

Article

Integrated Extractive Fermentation and Aqueous Two-Phase Systems Enable Efficient Production and Purification of an Extracellular Protease from *Aspergillus* sp. UCP1287

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Abstract

Proteases are among the most commercially important industrial enzymes, yet their large-scale production is often limited by complex and costly downstream processing. In this study, an integrated bioprocess was developed for the production, in situ recovery, and purification of an extracellular protease produced by *Aspergillus* sp. (SIS 22/UCP 1287) under submerged fermentation. Enzyme extraction was coupled directly to fermentation using a polyethylene glycol (PEG)–phosphate aqueous two-phase system (ATPS), aiming to enhance recovery while preserving enzymatic activity. The effects of PEG molecular weight, polymer and phosphate concentrations, and pH on enzyme partitioning were systematically investigated through a full factorial experimental design. Low-molecular-weight PEG and near-neutral pH conditions significantly favored enzyme migration to the PEG-rich phase. Under optimized conditions (15% PEG 3500, 20% phosphate, pH 7.0), the ATPS achieved a partition coefficient of 65.55, enzyme recovery of 209%, and a purification factor of 1.64. Subsequent purification by DEAE–Sephadex ion-exchange chromatography yielded a tenfold increase in specific activity, with optimal elution at 0.5 M NaCl. SDS–PAGE analysis confirmed the homogeneity of the purified protease, revealing a single band at approximately 59 kDa. Overall, the proposed integrated ATPS–chromatography strategy represents a robust, scalable, and environmentally friendly platform that significantly simplifies downstream processing while maintaining high enzyme activity, highlighting its potential for industrial and biotechnological applications.

Keywords: bioprocess; fungal biocatalysts; aqueous two-phase system; process integration; enzyme activity



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1. Introduction

Biotechnology has experienced remarkable growth in recent decades, consolidating itself as a multibillion-dollar global industry with applications in pharmaceuticals, agri-

culture, food technology, and industrial processes. The global biotechnology market was valued at approximately USD 295 billion in 2019 and exceeded USD 1 trillion in 2021, with projections indicating continued expansion driven by increased investment in research and development and growing demand for innovative and sustainable biotechnological solutions [1–3]. One of the most dynamic areas within biotechnology is the discovery and optimization of industrial enzymes, which increasingly depends on interdisciplinary approaches combining microbiology, biochemistry, chemical engineering, and molecular biology. Advances in these fields, together with improved screening methods that simulate industrial conditions, have enabled the rapid identification and development of tailor-made enzymes suited to specific industrial applications [4].

Enzymes are highly specific biocatalysts produced by all living organisms that accelerate biochemical reactions essential to life, including digestion, respiration, and metabolism. Their ability to operate efficiently under mild temperature and pH conditions makes them ideal catalysts for industrial processes, particularly in food technology, where they enable selective modification of raw materials without destroying essential nutrients [5,6]. Beyond the food industry, enzymes play vital roles in textile processing, pharmaceuticals, cosmetics, and bioenergy production [7,8].

Microbial enzymes have gained attention for their industrial potential, as they can be produced in large quantities through controlled fermentation processes and are more amenable to genetic manipulation compared to enzymes from plant or animal sources [9,10]. Proteases constitute the largest share of the global enzyme market, representing approximately 60% of total enzyme sales, followed by amylases [11]. Brazil, despite its vast biodiversity, still imports most of the enzymes it consumes, highlighting the need to explore native microbial strains as alternative enzyme producers [12,13].

Industrial enzymatic processes are generally simple, energy-efficient, and cost-effective, making them attractive for large-scale applications [14,15]. Microbial enzyme production is typically achieved by either submerged fermentation (SmF) or solid-state fermentation (SSF). SmF, the predominant technique in Western industries, offers advantages such as controlled pH and temperature, improved oxygen transfer, and easier recovery of extracellular enzymes [16,17]. In SmF, microorganisms grow in a liquid medium with soluble nutrients and high water content (>95%), favoring high enzymatic yields and reproducibility [18]. However, conventional SmF often requires additional downstream steps for product recovery, which has driven growing interest in extractive fermentation strategies that integrate enzyme production with in situ separation, improving productivity, stability, and overall process efficiency [19,20].

Enzyme purification is a critical and complex step in enzyme production, directly impacting enzyme stability, purity, and economic feasibility. The choice of purification method depends on the enzyme's intended application, required purity, stability, and cost considerations. Multiple strategies are often combined to achieve optimal results, as no single protocol fits all enzymes due to their heterogeneous nature [21,22]. Conventional purification techniques rely on differences in solubility, charge distribution, molecular weight, and hydrophobicity. Among these, ion-exchange chromatography is one of the most widely applied due to its high selectivity, simplicity, and scalability [23,24]. This method separates proteins based on electrostatic interactions between charged amino acid residues and oppositely charged groups immobilized on a stationary matrix. Adjusting the ionic strength and pH of the mobile phase enables efficient elution of target enzymes without compromising protein integrity [25,26].

Another efficient and environmentally friendly purification alternative is aqueous two-phase systems (ATPS), particularly those formed by polyethylene glycol (PEG) and inorganic salts such as phosphate. ATPS enables mild, non-denaturing partitioning of

biomolecules, offering high selectivity, recyclability, and compatibility with industrial-scale bioprocesses [27,28]. Recent studies have demonstrated the efficiency of PEG–phosphate systems in purifying proteases, lipases, and other hydrolases with significant recovery rates and preserved enzymatic activity [29,30]. The combination of ion-exchange chromatography and ATPS represents a robust, cost-effective approach for the recovery and concentration of microbial proteases with potential biotechnological applications [31,32].

In this context, the present study addresses a key limitation in industrial protease production by developing an integrated bioprocess that combines submerged fermentation with in situ recovery using a polyethylene glycol–phosphate aqueous two-phase system (ATPS). Although ATPS has been extensively investigated as a post-fermentation purification strategy, its direct incorporation into fungal fermentation systems for simultaneous production and extraction of extracellular proteases remains insufficiently explored. The originality of this work lies in the systematic evaluation of critical physicochemical parameters—PEG molecular weight, polymer and salt concentrations, and pH—through factorial experimental design to elucidate their influence on enzyme partitioning behavior and stabilization within an extractive fermentation framework. Furthermore, the study integrates optimized ATPS conditions with ion-exchange chromatography as a complementary purification step, aiming to streamline downstream processing. By bridging enzyme production and recovery within a unified operational strategy, this work contributes to the advancement of scalable and sustainable platforms for industrial protease manufacturing.

2. Results and Discussion

2.1. Growth of *Aspergillus* sp. and Protease Production Under Extractive Fermentation

The filamentous fungus *Aspergillus* sp. (SIS 22/UCP 1287) demonstrated effective adaptation to the MS-2 medium under extractive fermentation conditions. After 72 h of cultivation at 30 °C, the crude broth exhibited a protease activity of 20.80 U mL^{−1} and a specific activity of 163.8 U mg^{−1}. Based on the total cultivation time, the volumetric productivity reached 0.29 U mL^{−1} h^{−1}, indicating sustained extracellular enzyme secretion throughout submerged growth. These values confirm that the biphasic system did not impair fungal metabolism or secretory capacity [33,34].

The integration of a polyethylene glycol (PEG)–phosphate aqueous two-phase system (ATPS) directly into the cultivation medium enabled simultaneous enzyme production and primary recovery. In this configuration, the PEG-rich phase acted as a liquid extractant, favoring the partitioning of the secreted protease away from the biomass-containing phase. Although enzyme migration was quantitatively evaluated in Section 2.2, the maintenance of catalytic activity in the crude system suggests that the biphasic environment did not promote detectable denaturation during cultivation.

From a process engineering perspective, the in situ recovery strategy reduces the need for post-fermentation clarification steps and may contribute to process intensification by coupling upstream and primary downstream operations. The compatibility between fungal growth and phase formation was evidenced by the absence of macroscopic phase disruption or visible growth inhibition during the 72 h cultivation period. This behavior indicates that the selected PEG molecular weight and phosphate concentration were within a tolerance range that preserves fungal physiological stability.

The use of soybean filtrate as a complex nitrogen and carbon source likely contributed to protease biosynthesis, as plant-derived substrates are known to support robust extracellular enzyme production in *Aspergillus niger* and *Aspergillus oryzae* [35,36]. In such systems, protease secretion is frequently associated with nutrient availability and extracellular protein induction mechanisms, which may explain the consistent enzyme activity observed.

Previous studies employing PEG–phosphate and PEG–sulfate ATPS have reported 1.5- to 3-fold improvements in protease recovery relative to conventional extraction approaches [37,38]. In the present study, the integration of extractive fermentation with subsequent optimization of partition parameters (Section 2.2) provides experimental evidence that the ATPS can operate not merely as a downstream purification step but as a process-integrated recovery platform. This configuration aligns with current trends in bioprocess intensification, where phase-forming systems are used to enhance operational efficiency while preserving enzyme activity.

2.2. Partitioning Behavior in PEG–Phosphate Aqueous Two-Phase System

The partitioning of the protease in the PEG–phosphate aqueous two-phase system (ATPS) was investigated through a full 2^4 factorial design evaluating the effects of four variables—PEG molecular weight (MPEG), PEG concentration (CPEG), phosphate concentration (CFOSF), and pH—on enzyme recovery, partition coefficient (K), and purification factor (PF). Analysis of variance (ANOVA, $p < 0.05$) revealed that MPEG was the dominant factor influencing enzyme distribution, followed by pH and the MPEG $\times$ CFOSF interaction.

The complete experimental matrix and results are presented in Table 1, which summarizes the effects of these parameters on partition performance. Runs with low MPEG (400 g mol^{-1}) consistently produced the highest partition coefficients ($K > 40$) and recovery yields ($Y > 150\%$), whereas systems containing high-molecular-weight PEG (8000 g mol^{-1}) exhibited drastically reduced recovery ($Y < 60\%$). The central-point assays (runs 17–20) confirmed excellent model reproducibility, with PF values between 1.0 and 1.6, indicating consistent performance near the optimized region.

Table 1. Full 2^4 factorial design matrix with conditions and results for protease partitioning in the PEG–phosphate ATPS.

Assay	pH	MPEG ^a (g/mol)	CPEG (%) ^b	CFOSF (%) ^c	K ^d	Y ^e (%)	FP ^f	AE (U/mL) ^g
1	6	400	12.5	15	109.67	198.26	0.84	417.78
2	6	8000	12.5	15	1.20	60.34	0.27	133.65
3	6	400	17.5	15	20.41	164.53	0.57	280.80
4	6	8000	17.5	15	2.99	76.74	0.32	157.46
5	6	400	12.5	25	30.92	170.23	0.82	404.53
6	6	8000	12.5	25	19.80	37.08	0.18	87.04
7	6	400	17.5	25	23.92	155.84	0.85	423.37
8	6	8000	17.5	25	9.29	15.35	0.08	37.96
9 *	8	400	12.5	15	-	-	-	-
10	8	8000	12.5	15	2.52	134.38	0.51	254.87
11	8	400	17.5	15	18.54	141.33	0.31	156.87
12	8	8000	17.5	15	17.15	143.27	0.48	235.95
13	8	400	12.5	25	46.77	209.49	0.87	433.29
14	8	8000	12.5	25	22.60	57.97	0.23	116.90
15	8	400	17.5	25	10.36	36.60	0.08	37.91
16	8	8000	17.5	25	17.79	82.57	0.24	117.57
17 (C)	7	3350	15	20	39.12	162.46	1.26	625.88
18 (C)	7	3350	15	20	40.44	158.06	1.20	594.94
19 (C)	7	3350	15	20	50.30	122.88	1.01	503.05
20 (C)	7	3350	15	20	65.55	182.01	1.64	812.40

(a) MPEG = PEG molecular weight; (b) CPEG = PEG concentration; (c) CFOSF = phosphate concentration; (d) K = partition coefficient; (e) Y = recovery in PEG phase; (f) PF = purification factor; (g) AE = specific activity.

* Run 9 showed no phase separation under the tested conditions; therefore, partitioning parameters could not be determined.

The Pareto chart (Figure 1) quantitatively demonstrated that MPEG exhibited the most significant negative standardized effect (-3.43 ; $p < 0.05$), confirming that decreasing the polymer's molecular weight favored enzyme migration to the PEG-rich phase. This inverse relationship between PEG molecular weight and partition efficiency has been well documented for fungal hydrolases and attributed to the excluded-volume effect, as PEG chain length increases, water availability and enzyme diffusivity decrease, hindering solvation and mobility [39,40].

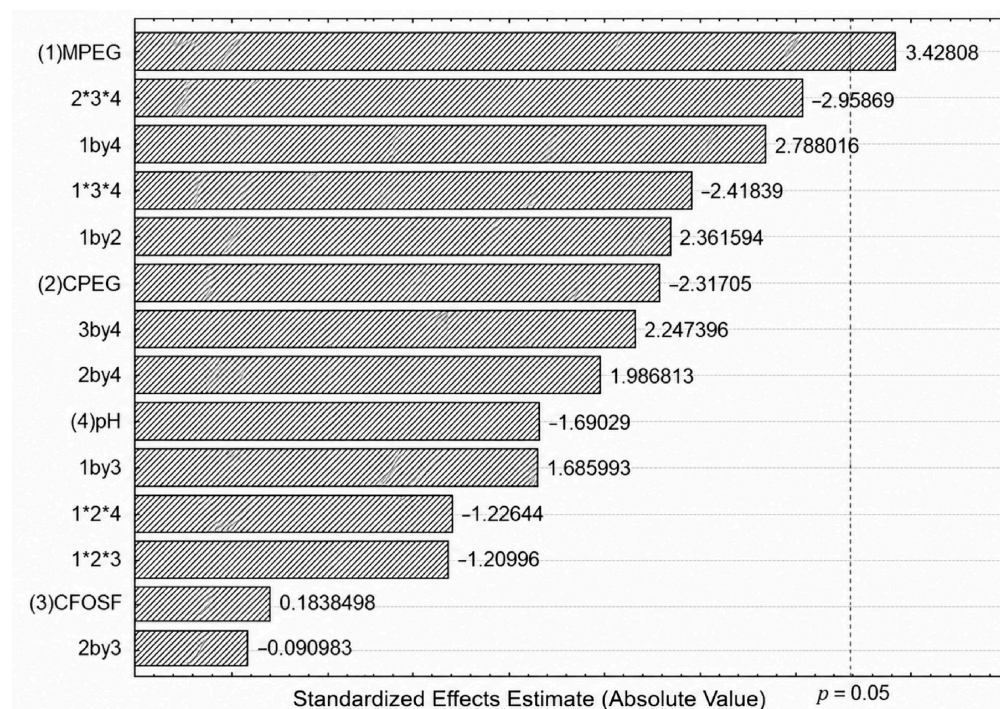


Figure 1. Pareto chart of standardized effects obtained from the 2^4 full factorial design. Factor coding: (1) PEG molecular weight (MPEG), (2) PEG concentration (CPEG), (3) phosphate concentration (CFOSF), and (4) pH. Interaction terms (e.g., 1×3 , 2×3 , $1 \times 2 \times 3$) represent combined effects among the corresponding coded variables.

Through multiple studies, low-molecular-weight polyethylene glycols (PEGs), such as PEG-400, have demonstrated the ability to form dynamic hydrogen-bonding networks with water and complementary polymers. This property results in less structured and more flexible aqueous environments compared with those generated by higher-molecular-weight PEGs or polyelectrolytes. Such a loosely organized molecular arrangement increases the mobility of the aqueous phase and facilitates more dynamic intermolecular interactions [41–47].

The structural flexibility and continuous hydrogen-bond formation explain the enhanced affinity of low-molecular-weight PEGs for enzymes and their ability to preserve catalytic conformation and biological activity. In contrast, high-molecular-weight PEGs tend to interact more strongly with hydrophobic protein surfaces, potentially inducing protein precipitation or denaturation [48]. Consequently, the combination of high free volume, increased molecular mobility, and dynamic hydrogen bonding renders low-molecular-weight PEGs particularly effective in systems that require reversible interactions and preservation of biomolecular functionality in aqueous environments [41,42].

Figure 2 shows that MPEG and its interactions with pH ($1 \times b \times 4$) and phosphate concentration ($2 \times 3 \times 4$) were also significant at the 95% confidence level, confirming the synergistic effects of electrostatics and polymer hydrodynamics. A decrease in PEG molecular weight combined with slightly acidic to neutral pH (6.0–7.0) enhanced both recovery and specific activity. These conditions likely favor the protonation state of charged

residues near the enzyme's surface, improving solubility and affinity for PEG via hydrogen bonding [49,50].

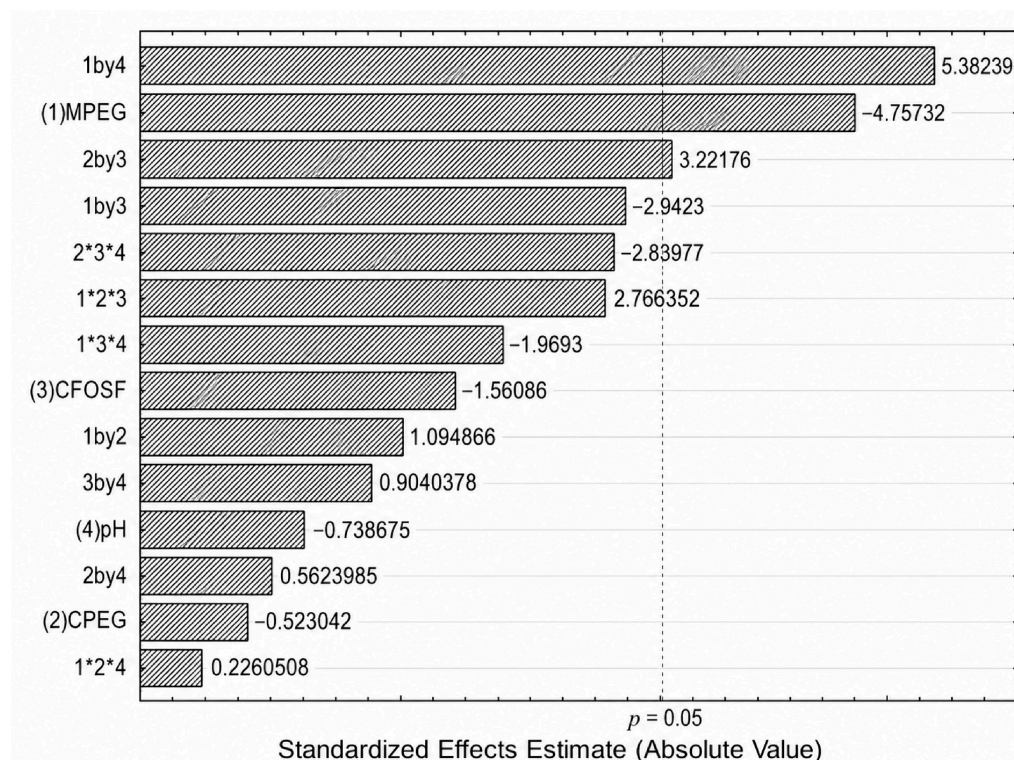


Figure 2. Pareto chart of standardized effects (absolute values) obtained from the 2^4 full factorial design for enzyme recovery (Y, %) in the PEG-rich phase. Factor coding: (1) PEG molecular weight (MPEG), (2) PEG concentration (CPEG), (3) phosphate concentration (CFOSF), and (4) pH. Interaction terms (e.g., 1×3 , 2×3 , $1 \times 2 \times 3$) represent combined effects among the corresponding coded variables. Bars extending beyond the vertical reference line indicate statistically significant effects at $p \leq 0.05$. The sign associated with each effect reflects its positive or negative influence on enzyme recovery.

To visualize these effects, the response-surface plot (Figure 3) illustrates the joint influence of pH and MPEG on enzyme recovery. The figure clearly shows that systems containing low-molecular-weight PEG (400 g mol^{-1}) yielded the highest recovery ($>180\%$), especially at pH 6.0–6.5, whereas higher MPEG (8000 g mol^{-1}) markedly reduced recovery regardless of pH. This pattern corroborates that the protease preferentially partitions into the PEG-rich phase, characteristic of enzymes with basic isoelectric points ($pI > 7$) that display hydrophobic patches exposed to the solvent. Such enzymes interact more strongly with the neutral polymer phase through hydrophobic and van der Waals contacts [51,52].

Under optimized conditions—15% PEG 3500, 20% phosphate, and pH 7.0—the system achieved a partition coefficient (K) = 65.55, recovery yield (Y %) = 209, and purification factor (PF) = 1.64. Recovery yields above 100% are frequently attributed to the removal of reversible inhibitors or to conformational activation caused by preferential hydration of PEG around the protein surface [53–55].

These findings confirm that the PEG–phosphate ATPS provides an energetically favorable microenvironment for protease stabilization and enrichment, delivering extraction efficiencies comparable to multi-step chromatographic processes while maintaining mild physicochemical conditions [56,57].

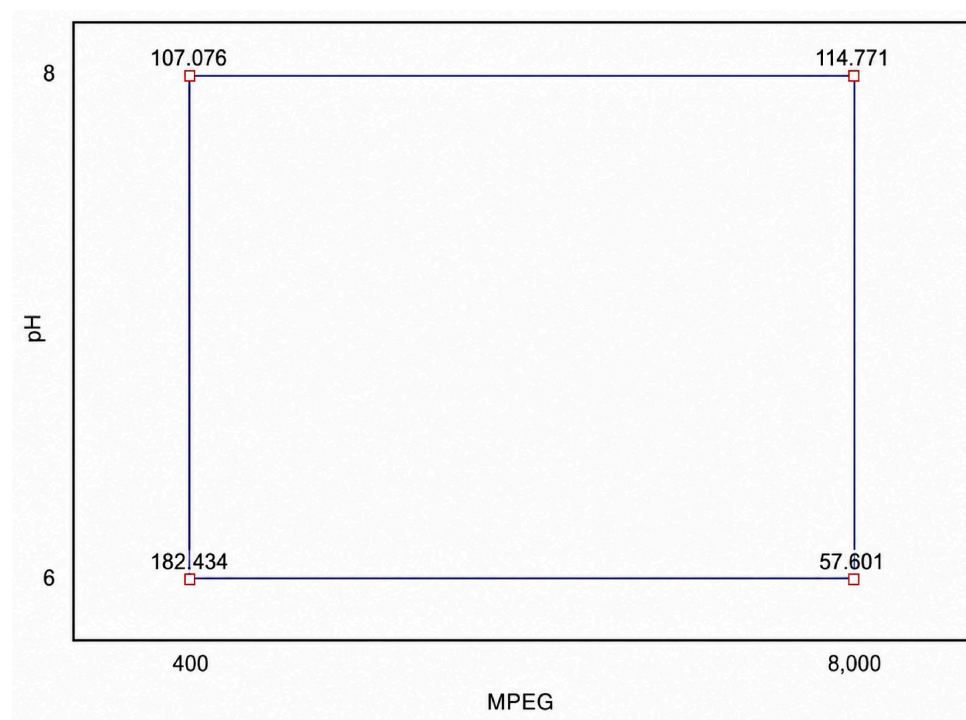


Figure 3. Response-surface plot showing the interactive effect of PEG molecular weight (MPEG) and pH on protease recovery in the PEG–phosphate aqueous two-phase system.

2.3. Ion-Exchange Chromatography and Active Fraction Identification

To refine purification, the PEG-rich extract was applied to a DEAE–cellulose ion-exchange column (Figure 4). Three major peaks were detected at NaCl concentrations of 0.1 M, 0.3 M, and 0.5 M. Proteolytic activity was systematically evaluated for all collected fractions across the entire elution profile using the azocasein assay, including fractions exhibiting low absorbance at 280 nm. This comprehensive analysis allowed full mapping of enzymatic activity distribution throughout the chromatographic process.

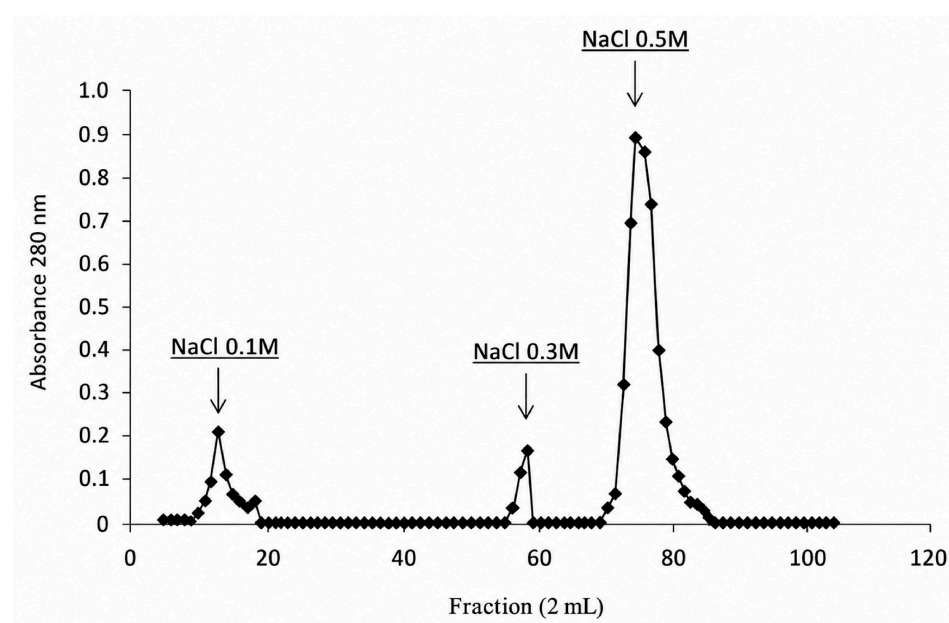


Figure 4. Chromatogram of protease from *Aspergillus* sp., obtained by ion-exchange chromatography using DEAE–Sephadex resin. Elution was performed using a stepwise NaCl gradient (0.1 M, 0.3 M, and 0.5 M) in 0.1 M Tris–HCl buffer (pH 8.0).

Among the detected fractions, the 0.5 M NaCl fraction (Fraction II) exhibited the highest absorbance at 280 nm (≈ 0.9 AU) and corresponded to the fraction with maximum proteolytic activity. Fractions eluted at 0.1 M and 0.3 M NaCl showed minimal or residual enzymatic activity, indicating that the majority of catalytically active protease was selectively retained and eluted at higher ionic strength. The selective elution of active protease at 0.5 M NaCl suggests moderate anionic affinity, compatible with its alkaline nature and slightly basic isoelectric point. Similar elution profiles have been reported for *Aspergillus fumigatus* alkaline protease [58] and *Aspergillus parasiticus* thermolabile protease [59].

The narrow elution peak at 0.5 M NaCl reflects enhanced resolution and purity, as weaker protein–matrix interactions are disrupted at this ionic strength. This step achieved a 10.23-fold increase in specific activity relative to the PEG-phase crude extract, calculated as the ratio between the purified fraction and the crude extract specific activities (Table 2), demonstrating the complementarity between ATPS and ion-exchange chromatography.

Table 2. Enzymatic and protein analyses derived from the protease production process by *Aspergillus* sp.

Sample	Protein Concentration ($\mu\text{g mL}^{-1}$) ($\mu\text{g/mL}$)	Protease Activity (U/mL)	Specific Activity (U)
Crude Extract	127.25	20.80	163.80
DEAE–Sephadex Fraction (0.5 M NaCl)	53.50	89.70	1676.6

Comparable enrichment factors ($8\text{--}12\times$) were reported by Yao et al. [60] and Zheng et al. [61], confirming that DEAE-based separation remains one of the most reliable approaches for fungal protease purification.

Industrial alkaline proteases produced by *Bacillus licheniformis*, *Bacillus subtilis*, and filamentous fungi such as *Aspergillus* sp. have been widely reported to exhibit specific activities ranging from several hundred to a few thousand U mg^{-1} , depending on substrate and assay conditions [62–64]. Under casein-based or chromogenic substrate assays, reported values commonly fall within the $500\text{--}3000$ U mg^{-1} range for industrially relevant formulations. The specific activity obtained in the present study (1676.6 U mg^{-1}) falls within this reported interval, indicating catalytic performance comparable to commercially relevant alkaline proteases.

2.4. Molecular Characterization by SDS–PAGE

SDS–PAGE analysis (Figure 5) was employed to confirm the homogeneity and to estimate the molecular mass of the purified protease, as this technique is widely used to assess enzyme purity and structural integrity, where the presence of a single band indicates a homogeneous preparation [65–68]. Lane (1) corresponds to the crude PEG extract, showing multiple faint protein bands, indicative of contaminant proteins. Lane (2) represents an intermediate fraction obtained after ATPS, evidencing partial enrichment of the target enzyme. In contrast, Lane (3) exhibited a single, well-defined band at approximately 59 kDa, confirming the successful purification of the protease. The observed molecular mass falls within the range commonly reported for extracellular alkaline proteases from *Aspergillus* species, which typically vary between 33 and 65 kDa, although broader molecular mass distributions (23–124 kDa) have been described in the literature [68,69]. Such variability in molecular mass among *Aspergillus* proteases is frequently attributed to genetic diversity and post-translational modifications, including glycosylation and differential processing of pre-proenzymes [68–72]. Slight variations in apparent molecular weight can result from glycosylation or different processing of the pre-proenzyme [68,73]. The disappearance

of lower-intensity bands after the chromatographic step indicates efficient removal of contaminating proteins, confirming the synergistic purification effect of the integrated ATPS + DEAE system.

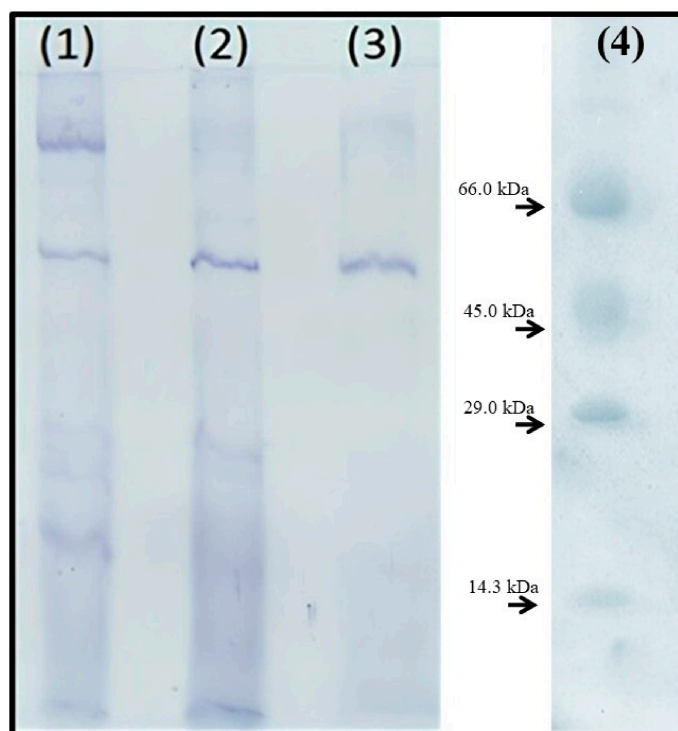


Figure 5. SDS–PAGE profile of protease purification. Lane (1): crude extract obtained from the PEG-rich phase; Lane (2): partially purified fraction recovered after the aqueous two-phase system (ATPS); Lane (3): purified protease, exhibiting a single protein band, suggesting successful purification. Lane (4): molecular weight marker. Arrows indicate the position of standard proteins corresponding to bovine serum albumin (69.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (29.0 kDa), and lysozyme (14.0 kDa).

2.5. Integrated Process Performance and Mechanistic Insights

The overall bioprocess integrating extractive fermentation, PEG–phosphate ATPS, and ion-exchange chromatography proved highly effective in combining high productivity with simplified downstream recovery. The preferential migration of the protease to the PEG-rich phase is attributed to hydrophobic and electrostatic interactions influenced by the enzyme’s pI. The observed negative effect of PEG molecular weight on partitioning supports a model where smaller PEG molecules reduce steric hindrance, enhancing enzyme–polymer contact and diffusivity.

This interpretation aligns with the preferential hydration theory, in which PEG acts as a stabilizing agent by excluding water molecules from the enzyme’s immediate environment, promoting compact folding and enhanced catalytic activity [74]. Furthermore, the 0.5 M NaCl elution peak (Figure 1) indicates moderate anionic affinity, characteristic of alkaline proteases with partially exposed acidic residues.

High recovery values (>200%) observed in the ATPS step may reflect the elimination of reversible inhibitors and conformational stabilization induced by PEG–protein interactions [38,72]. Similar activation phenomena have been reported for proteases from *Clostridium*, *Aspergillus tamarii*, and *Bacillus* species, where PEG polymers promoted increased catalytic efficiency due to microenvironmental modulation of surface charge distribution and structural stabilization [75].

From an economic perspective, the proposed integrated ATPS + DEAE strategy demonstrates competitive feasibility when compared to commercially available alkaline proteases. Industrial proteases represent approximately 60% of the global enzyme market, with a market value exceeding USD 3 billion, largely driven by their extensive application in detergents and other biotechnological sectors [76]. Although direct commercial pricing data are rarely detailed in scientific literature, techno-economic analyses indicate that large-scale production of extracellular proteases can result in very low costs when normalized by activity unit. For example, optimized industrial systems have reported production costs of approximately USD 0.24 per 10^6 U of protease activity, highlighting the potential for highly cost-effective manufacturing at scale [77]. Furthermore, overall enzyme production costs per kilogram are strongly influenced by downstream processing intensity, with simpler purification schemes and higher extracellular yields substantially reducing final unit costs [78].

In the present study, the cost estimation of the purification process indicates a production cost per activity unit that falls within or below the lower range reported for commercial fungal proteases, particularly when considering the integration of extractive fermentation with primary recovery in a single ATPS step. The reduction in downstream processing stages, lower solvent consumption, and partial in situ purification contribute significantly to cost minimization.

Ultimately, this integrated strategy not only improves economic performance but also enhances enzyme yield, catalytic activity, and purity under mild physicochemical conditions. By avoiding thermal or harsh chemical treatments commonly associated with conventional purification methods, the process preserves structural integrity and functional stability of the protease. Such characteristics reinforce its industrial scalability and environmental compatibility, while expanding its potential application in detergent, biomedical, and food biotechnology sectors, where enzyme stability and activity are critical parameters [79–81].

3. Materials and Methods

3.1. Microorganism, Sporulation, and Maintenance

The filamentous fungus *Aspergillus* sp. (SIS 22/UCP 1287) was employed in this study as the biological source for protease production. The strain was obtained from the North–Northeast Network of Filamentous Fungi from Caatinga Soils (RENNORFUN/SISBIOTA) project and was kindly provided by Professor Galba Maria de Campos Takaki from the Catholic University of Pernambuco (Recife, Brazil). For strain maintenance and sporulation, Czapek Dox Agar (CDA) (Sigma-Aldrich, St. Louis, MO, USA) medium was used, prepared and sterilized in an autoclave at 121 °C and 1 atm for 20 min prior to inoculation. The cultures were incubated at 30 °C until complete sporulation was achieved, ensuring the physiological integrity, purity, and viability of the fungal strain for subsequent fermentation and enzyme production assays.

3.2. Inoculum Preparation

The fungus was subcultured on Czapek Dox Agar plates and incubated for seven days at 30 °C to promote conidia formation. For spore collection, 10 mL of a sterile NaCl (0.9%, *w/v*) and Tween 80 (0.01%, *v/v*) solution was added to the plates. Spore concentration was determined using a Neubauer counting chamber and adjusted to a final concentration of 1×10^4 spores/mL for inoculation.

3.3. Protease Production by Extractive Fermentation

Protease production was carried out using extractive fermentation in the MS-2 culture medium, previously described by Porto et al. [82]. The composition of the medium per

100 mL of distilled water was as follows: 50 mL soybean filtrate (4%, *w/v*), NH_4Cl (0.1%, *w/v*), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.06%, *w/v*), yeast extract (0.1%, *w/v*), glycerol (1%, *w/v*), K_2HPO_4 (0.435%, *w/v*), and 1 mL of a mineral solution containing 100 mg each of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, and $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$. The fermentation was performed under agitation at 30 °C for 72 h. After cultivation, the fermented broth was subjected to in situ extraction using a polyethylene glycol (PEG)/phosphate aqueous two-phase system, enabling simultaneous enzyme production and recovery while maintaining protease stability under mild, non-denaturing conditions.

3.4. 2^4 -Full Factorial Design

An aqueous two-phase system (ATPS) composed of polyethylene glycol (PEG) and phosphate salt was employed for further enzyme extraction. The coded and actual values of the independent variables evaluated in the experimental design are presented in Table 3. The effects of PEG molecular weight (400, 3350, and 8000 g/mol), PEG concentration (12.5–17.5%, *w/w*), phosphate concentration (15–25%, *w/w*), and pH (6.0, 7.0, and 8.0) were evaluated using a full 2^4 factorial design. Systems were prepared in 15 mL conical tubes containing the specified PEG and salt concentrations, to which 20% (*w/w*) of the crude enzyme extract was added. The total system mass was adjusted to 10 g with distilled water. After vortexing for 1 min, the mixtures were left to separate by decantation for 60 min at 25 ± 1 °C. Each phase was analyzed for protein concentration and protease activity to determine partition coefficient (K), purification factor (PF), and enzyme recovery (Y%). The experimental conditions under which each system was studied, as described by Porto et al. [83].

Table 3. Coded and real values of the independent variables used in the 2^4 full factorial design for protease partitioning in the PEG–phosphate aqueous two-phase system.

Factors	Coded Levels		
	+1	0	−1
PEG Molecular Weight (MPEG)	400	3350	8000
PEG Concentration (CPEG)	12.5	15	17.5
Phosphate Concentration (CFOSF)	15	20	25
pH	6	7	8

3.5. Determination of Partition Coefficient, Recovery Yield, and Purification Factor

The partition coefficient, K (dimensionless parameter), in the aqueous two-phase system was defined as the ratio between the specific activity (AE, U mL^{-1}) in the PEG-rich phase (AE_p) and that in the salt-rich phase (AE_s) (Equation (1)).

$$K = \frac{\text{AE}_p}{\text{AE}_s} \quad (1)$$

The purification factor (PF) was calculated as the ratio between the specific activity of the target phase and the specific activity of the crude extract. The specific activity (U mg^{-1}) was defined as the ratio of the enzymatic activity in the target phase (U mL^{-1}) to the corresponding protein concentration in that phase (mg mL^{-1}) (Equation (2)).

$$FP = \frac{AE}{AE_{crude}} \quad (2)$$

Enzyme recovery (Y%) was determined as the ratio between the product of the phase volume (Vf) and its specific activity (AEf) and the product of the crude extract volume (Ve) and its specific activity (AEcrude), expressed as a percentage (Equation (3)).

$$Y(\%) = \left(\frac{AEf \cdot Vf}{AEcrude \cdot Ve} \right) \times 100 \quad (3)$$

3.6. Purification by Ion-Exchange Chromatography

The protease-rich extract obtained after the aqueous two-phase system was subjected directly to ion-exchange chromatography using a DEAE–cellulose column (Sigma-Aldrich, St. Louis, MO, USA). The column was equilibrated with 0.1 M Tris–HCl buffer (pH 8.0) at room temperature (25 °C) and operated at a flow rate of 20 mL h^{−1}. Adsorbed proteins were eluted using stepwise NaCl solutions with increasing ionic strength (0.1–0.5 M). Fractions of 2 mL were collected, and protein elution was monitored by measuring absorbance at 280 nm. Fractions exhibiting proteolytic activity were pooled and subsequently analyzed for protein concentration and enzymatic activity.

3.7. Protease Activity Assay

Protease activity was determined following the modified method of Ginther [84], with 1% azocasein as a substrate. Briefly, 0.8 mL of the enzyme sample was incubated with 0.2 mL of 1.8 N NaOH, and the absorbance was measured at 420 nm using a UV–Vis spectrophotometer (Shimadzu UV-1800, Shimadzu Corporation, Kyoto, Japan). Enzymatic activity was expressed in units per milliliter (U/mL), where one unit corresponds to the amount of enzyme required to produce a 0.01 increase in absorbance under the assay conditions.

3.8. Protein Concentration Determination

Total protein concentration was measured using the Bradford [85] method with bovine serum albumin (BSA) as a standard. Calibration curves were established using protein concentrations ranging from 0.1 to 1.0 mg/mL. All measurements were conducted in triplicate.

3.9. Polyacrylamide Gel Electrophoresis (SDS–PAGE)

The molecular characterization of the purified protease was carried out using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli [86]. A 5% stacking gel and a 12.5% resolving gel were used. Following electrophoresis, gels were stained with Coomassie Brilliant Blue G-250 to visualize protein bands. Molecular weights were estimated using standard protein markers (Bio-Rad Laboratories, Hercules, CA, USA).

3.10. Data Analysis

The quality of model fitting was evaluated using the coefficient of determination (R²) and multiple regression analysis to validate the predictive accuracy of the model. Statistical significance was assessed by ANOVA at a 95% confidence level ($p < 0.05$). All analyses were performed using Statistica 8.0 software [87].

4. Conclusions

In conclusion, this work demonstrates that the integration of extractive fermentation with downstream purification provides an effective and scalable approach to produce fungal proteases. The use of a polyethylene glycol–phosphate aqueous two-phase system enabled efficient *in situ* recovery of the target enzyme from the fermented broth, yielding

high partition coefficients while preserving enzymatic activity. Subsequent purification by DEAE–Sephadex ion-exchange chromatography, with optimal elution at 0.5 M NaCl, resulted in a homogeneous protease preparation with an apparent molecular mass of approximately 59 kDa, as confirmed by SDS–PAGE analysis. The strong influence of PEG molecular weight on enzyme partitioning highlights the importance of system design for maximizing purification efficiency. Overall, the combined strategy represents a robust, cost-effective, and industrially relevant bioprocess aligned with principles of process intensification. By integrating fermentation and primary recovery into a single operation, the platform reduces downstream unit operations, solvent consumption, processing time, and energy demand, thereby improving overall process efficiency and sustainability. The mild physicochemical conditions employed during ATPS partitioning preserve enzyme structure and functionality, avoiding thermal or harsh chemical treatments typical of conventional purification routes.

From an application standpoint, the purified alkaline protease exhibits strong potential for use in detergent formulations, protein hydrolysis in food biotechnology, and other industrial sectors where catalytic efficiency and operational stability are critical. Beyond this specific enzyme, the proposed extractive fermentation framework can be extended to other extracellular biocatalysts, contributing to the development of sustainable, scalable, and economically optimized enzyme manufacturing systems. Thus, aqueous two-phase systems may serve not only as purification tools but also as strategic components in next-generation integrated biorefineries and intensified bioprocess platforms.

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