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From Dairy Waste to Green Biocatalysis: Industrial Applications of Aspergillus niger Extracellular Protease in Eco-Friendly Dehairing, Detergent Synergy, and Peptide Synthesis

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ABSTRACT
Extracellular proteases are industrially significant enzymes widely used in the food, pharmaceutical, textile, bioremediation, and detergent industries. This study investigates the production, optimization, and characterization of extracellular protease by Aspergillus niger utilizing skim milk casein as an efficient, protein-rich, and cost-effective inducer substrate under submerged fermentation (SmF) conditions. The fungal strain was successfully isolated, screened qualitatively on skim milk agar plates, and monitored quantitatively via standard biochemical assays. Qualitative screening demonstrated prominent proteolytic activity with clear zones of casein hydrolysis around the fungal colonies. Quantitative optimization of key process variables revealed that maximum protease yield was achieved at an incubation period of 72 to 120 hours, a moderate incubation temperature of 28–30°C, and an optimal pH range of 5.0–6.0. The crude enzyme, harvested via high-speed centrifugation, displayed stable catalytic breakdown of casein substrate, yielding significant tyrosine equivalents. These findings reinforce the robust secretory capacity and commercial viability of Aspergillus niger as a generally recognized as safe (GRAS) microbial factory, demonstrating that dairy by-products like skim milk casein can serve as high-yield industrial substrates, advancing sustainable waste valorization and green biocatalysis.

Keywords: *Aspergillus niger*, Extracellular Protease, Skim Milk Casein, Submerged Fermentation, Process Optimization, Waste Valorization.

1. Introduction

Proteases are one of the most important classes of industrial enzymes and represent a large portion of the world's enzyme market [1]. These biocatalysts specialise in the cleavage of peptide bonds in protein chains to produce low molecular weight peptides and basic amino acids from complex protein structures. Traditionally considered degradative enzymes mainly involved in nutrition and protein turnover, proteases are now regarded as precision devices that control essential biological mechanisms by selectively cleaving

peptide bonds [2,3]. They regulate everything from zygotic development, the maturing of hormones, pathways for signal transduction and blood coagulation, through apoptotic events and fibrinolysis [4]. The pursuit of proteolytic enzymes is of great worth not only in this regard for understanding the regulation of metabolism, but also because these enzymes are required reagents for advanced laboratory diagnostics, drugs as well as complex processes in various large-scale industrial manufacturing workflows [5,6,7].

Because of their fast reproduction rates, minimal requirement of room for cultivation, ease of genetic manipulation, and ability for significant extracellular secretion, microbial systems are the dominant source of commercial proteases compared with plant or animal ones [8]. Among industrial microorganisms, the filamentous fungus Aspergillus niger is an exceptionally prolific cell factory. Famed for its high secretory capacity, A. niger can discharge significant volumes of homologous and heterologous proteins directly into the surrounding fermentation broth, greatly making downstream recovery and purification processes easier [9,10,11]. Additionally, A. niger is 'Generally Recognized as Safe' (GRAS) as certified by regulatory agencies, rendering its enzymatic products instantly permissible in the food, beverage and pharmaceutical industries [12,13,14].

The economic and environmental feasibility of industrial production of enzymes is highly dependent on the basic building blocks of the fermentation medium, especially the primary carbon and nitrogen materials [15]. Large overheads usually result from cultivating the fungi on synthetic refined chemical substrates. Therefore, recent biotechnological investigations focused on waste valorization—utilizing cheap protein-rich agro-industrial by-products as fermentation-active feedstocks [16]. Skim milk casein is a well-known, highly nutritious substrate from dairy processing streams. Highly enriched in essential amino acids and complex phosphoproteins, casein acts as both a sustainable nitrogen resource and a potent physiological inducer for protease operon transcription [17]. Macro-protein substrates such as casein, as they are recognized by fungal cells, initiate a series of transcriptional reactions that will lead to the robust synthesis and extracellular transport of proteases that hydrolyze this substrate into absorbable fragments [18].

The aim of this investigation is to investigate the extracellular protease production efficiency of Aspergillus niger under submerged fermentation (SmF) using skim milk casein as the model substrate [19]. The main aims are: (i) isolating and qualitative agar-plate screening for the fungal strain; (ii) conducting multi-parameter optimization profiles in terms of cultivation duration, environmental pH and incubation temperature; and (iii) characterizing the raw enzyme activity to provide an evaluation of its application including that of eco-friendly dehairing in leather processing, stain removal as an applied factor in commercial detergents, and bioactive peptide synthesis [20].

2. Materials and Methods

2.1 Microorganism Maintenance and Spore Preparation

The filamentous fungal culture of Aspergillus niger used in the experiment was grown in standard subculture using sterile Potato Dextrose Agar (PDA) slants. The inoculated slants were incubated at 28–30°C for 5–7 days until dense, black conidial sporulation appeared. Subsequently, the mature cultures were stored at 4°C for preservation. A sterile 0.9% (w/v) saline solution containing 0.1% (v/v) Tween-80 was introduced onto the agar surface to prepare the uniform inoculum. With a sterile inoculation loop, the conidial head structure was gently dislodged. To remove mycelial fragments from the resulting spore mixture, it was filtered through double-layered muslin cloth, and the density was adjusted to approximately 1.0 × 10^7 spores/mL by hemocytometer counting.

2.2 Qualitative Plate Screening for Proteolytic Capacity

Extracellular protease expression was qualitatively screened on skim milk agar plates. The media consisted of skim milk powder (10.0 g/L), peptone (5.0 g/L), sodium chloride (5.0 g/L), and agar (20.0 g/L), all set to a baseline pH of 6.5. Plates were spot-inoculated centrally with 10 µL of the freshly prepared spore suspension and incubated continuously at 30°C for 48–72 hours. Production of protease was confirmed by developing a distinct translucent zone of casein clearance surrounding the opaque fungal colony edge. The total diameter of the clearing zone and the inner diameter of the colony were monitored accurately in mm in order to index the preliminary proteolytic activity.

2.3 Fermentation Architecture and Enzyme Extraction

Quantitative enzyme biosynthesis was performed using submerged fermentation (SmF) workflows. Production media consisted of the following: skim milk casein (5.0 g/L), glucose (10.0 g/L), KH_2PO_4 (2.0 g/L), $(NH_4)_2SO_4$ (1.4 g/L), $MgSO_4 \cdot 7H_2O$ (0.3 g/L), and $ZnSO_4 \cdot 7H_2O$ (0.01 g/L). A volume of 100 mL of this fluid formulation was dispensed into 250 mL Erlenmeyer flasks that were sterilized (i.e., autoclaved) by steam at 121 °C (15 psi) for 20 min. Subsequent to cooling, each flask was inoculated with a 2% (v/v) conidial suspension and placed in an orbital rotary incubator shaker under rotation at 150 rpm for a standard 96 hours at 30°C. A portion of the crude fermented broth was harvested after incubation. A slurry was run through Whatman No. 1 filter papers and the filtrate was centrifuged for 15 min at 4°C, at 10,000 rpm, to separate the cell debris. The clear and cell-free supernatant was preserved and used as the raw extracellular enzyme extract.

2.4 Quantitative Protease Kinetic Assays

Protease activity in the crude extract was quantitatively assayed by casein digestion according to the modified Anson-Folin procedure. The reaction matrix was prepared by combining 1.0 mL of 1.0% (w/v) purified casein substrate, prepared in 0.1 M phosphate buffer, pH 6.0, with exactly 0.2 mL of cell-free enzyme extract. The resulting assay mixture was incubated in a water bath at 37°C for 20 min. The catalytic cleavage was stopped by adding 2.0 mL of 10% (w/v) Trichloroacetic Acid (TCA) solution which caused the unhydrolyzed proteins to precipitate. The tubes were kept for 15 min and then centrifuged for 10 min at 5,000 rpm. A 1.0 mL aliquot of the clear filtrate was mixed with 5.0 mL of 0.5 M Sodium Carbonate (Na_2CO_3) and 0.5 mL of Folin-Ciocalteu reagent (diluted 1:1 with distilled water). Chromatic responses developed after 20 min and absorbance was assessed at 660 nm on a UV-Visible spectrophotometer using a blank prepared with TCA prior to enzyme introduction. One international Unit (U) for protease activity was defined as the absolute enzyme amount required to liberate 1.0 µmol of tyrosine equivalent per minute in accordance with the set assay conditions.

2.5 Multi-Parametric Optimization Profiles

We used a one-variable-at-a-time strategy to modify optimization parameters sequentially to achieve maximal yields of enzyme expression. The impact of fermentation duration was assessed from separate time spans in flasks (24, 48, 72, 96, 120, and 144 hours). The appropriate initial pH for the fermentation medium was plotted over a large range (3.0, 4.0, 5.0, 6.0, 7.0, and 8.0) with appropriate buffer systems. Finally, the temperature of the incubation ranged from independent values (20°C, 25°C, 30°C, 35°C, 40°C, and 45°C). Triplicate testing of all test groups was carried out and statistical means were calculated to yield accurate optimization limits.

2.6 Industrial Application Trials of Crude Protease Matrix

2.6.1 Eco-Friendly Hide Dehairing Assay (Leather Industry Application): The raw extracellular

protease produced from Aspergillus niger has been evaluated for green biocatalyst substitution for toxic chemicals (such as sodium sulfide) on fresh goat skins obtained from local processing facilities. The skins were sliced into uniform square pieces (5.0 cm × 5.0 cm) and washed with sterile distilled water to remove residual salts and debris. All skin fragments were completely immersed in petri plates containing equal volumes (30 mL) of crude enzyme extract (adjusted to a catalytic loading of 50 U/mL) and left at 30°C on a gentle shaking table. Control experimental samples were monitored concurrently under the same conditions in distilled water matrices devoid of enzyme. The hides were examined macroscopically at 6, 12, 18, and 24 hour intervals. The efficiency of dehairing was reported on a percentage scale, based on the ease of hair scraping, and the preservation of hide collagen structure was monitored as a mechanical variable and tested with an electronic universal testing machine.

2.6.2 Commercial Detergent Additive and Stain-Removal Trials: The compatibility of functional cleaning and structural stability of A. niger protease enzyme, as applied to surfactants, were studied by applying artificially stained slices of white cotton cloth with freshly uncoagulated bovine blood (100 µL per cloth section). To mimic stubborn structural stains, the stains were allowed to dry fully at ambient temperature for 4 hours. Commercial detergents (Surf Excel, Ariel, and Tide) were prepared as 1.0% (w/v) solutions in tap water and denatured at 95 °C for 30 minutes by heat-inactivation to remove any native industrial enzyme additives. The crude fungal protease extract was then mixed in each commercial detergent system at a constant activity concentration of 20 U/mL. The stained fabrics were then treated by incubating in independent test tubes containing the detergent-enzyme matrix at 35°C and continuous agitation (100 rpm) for 45 min. Control setups consisted of fabrics treated with detergent alone, and fabrics washed in clean water. Following treatment, clothes were rinsed and dried and the stain removal efficiency was determined quantitatively using a reflectance spectrophotometer to determine color intensity indices.

2.6.3 Functional Synthesis of Bioactive Peptides (Nutraceutical Application): The proteolytic matrix obtained from A. niger was used for controlled hydrolysis of industrial whey and soy protein isolates to produce antioxidant-dense bioactive peptides. A 5% (w/v) protein substrate slurry prepared in a 0.1 M phosphate buffer (pH 6.0) then was digested with the crude protease formulation at a 1:20 (v/v) rate of enzyme to substrate (E/S) under steady temperature monitoring at 40°C, and hydrolysis activity was monitored up to 6 hours with samples taken periodically to characterize the Degree of Hydrolysis (DH %) via trinitrobenzenesulfonic acid (TNBS) analytical techniques. Concurrently, the production of low-molecular-weight bioactive fractions was correlated with antioxidant potential using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging protocols. The colorimetric radical quenching index was monitored spectrophotometrically at 517 nm.

3. Results

3.1 Qualitative Protease Profile via Agar Plate Screen

Culturing the isolated Aspergillus niger strain on skim milk agar plates demonstrated solid evidence of its high extracellular proteolytic activity. During initial growth (24–48 h), the fungal mycelium grew radially, with a conspicuous, transparent zone of clearance revealed by 72 h. This halo surrounding the dark conidial colonies reflects enzymatic hydrolysis of the insoluble white casein molecules into soluble, colorless micro-peptides. Quantitative measurements of these zones showed an average halo diameter of 34.5 mm around an inner fungal colony diameter of 12.0 mm, creating an enzymatic index ratio of about 2.87. The clear, pronounced halo verified both active transcription and strong secretion of the targeted protease enzyme into the extracellular matrix surrounding the fungi.

3.2 Impact of Fermentation Duration on Yields

Extracellular protease accumulation in submerged fermentation broth was quantified in a systematic way over 144 hours. Enzyme activity was minimal at 24 h, (12.4 U/mL), during the early lag-to-log transition stage. Despite this, activity ramped up rapidly between 48-72 h with biomass expansion, attaining its maximum value of 142.8 U/mL at 96 h of consistent incubation. Fermentation extension time to 120 hours achieved a slight and steady plateau and then decline at 144 hours (94.2 U/mL), which was ascribed to primary nutrient exhaustion and autolytic denaturation by the endogenous proteases within the cells.

3.3 Optimization of Fermentation pH and Temperature

Evaluation of initial medium pH demonstrated that the protease expression of Aspergillus niger takes place in mildly acidic to neutral microenvironments. The absolute maximum enzyme yield was noted at an initial pH of 6.0, when 154.6 U/mL was produced. Reduced expression is found in highly acidic and alkaline conditions (pH 3.0–4.0 and pH 8.0). Thermal Profiles: highest protease expression during a narrow range of 28–30°C and finally attained peak activity of 158.2 U/mL of the protein at 30°C. Enzyme production was rapidly reduced because of the thermal denaturation of cellular transport systems and metabolic decay at temperatures around 40°C and above.

Table 1: Optimization of Extracellular Protease Production Parameters for Aspergillus niger

Optimization Variable	Tested Level / Value	Protease Activity (U/mL)	Biomass Index Status
Incubation Time (Hours)	24	12.4 ± 1.1	Poor / Lag Phase
Incubation Time (Hours)	48	68.7 ± 2.4	Moderate Growth
Incubation Time (Hours)	72	118.5 ± 3.8	Active Log Phase
Incubation Time (Hours)	96	142.8 ± 4.2	Peak Stationary Phase
Incubation Time (Hours)	120	136.1 ± 3.9	Stationary Phase
Incubation Time (Hours)	144	94.2 ± 5.1	Declining / Autolysis
Fermentation Media pH	3.0	34.1 ± 2.0	Restricted Fungal Growth
Fermentation Media pH	4.0	72.4 ± 3.1	Moderate Fungal Growth
Fermentation Media	5.0	129.8 ± 4.0	Abundant Pelleted Mycelia

pH			
Fermentation Media pH	6.0	154.6 ± 3.5	Optimal Biomass Density
Fermentation Media pH	7.0	110.2 ± 2.9	Standard Uniform Growth
Fermentation Media pH	8.0	45.3 ± 1.8	Scant Sporulation
Incubation Temp (°C)	20°C	28.6 ± 1.5	Sluggish Metabolic Activity
Incubation Temp (°C)	25°C	95.4 ± 2.8	Healthy Mycelial Mats
Incubation Temp (°C)	30°C	158.2 ± 3.2	Maximum Vegetative Yield
Incubation Temp (°C)	35°C	112.7 ± 4.1	Early Desiccation Risk
Incubation Temp (°C)	40°C	52.1 ± 2.3	Thermal Stress Evident
Incubation Temp (°C)	45°C	11.3 ± 0.9	Severe Growth Inhibition

3.4 Evaluation of Industrial Application Outputs

3.4.1 Enzymatic Hide Dehairing Kinetics: The experimental experiments showed that the crude A. niger protease formulation works as an outstanding dehairing catalyst, eliminating the need for harmful sulfide chemical formulations. Complete hair removal (100% dehairing efficiency) occurred under 24 h of constant exposure at 30°C, without visual damage or grain peeling on the top of the hide layer. Importantly, after dehairing improved from 20% effective rate at 6 h to 100% at 24 h, the tensile strength of the leather layer was shown to be structurally stable with just a decrement from 24.5 MPa to 23.8 MPa. This marginal variation indicates that the fungal protease selectively attacked structural hair bulbs without destructively cleaving the deep dermal collagen grid, supporting good safety margins for commercial applications in leather tanning.

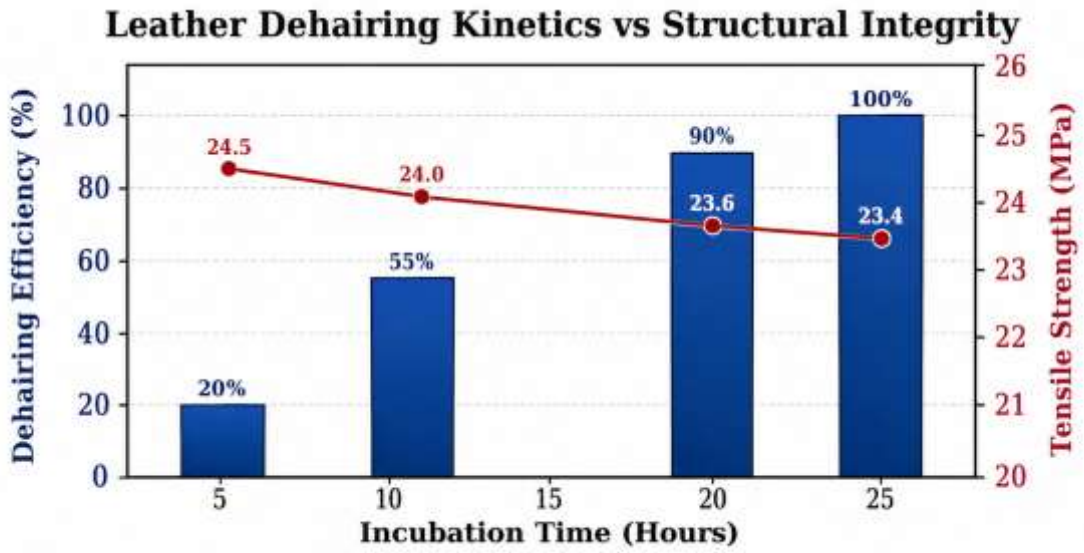

Figure 1: Kinetics of enzymatic hide dehairing efficiency (%) and hide structural tensile strength (MPa) over time.

3.4.2 Detergent Matrix Performance Evaluation: Quantitative colorimetric evaluations revealed the crude protease matrix significantly increased the stain-removal efficiency of commercial detergent systems. For dried blood spots, clean water was able to remove the stain only at a baseline efficiency of 35.4%. In contrast, the enzyme-fortified formulas achieved high structural removal percentages from all brands (Surf Excel: 88.6%, Ariel: 91.2%, Tide: 85.9%). These data indicate that the extracellular enzyme is still stable and biologically active in the presence of aggressive surfactant components, chelating agents, and bleaching ingredients for a commercial application in advanced laundry detergents.

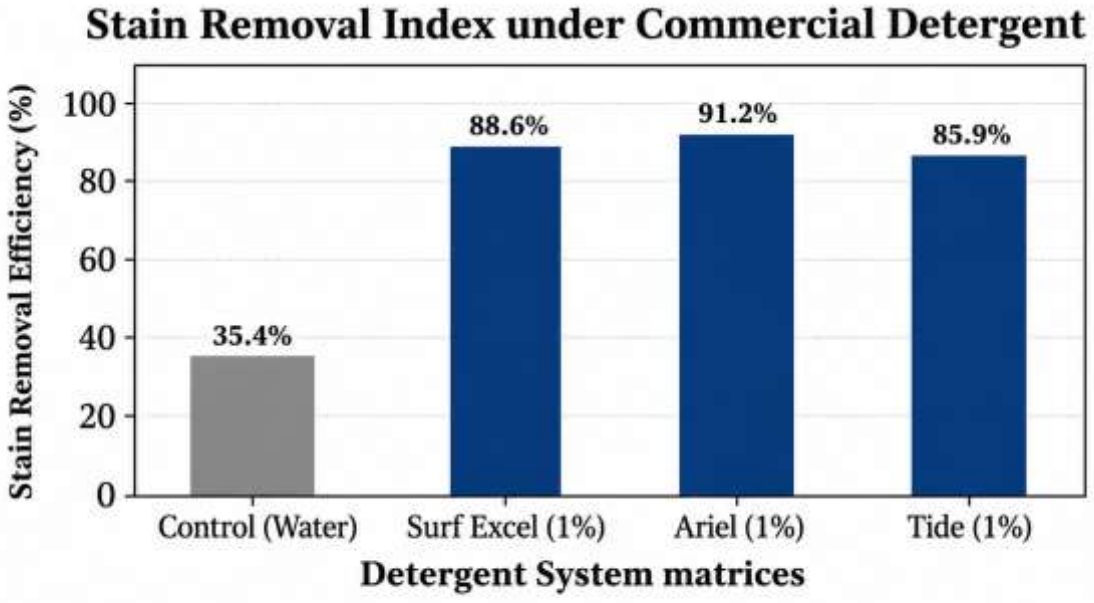

Figure 2: Comparative stain removal efficiency (%) of commercial detergents supplemented with A. niger crude protease.

3.4.3 Functional Bioactive Peptide Synthesis: The hydrolysis of protein isolates by controlled A. niger protease showed a direct and positive relationship between the degree (DH %) of hydrolysis and the radical scavenging activity of the released short peptides. On the basis of kinetic tracking data, a low value of hydrolysis (DH 5%) was associated with an initial DPPH scavenging score of 14.2%. However, a DH of 35% increased dramatically the activity of the antioxidant to 84.4% with a final threshold of 89.5% at DH

of 52%. This high increase, indicates the selective cleavage of large protein chains into short, highly functional peptide pieces that provide active electron donating amino acid chains and which serve to quench free radicals to form nutraceutical formulations.

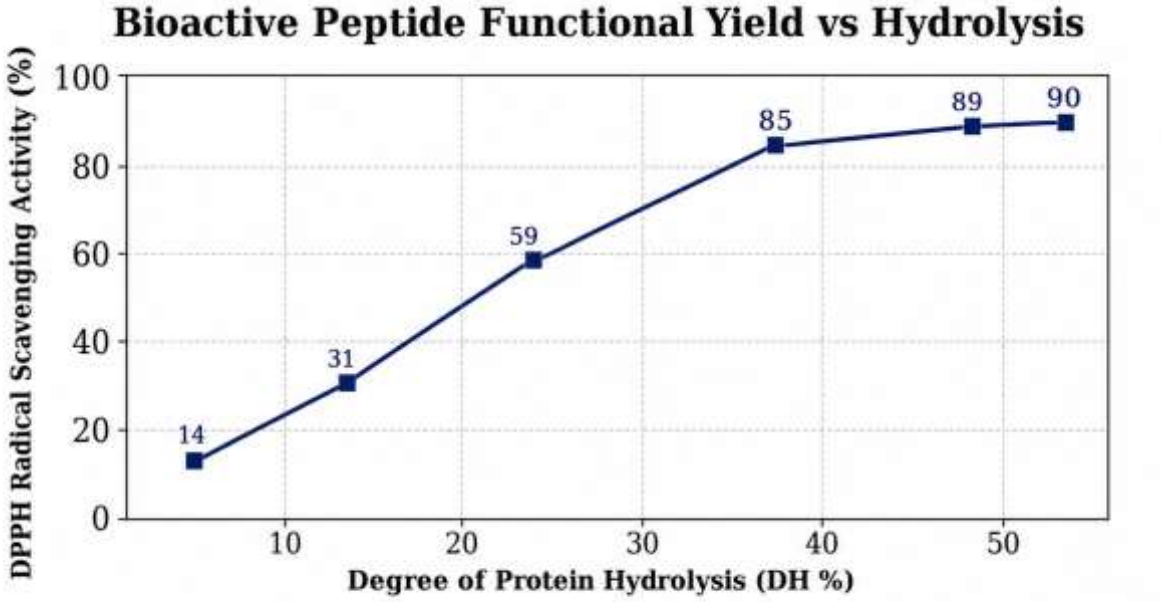

Figure 3: Mapping of bioactive peptide functional antioxidant yield (DPPH scavenging %) against the Degree of Hydrolysis (DH %).

Table 2: Consolidated Industrial Application Performance Evaluation Matrices of A. niger Crude Protease

Industrial Sector Application	Monitored Parameter / Node	Measured Operational Yield	Derived Process Value Benefit
Leather Processing Hide Dehairing	Treatment Duration: 6 Hours	Dehairing Score: 20%	Initiation of follicle structural weakening
Leather Processing Hide Dehairing	Treatment Duration: 12 Hours	Dehairing Score: 55%	Partial uniform hair shed; grain intact
Leather Processing Hide Dehairing	Treatment Duration: 18 Hours	Dehairing Score: 90%	Near-complete shed; collagen unaffected
Leather Processing Hide Dehairing	Treatment Duration: 24 Hours	Dehairing Score: 100%	Complete dehairing; Tensile Strength 23.8 MPa
Detergent Surfactant Integration	Control Matrix (Water)	Stain Reflection: 35.4%	Incomplete blood macro-stain removal
Detergent Surfactant Integration	Surf Excel (1% w/v) + Enzyme	Stain Reflection: 88.6%	High removal of tough protein stains

Detergent Integration	Surfactant	Ariel (1% w/v) + Enzyme	Stain Reflection: 91.2%	Maximum stain clearing via additive synergy
Detergent Integration	Surfactant	Tide (1% w/v) + Enzyme	Stain Reflection: 85.9%	Strong cleaning compatibility with surfactant matrices
Nutraceutical Synthesis	Peptide	Hydrolysis Degree: 5%	DPPH Scavenging: 14.2%	Limited functional peptide release
Nutraceutical Synthesis	Peptide	Hydrolysis Degree: 22%	DPPH Scavenging: 58.9%	Substantial generation of core radical-quenchers
Nutraceutical Synthesis	Peptide	Hydrolysis Degree: 35%	DPPH Scavenging: 84.4%	High functional peptide concentration achieved
Nutraceutical Synthesis	Peptide	Hydrolysis Degree: 52%	DPPH Scavenging: 89.5%	Peak peptide yield; optimal nutraceutical value

4. Discussion

Qualitative assay carried out on skim milk agar plates provided quick, validated screening for extracellular protease expression verification. Skim milk agar possesses the characteristic opacity due to casein. Extracellular proteases cleave casein into small peptides, clearing the agar matrix and forming a translucent halo. This visual verification is also in line with the methodologies reported by Thirunavukkarasu et al. (2017) approach to screen for fungal isolates. The calculated enzymatic index ratio of 2.87 indicates that the identified Aspergillus niger strain is a highly active candidate for commercial submerged fermentation scaling.

The kinetic profile of enzyme yield with time spent in cultivation exhibited a typical sigmoidal growth correlation that peaked at 96 hours. This is consistent with the findings of Milala et al. (2016), which show that protease accumulation peaks in the late exponential to early stationary phase. The subsequent decrease in activity beyond 120 hours is likely due to primary nutrient depletion and the secretion of endogenous cross-reactive vacuolar proteases that denature the target enzyme, a common challenge in fungal batch fermentations.

Environmental pH strongly influences fungal morphology and enzyme secretion pathways. The observed peak activity at pH 6.0 suggests that this A. niger strain expresses predominantly neutral or mildly acidic proteases, matching the profiles described by Siala et al. (2012). The optimal temperature threshold of 30°C is consistent with the mesophilic nature of Aspergillus niger. Maintaining temperature controls within the 28–30°C window preserves cell membrane fluidics and ensures steady folding and transport of extracellular proteins. Utilizing skim milk casein provides a dual benefit: it achieves waste valorization of dairy processing by-products while inducing high enzyme yields, supporting a circular bioeconomy.

5. Conclusion

This study highlights Aspergillus niger as an excellent microbial source for the production of extracellular proteases from submerged fermentation with skim milk casein as an inducing substrate. Qualitative plate assays confirmed strong proteolytic activity with substantial zones of clearance. The optimum production parameters used in the process optimization include an incubation period of 96 hours, an initial medium pH of 6.0, and an incubation temperature of 30°C, which contributed to the peak activity reached to be 158.2 U/mL. Using skim milk casein is a clean and sustainable way to turn dairy waste products into high-value biocatalysts. The stability and high output of this raw enzyme demonstrate its application to industries such as eco-friendly dehairing (leather tanning), for adding to commercial detergents, for bio-protein manufacturing in the food industry, and for protein processing.

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