteolytic activity

Protease activity was determined by the modified method of Leighton et al. (1973). Reaction was initiated by mixing 150 µL of crude enzyme extract with 250 µL of 1% azocasein (w/v) dissolved in Tris-HCl buffer (0.2 M; pH 7.2) and incubated in a water bath at 40 °C for 30 minutes. The reaction was stopped by adding 500 µL of 10% trichloroacetic acid (TCA), followed by centrifugation at 15,000 rpm for 5 minutes at 4 °C. A total of 800 µL of the reaction mixture was then added to 1.4 mL of 1 M NaOH. The intensity of the color developed was measured in a spectrophotometer at 420 nm. For each sample, a blank was prepared by immediately adding 10% TCA to the reaction mixture.

The experiment was performed in triplicate. One unit of enzyme activity was defined as the amount of enzyme required to cause an increase in absorbance of 0.01 unit in 1 hour, expressed in U/mL.

Factorial design

A 2^3 full factorial design with 4 centers was used to analyze the main effects and interactions of the independent variables (i.e., pH, temperature, and whey concentration) on the response variable (i.e., proteolytic activity) in submerged fermentation at 150 rpm for 96 hours. Independent variables were evaluated at three levels: minimum (-1), central (0), and maximum (+1) (Table 1).

Table 1 Levels of 2^3 factorial design variables for protease production by *Aspergillus* sp. UCP 1290 in whey medium

Variables	Levels		
	-1	0	+1
Cheese whey (%, v/v)	20	60	100
Temperature (°C)	24	28	32
pH	4	6	8

Source: Authors (2024)

Determination of the effect of pH and temperature on proteolytic activity

Assessment of ideal pH and temperature for the catalytic activity of enzyme was performed by using the crude extract, following the method of Gimenez et al. (2019), with modifications to the pH and temperature ranges. Enzymatic reaction was performed at different pH values (5.0, 6.0, 7.0, 8.0, 9.0, and 10.0) and temperatures (20, 30, 40, 50, 60, 70, 80, and 90 °C). Different buffers were used to maintain pH at different levels: citrate-phosphate (pH 5.0), sodium phosphate (pH 6.0), Tris-HCl (pH 7.0 and 8.0), and carbonate-bicarbonate (pH 9.0 and 10.0). Proteolytic activity was determined as described in section 2.5.

Statistical analysis

Statistica® 12.0 software (StatSoft, Inc., Tulsa, Oklahoma, USA) was used to analyze the results of the experimental tests and to analyze interactions and multiple effects that were considered statistically significant at $p < 0.05$. The experimental error estimate was calculated based on the four replicates of the central point.

Results and Discussion

Screening to select highest protease producers

The search for new microorganisms and low-cost substrates for efficient enzyme production is an ongoing process (Ravindran et al., 2018 and Souza et al., 2015). Aspergillus species are among the best fungi for protease production (Mamo et al., 2022 and Othman et al., 2023).

Six Aspergillus spp. strains were screened for protease production in submerged fermentation using a conventional medium containing gelatin as an inducing substrate and an alternative medium based on cheese whey (Table 2). All *Aspergillus* spp. strains exhibited proteolytic activity in both media, with higher enzymatic activity observed in the alternative medium based on cheese whey. Aspergillus sp. UCP 1290 was selected for its higher proteolytic activity of 37.33 U/mL in the alternative medium. Radha et al. (2011) were observed the proteolytic activity of Aspergillus spp. in whey and molasses media and suggested the use of whey as a nitrogen source for protease production. Similarly, Gul et al. (2012) demonstrated excellent protease production by Rhizopus oryzae using whey as the sole substrate.

Besides, biomass production by all Aspergillus spp. strains was higher in the alternative medium containing whey.

Vamvakak et al. (2010) observed significant biomass production by Zygomycete species when grown in whey supplemented with salts only. Similarly, Ibarruri and Hernández (2019) reported biomass production by Rhizopus spp. in submerged fermentation using whey with low protein content, indicating the need for supplementation with nitrogen and carbon sources.

Cheese whey, a byproduct of cheese production, has a high nutritional value, consisting mainly of lactose (4.5-5% w/v), soluble proteins (0.6-0.8% w/v), lipids (0.4-0.5% w/v), and mineral salts (8-10% w/v of dry extract), which can support microbial growth and induce good proteolytic activity (Spalatele at el., 2012).

Biotechnological valorization of whey involves its conversion into bioethanol, bioplastic, lipids, organic acids, and enzymes, mainly by bacteria and fungi.

Therefore, the selection of microorganisms capable of fermenting whey is crucial, as not all microorganisms can utilize lactose as a carbon source for enzyme production at usable levels (Pescuma et al., 2015)

Table 2 Proteolytic activity and biomass production by *Aspergillus* spp. UCP 0076, 1064, 1132, 1177, 1290, and 1461 in conventional and alternative medium

Aspergillus strains	Alternative medium		Conventional medium	
	PA (U/mL)	Biomass (g/L)	PA (U/mL)	Biomass (g/L)
UCP 0076	35.49 ±0.03	3.40 ±0.10	9.42 ±0.03	0.50 ±0.06
UCP 1064	7.48 ±0.00	1.62 ±0.07	3.96 ±0.01	0.52 ±0.02
UCP 1132	19.43 ±0.02	1.17 ±0.04	4.84 ±0.07	0.50 ±0.02
UCP 1177	14.19 ±0.01	0.88 ±0.05	13.75 ±0.10	0.52 ±0.05
UCP 1290	37.33 ±0.06	0.88 ±0.38	28.75 ±0.01	0.77 ±0.03
UCP 1461	10.78 ±0.03	1.52 ±0.04	6.67 ±0.01	0.44 ±0.04

Source: Authors (2024)

Table 3 Production of protease by *Aspergillus* sp. UCP 1290 by using a 2^3 factorial planning for 96 h at 150 rpm in culture medium with whey

Runs	pH	Temperature (°C)	Substrate (%)	Proteolytic activity (U/mL)
1	4.0	24	20	47.15
2	8.0	24	20	20.09
3	4.0	32	20	106.33
4	8.0	32	20	129.80
5	4.0	24	100	35.93
6	8.0	24	100	30.95
7	4.0	32	100	26.18
8	8.0	32	100	23.03
9	6.0	28	60	28.16
10	6.0	28	60	23.98
11	6.0	28	60	22.81
12	6.0	28	60	20.97

Source: Authors (2024)

Protease production by *Aspergillus* sp. UCP 1290 using whey in submerged fermentation

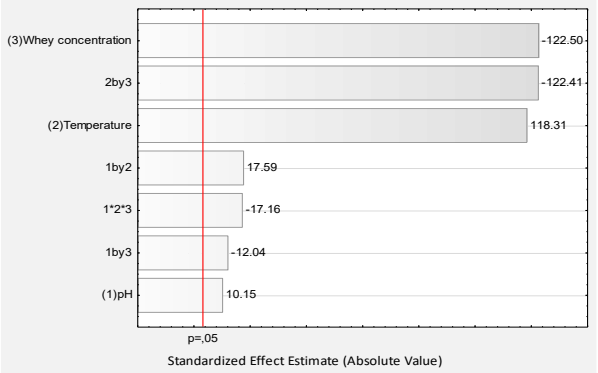
Composition of growth medium and environmental factors such as temperature, pH, inoculum size, and incubation time are fun
1290 were evaluated using a 23-factorial design (Table 3). Optimal conditions for proteolytic activity were found in run 4 with pH 8.0, temperature of 32 °C, and whey concentration of 20%, which maximized proteolytic activity from 37.33 U/mL to 129.80 U/mL, representing a 71.24% increase in production. By using a 2^3-factorial design. Nascimento et al. (2015) reported a maximum protease production of 48.33 U/mL with Mucor subtillissimus UCP 1262 using wheat bran as a substrate. Although the optimal concentrations of control brs for wheat bran production depe
(2015) reported maximum protease production for Aspergillus flavus and *Aspergillus niger* at pH 8 and 7 and temperatures of 30 °C and 35 °C, respectively. Therefore, Aspergillus sp. UCP 1290 used in this study showed maximum proteolytic activity under environmental conditions similar to those reported in the literature.

The ratio of carbon to nitrogen is essential for microbial growth and maximum enzyme production. However, the specific requirements for carbon and nitrogen sources vary between microbial species and even between different strains of the same species (Sharma et al., 2017). The use of agroindustrial residues to produce proteases by Aspergillus species has emerged as a promising alternative to conventional carbon and nitrogen sources (Ielizek et al., 2020 and Revindran et al., 2018).

We demonstrated *Aspergillus* sp. UCP 1290 produced a high proteolytic activity of 129.8 U/mL in a whey-based culture medium by using whey as the sole carbon and nitrogen source. This strain used lactose and whey proteins as carbon and nitrogen sources, respectively, to support its growth and protease production, which makes Aspergillus sp. UCP 1290 a promising candidate for industrial bioprocesses involving the conversion of whey to proteases.

Statistical analysis showed that all variables and their interactions were significant at the 95% confidence level ($p \leq 0.05$) (Figure 1). However, cheese whey concentration was the most significant variable, showing a negative effect on proteolytic activity, this suggests that reducing the whey concentration may result in higher protease activity. Arumugam et al. (2020) observed increased production of alkaline protease by Aspergillus tamarii at optimal concentrations of control brs, wheat bran, skim milk, and soybean flour. Cheese whey and other agrosubstrates can be used for biomass and enzyme production by microorganisms Ieliszek et al. (2020) and Marzo et al. (2019). In addition, temperature is a critical factor to control in bioprocesses, and its optimal value for growth and enzyme production varies from species-to-species (Sharma et al., 2019). The interaction between the independent variables of maximum temperature and minimum substrate concentration was antagonistic, negatively affecting protease production. Although pH also showed statistical significance, its effect on protease production was less pronounced within the values evaluated. Therefore, whey is an efficient and promising substrate for protease production in submerged fermentation.

Figure 1 Pareto diagram of the effect of independent variables on proteolytic activity (U/mL) of *Aspergillus* sp. UCP 1290 in submerged fermentation using whey as substrate for 96 h.



Source: Authors (2024)

Effect of pH and temperature on protease activity

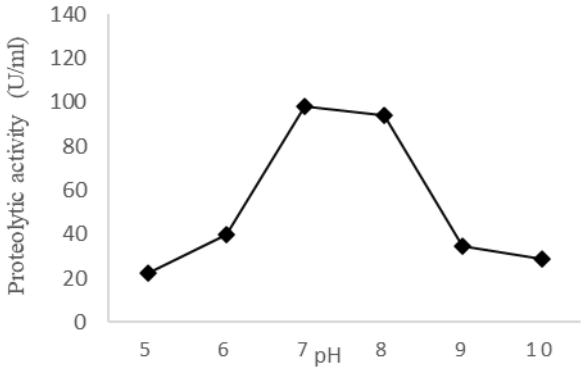
To evaluate the optimal pH of the protease produced by *Aspergillus* sp. UCP 1290, proteolytic activity was determined using different buffers over a pH range of 5 to 10 (Figure 2). Neutral proteases are defined as those that are active at neutral, slightly alkaline, or acidic pH (Razzaq et al., (2019).

In this study, maximum proteolytic activity was observed at pH 7.0 and 8.0 with values of 97.97 and 93.92 U/mL, respectively, indicating the presence of a neutral protease. Similarly, Wajeeha et al. (2021) reported higher protease activity in *Aspergillus flavus* in the pH range of 7.0 to 8.0. Ao et al. (2018) also reported a neutral protease produced by *Aspergillus oryzae* Y1, neutral proteases are valuable in the food industry because they generate less bitterness in protein hydrolysis. Additionally, neutral and alkaline fungal proteases are important in the processing of soy sauces and other soybean products (Razza et al. (2020).

Figure 3. shows the effect of temperature on the protease activity of *Aspergillus* sp. UCP 1290. Proteolytic activity was highest at 60 °C with a value of 119.58 U/mL, while temperatures above 60 °C resulted in a significant decrease in activity. Alkaline proteases from fungi and bacteria show optimal activity at temperatures between 50 °C and 70 °C (Sharma et al., 2019).

Osman. et al. (2014) reported the optimal temperature for protease produced by *Aspergillus terreus* was 55 °C. In contrast, Rukmi and Purwantisari (2020) observed higher protease activity in *Aspergillus flavus* at temperatures of 40 °C and 45 °C. High catalytic activity and thermal stability are highly desirable properties for the industrial use of proteases (Navved et al., (2021).

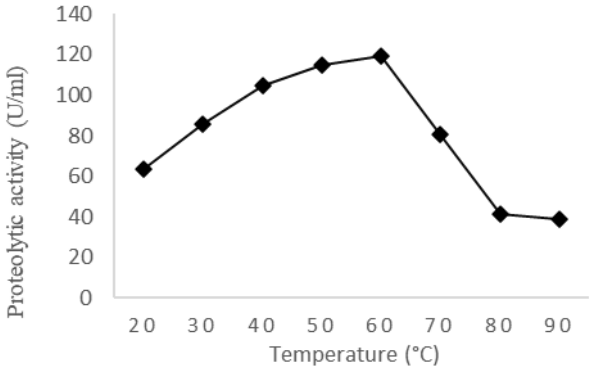
Figure 2. pH effect on protease activity produced by *Aspergillus* sp. UCP 1290 in submerged fermentation using whey as substrate for 96 h.



Source:Authors 2024

The results of this study showed good proteolytic activity of the protease produced by *Aspergillus* sp. UCP 1290 at relatively high temperatures, making it a promising candidate for exploration in various industries.

Figure 3. Temperature effect on protease activity produced by *Aspergillus* sp. UCP 1290 in submerged fermentation using whey as substrate for 96 h.



Source:Authors 2024

Conclusions

Aspergillus sp. UCP 1290 is an effective producer of protease in a whey-based culture medium. The factorial design facilitated an increase in protease production by *Aspergillus* sp. UCP 1290, identifying temperature, whey concentration, and their interaction as the most significant variables. The neutral protease produced is active at 60 °C, which is advantageous for industrial applications. Cheese whey can be used as a low-cost substrate as an alternative to synthetic media for protease production in submerged fermentation.

Acknowledgments

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE).

References

Ao Xl, Yu X, Wu, Dt. et al. Purification and characterization of neutral protease from *Aspergillus oryzae* Y1 isolated from naturally fermented broad beans. AMB Express. 2018 Jun; 8:96, doi: 10.1186/s13568-018-0611-6.

Arumugam N et al. Optimized production of extracellular alkaline protease from *Aspergillus tamarii* with natural by-products in a batch stirred tank bioreactor. Preparative Biochemistry & Biotechnology. 2020 Jun; 50, doi: 10.1080/10826068.2020.1777426.

Banerjee G. and Ray Ak. Impact of microbial proteases on biotechnological industries. Biotechnology and Genetic Engineering Reviews. 2017; 33(2), 119-143, doi: 10.1080/026 48725.2017.1408256.

Gimenes NC, Silveira E, Tambourgi EB. An overview of proteases: production, downstream processes and industrial applications. Separation & Purification Reviews. 2019; 1-21.

Gul A, et al. Efficient Utilization of Dairy Industry Waste for Hyper-Production and Characterization of a Novel Cysteine Protease. Pakistan Journal of Zoology. 2012; 44(3), 713-721.

Ibarruri j, Hernández I. Valorization of cheese whey and orange molasses for fungal biomass production by submerged fermentation with *Rhizopus* sp. Bioprocess and Biosystems Engineering. 2019; 42(8), 1285–1300, doi: 10.1007/s00449-019-02127-4.

Ieliszek M. et al. The aspects of microbial biomass use in the utilization of selected waste from the agro-food industry. Open Life Sciences. 2020;15(1), 787–796, doi: 10.1515/biol-2020-0099.

Leighton TJ. et al. The relationship of serine protease activity to RNA polymerase modification and sporulation in *Bacillus subtilis*. Journal of Molecular Biology. 1973; 76(1), 103–122, doi: 10.1016/0022-2836(73)90083-1.

Manachini PL, Fortina MG, Parini C. Purification and properties of an endopolygalacturonase produced by *Rhizopus stolonifer*. Biotechnology letters. 1987; 9(3), 219-224.

Marzo C. et al. Valorization of agro-industrial wastes to produce hydrolytic enzymes by fungal solid-state fermentation. Waste Management & Research. 2019; 37(2), 149-156.

Matkawala F. et al. Microbial alkaline serine proteases: Production, properties and applicati ons. World Journal of Microbiology and Biotechnology. 2021; 37(4), 63, doi: 10.1007/s112 74-021-03036-z.

Menezes BS. et al. Biomass of unconventional plants from Brazilian semiarid as substrate for hydrolytic enzymes production by *Aspergillus niger* under solid and submerged fermentation. Acta Scientiarum. Biological Sciences. 2021; 43, e48257-48257, doi: 10.4025/actas cibiolsci. v43i1.48257.

Nascimento T. et al. Production and Characterization of New Fibrinolytic Protease from *Mucor subtillissimus* UCP 1262 in Solid-State Fermentation. Advances in Enzyme Research, 2015; 03, 81–91, 2015, doi: 10.4236/aer.2015.33009.

Naveed M, et al. Protease—A Versatile and Ecofriendly Biocatalyst with Multi-Industrial Applications: An Updated Review. Catalysis Letters. 2021; 151(2), 307–323, doi: 10.1007/s 10562-020-03316-7.

Osman ME, Yasmin E, Khattab. *Aspergillus terreus* proteases: characterization and applications. Journal of Chemical, Biological and Physical Sciences. 2014; 4, 2333 –2346.

Pescuma M, De Valdez GF, Mozzi F. Whey-derived valuable products obtained by microbial fermentation. Applied Microbiology and Biotechnology. 2015; 99(15), 6183–61 96, doi: 10.1007/s00253-015-6766-z.

Radha S. et al. Production and optimization of acid protease by *Aspergillus* spp. under submerged fermentation. Arch Appl Sci Res. 2011; 3(2), 155–63.

Ravindran R. et al. A Review on Bioconversion of Agro-Industrial Wastes to Industrially Important Enzymes. Bioengineering. 2018; 5(4), 93, doi: 10.3390/bioengineering 5040093.

Razzaq A. et al. Microbial Proteases Applications. Frontiers in Bioengineering and Biotechnology. 2019; (7), 110, doi: 10.3389/fbioe.2019.00110.

Rukmi I, Purwantisari S. The production of alkaline protease from *Aspergillus flavus* DUCC K225 on rice bran containing medium. Journal of Physics: Conference Series. 2020; 1524(1), 012058, doi: /1088/1742-659611524/1/012058.

Ryan MP, Walsh G. The biotechnological potential of whey. Reviews in Environmental Science and Bio/Technology. 2016; 15 (3), 479–498, doi: /10.1007/s11157-016-94 02-1.

Sharma KM. et al. Microbial alkaline proteases: Optimization of production parameters and their properties. Journal of Genetic Engineering & Biotechnology. 2017; 15(1), 115–126, doi: 10.1016/j.jgeb.2017.02.001.

Sharma KM. et al. A Review on Microbial Alkaline Protease: An Essential Tool for Various Industrial Approaches. Industrial Biotechnology. 2019; 15, 69–78, doi: 10.1089/ind.20 18.0032.

Souza PM, et al. A biotechnology perspective of fungal proteases. Brazilian Journal of Microbiology. 2015; 46(2), 337–346, doi: /10.1590/S1517-838246220140359.

Spalatelu C. Biotechnological valorization of whey. Innovative Romanian Food Biotechnology. 2012; 10, 1.

Sudarkodi C, Sundar SK, Murugan M. Production and Optimization of Protease by Filamentous Fungus Isolated from Paddy Soil in Thiruvarur District Tamilnadu. J App Biol Biotech. 2015; 3(06), 066-069, doi: 10.7324/JABB.2015.3610.

Trindade MB, et al. Cheese whey exploitation in Brazil: a questionnaire survey. Food Science and Technology. 2019; 39(3), 788–791, doi: /10.1590/fst.07419.

Vamvakak IAN. et al. Cheese whey as a renewable substrate for microbial lipid and biomass production by Zygomycetes. Engineering in Life Sciences. 2010; 10(4), 348–360, doi: /10.1002/elsc.201000063.

Wajeeha AW. et al. Production, Purification, and Characterization of Alkaline Protease from *Aspergillus flavus* and its Compatibility with Commercial Detergents. BioResources. 2021; 16(1), 291–301.

Mamo, J., Kangwa, M., Suarez Orellana, J.F. *et al.* Purification and Characterization of Aspartic Protease Produced from *Aspergillus oryzae* DRDFS13 MN726447 Under Solid-State Fermentation. *Catal Lett* **152**, 2033–2046 (2022).

Pawar Kadambari Subhash, Singh Paras Nath, Singh Sanjay Kumar. Fungal alkaline proteases and their potential applications in different industries. Frontiers in Microbiology. VOL. 14 (2023).

Othman B., Sebo N H. Utilization of Some Agro Wests for the Production of Acid Protease by *Aspergillus niger*. Journal of Survey in Fisheries Sciences. 10(3S) 4319-4331 (2023).