

# Cost-Effective Production and Process Optimization of Protease Enzyme from *Bacillus subtilis*

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## Abstract

**Background:** The present study focuses on developing an economical and efficient process for producing protease from *Bacillus subtilis* using low-cost agro-industrial residues. Rice bran, groundnut cake, and eggshell powder were selected as nutrient sources and evaluated in submerged fermentation to assess their suitability for enzyme production. **Methodology:** The fermentation conditions were systematically optimized by varying inoculum size, substrate concentration, and nutrient supplementation. **Results:** Among the conditions tested, a 3% inoculum and a 5% substrate concentration yielded the highest protease activity. Supplementation with yeast extract produced only a marginal improvement, indicating that the agro-residues alone were sufficient to support robust enzyme synthesis. The crude enzyme recovered from the broth was partially purified through ammonium sulfate precipitation, dialysis, and cation exchange chromatography, resulting in a preparation with enhanced specific activity of 310 IU/mL. **Conclusion:** The results demonstrate that inexpensive agricultural by-products can effectively replace conventional media components, significantly reducing production costs without compromising enzyme yield. This study supports the feasibility of adopting cost-efficient raw materials and optimized fermentation strategies for sustainable, scalable protease production.

**Key words:** Agro-industrial waste, enzyme optimization, industrial enzymes, protease production, submerged fermentation

## INTRODUCTION

Proteases (peptidases) are hydrolytic enzymes that catalyze the cleavage of peptide bonds and constitute one of the largest classes of industrial enzymes. Microbial proteases – particularly those from *Bacillus* species – dominate the global enzyme market because of their broad substrate specificity, catalytic efficiency, and suitability for large-scale fermentation. They are integral to diverse industrial sectors, including detergents, leather processing, food and feed, pharmaceuticals, and waste treatment, and account for a substantial fraction of commercial enzyme sales.<sup>[1]</sup>

Among microbial producers, *Bacillus subtilis* is widely regarded as an industrial workhorse. Its advantages include a generally recognized as safe status for many strains, robust growth

on inexpensive media, capacity to secrete large quantities of extracellular enzymes directly into culture broth, and amenability to genetic and process improvements that facilitate scale-up. These attributes make *B. subtilis* a preferred source for alkaline and broad-spectrum proteases used in formulations that require activity under harsh conditions (e.g., high pH, presence of surfactants).<sup>[2,3]</sup>

To meet rising commercial demand while keeping production economically viable, contemporary research emphasizes

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two complementary strategies: (1) replacement of costly refined media components with agro-industrial residues (e.g., wheat bran, oil cakes, and pulse husks) or low-cost carbon/nitrogen sources, and (2) systematic process optimization using statistical experimental designs (screening designs and response surface methodology [RSM]) to identify and tune critical variables such as pH, temperature, substrate concentration, inoculum size, and incubation time. Solid-state fermentation and optimized submerged fermentation (SmF) strategies that use agricultural by-products have repeatedly demonstrated substantial reductions in production cost while maintaining or enhancing protease yields.<sup>[4]</sup>

Despite numerous reports of high-yield protease production, translating laboratory gains into cost-effective, reproducible industrial processes remains challenging because of variation in substrate composition, downstream recovery costs, and scale-dependent changes in oxygen transfer and heat removal. Therefore, studies that combine low-cost substrates with rigorous statistical optimization and pilot-scale validation are essential to produce reliable, economically attractive enzyme processes. The present study aims to (i) isolate and characterize a robust *B. subtilis* protease producer, (ii) evaluate inexpensive agro-residues as substrate(s) for enzyme production, and (iii) apply a stepwise experimental design (screening followed by RSM) to maximize protease yield under conditions amenable to scale-up and industrial application.<sup>[5]</sup>

## MATERIALS AND METHODS

The *B. subtilis* strain was used to produce the protease enzyme. The bacterial strain was grown on a nutrient agar broth containing peptone, yeast extract, and sodium chloride. The cultures were incubated at 30°C for 96 h to promote vigorous growth. After achieving sufficient biomass development, the strain was transferred to a liquid nutrient medium formulated with agro-industrial waste substrate to facilitate further growth and enzyme production.

### Preparation of agro-industrial substrates

**Eggshell powder:** Eggshell was first collected and gently washed to remove any impurities present in it. Later, it was dried in the hot air oven for a few minutes. After it had completely dried, it was transferred to a grinder and ground into a fine powder, and was stored in an air-tight container to prevent any contamination. Groundnut cake was collected from an oil mill, and it was completely dried under the sun for a few days to remove any moisture. It was then ground into powder, milled, and then sieved. Rice bran was collected from rice mills and dried in a hot air oven to completely remove any moisture present, sieved, and stored in a container.

### Enzyme production by SmF

SmF was selected as the technique for producing proteases using *B. subtilis*. Rice bran (2%), groundnut cake (1%), and eggshell powder (1%) were used as cost-effective substrates. These substrates were chosen for their high carbohydrate and protein content. The fermentation was done in an orbital shaker under controlled laboratory conditions at 37°C. Samples were collected at regular 4-h intervals during the fermentation process to evaluate protease activity, yield, and overall productivity. The use of SmF facilitated efficient nutrient uptake by the bacterial culture and allowed better regulation of critical process parameters.

### Protease purification

The culture was then processed for proteases by filtering the broth, removing the biomass; the supernatant, which was clear and contained extracellular enzymes, was collected and used as a crude extract of proteases. The process for partial purification began with ammonium sulfate was slowly added to the extract to saturation at 75%, precipitating proteins. The solution was centrifuged for 10 min at 6000 revolutions per minute after the ammonium sulfate was added to separate the precipitated proteins from the solution. The pellet of protein was very gently resuspended in 10 mL of the 10 mM potassium phosphate buffer. The resuspended sample was dialyzed against the same buffer to remove free salts and small molecular weight impurities. After dialysis, the enzyme solution was further concentrated with the addition of sucrose powder. The concentrated enzyme solution was purified further by cation exchange chromatography. Separating proteases from the other contaminants in the crude extract through cation exchange chromatography. The purification process enhanced the purity of the enzyme preparation sufficiently to be used in subsequent biochemical assays and characterized the individual properties of proteases.

### Protease activity assay

The protease assay was performed to quantify the proteases present in the sample. The assay was done using casein as a substrate. Fifty microliters of enzyme were mixed with casein solution and incubated at 50°C for 10 min. Proteases break down the casein into smaller peptides, releasing the amino acid tyrosine. After 1 min, the enzyme activity was stopped with the help of TCA, and the absorbance was measured at 280 nm. The tyrosine standard curve was plotted to quantify the protease activity of purified bacterial protease.

### Statistical analysis

The data were presented as mean  $\pm$  standard deviation. Every outcome was determined to be statistically significant at  $P < 0.05$ .

## RESULTS AND DISCUSSION

### Protease production

The production of protease was carried out using *B. subtilis* in SmF to optimize enzyme production. Several fermentation parameters were systematically optimized to achieve maximum protease production. Different inoculum concentrations of *B. subtilis* (1%, 3%, and 5% v/v) were evaluated to determine the biomass level that yielded the highest enzyme activity. Rice bran and eggshell powder were used as an inexpensive agro-industrial residue, serving as the main carbon source, replacing conventional substrates. Its concentration was varied to assess its effect on substrate utilization and enzyme productivity. The SmF process was carried out for 7 days under controlled incubation. After fermentation, the culture broth was filtered to separate the biomass from the liquid phase. The filtrate, containing extracellular enzymes, i.e., proteases, was subjected to ammonium sulfate precipitation to obtain a concentrated crude enzyme extract for further purification and activity assays.

### Purification of protease

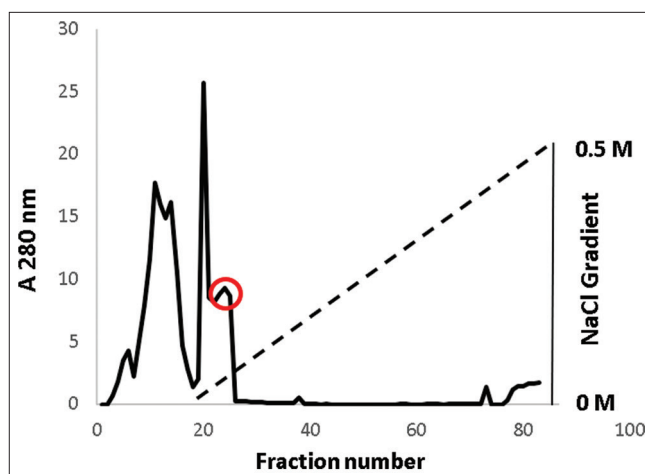
Following the initial concentration of extracellular proteins by ammonium sulfate precipitation, proteases were subjected to further purification using ion exchange chromatography to enhance both their purity and enzymatic activity. The protein pellet obtained from precipitation was carefully resuspended in an appropriate buffer and dialyzed extensively to remove residual salts that could interfere with subsequent chromatographic separation.

The dialyzed enzyme solution was then applied to a CM-Sephadex C-50 cation exchange column. The column was first equilibrated with a 10 mM potassium phosphate buffer to facilitate the binding of positively charged proteins. Upon loading the enzyme mixture, the enzyme adsorbed to the resin based on its net charge, while unbound proteins and other contaminants were removed through washing with the 10 mM potassium phosphate buffer equilibration buffer. The bound protease was subsequently eluted by gradually increasing the ionic strength of the buffer, using a 0 M to 0.5 M NaCl gradient, resulting in a purified enzyme suitable for activity assays and further characterization.

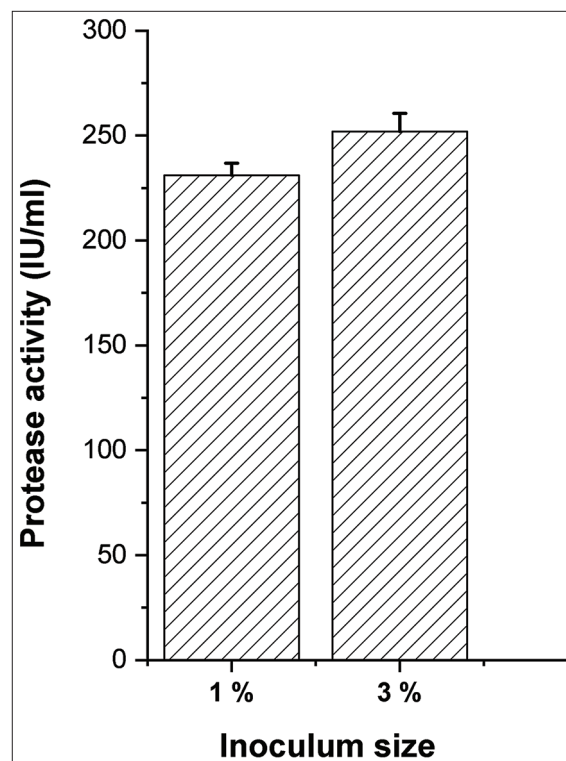
During elution, fractions were collected and analyzed spectrophotometrically, and those showing glucose oxidase activity were pooled for further assessment. This purification step substantially enhanced the specific activity of the enzyme by effectively removing contaminating proteins, as shown in Figure 1.

### Impact of inoculum size on enzyme production

Inoculum size plays a crucial role in determining the efficiency of microbial fermentation, as it influences



**Figure 1:** Chromatogram of protease enzyme purification using cation exchange chromatography on CM Sephadex C-50 resin



**Figure 2:** Culture initial concentration on protease productivity

biomass development, substrate utilization, and enzyme synthesis. In this study, the effect of varying inoculum sizes of *B. subtilis* (1%, 3% v/v) on protease production was evaluated under SmF, with rice bran powder serving as the principal carbon source. The results demonstrated a clear influence of inoculum size on enzyme production. At the lowest inoculum level (1%), protease yield was limited, likely due to insufficient biomass. In contrast, the highest inoculum level (3%) resulted in the highest protease activity, indicating that this concentration provided adequate bacterial growth while maintaining effective nutrient and oxygen availability. Consequently, it was found that a 3%

inoculum size was ideal for optimizing *B. subtilis* production of proteases Figure 2.

### Impact of substrate concentration

The amount of protease produced by SmF significantly increased when the initial concentration of substrate was increased from 1% to 5%. Optimal productivity of protease was observed with 5% substrate powder with an activity of 262 IU/mL (Figure 3).

### Impact of yeast extract on enzyme productivity

Adding yeast extract showed minimal effect on the overall productivity of proteases. We observed a slightly higher concentration of protease produced with the addition of yeast extract into the broth. These results indicate addition of yeast extraction has a minimal effect on overall protease productivity [Figure 4].

### Discussion

The present work demonstrates that *B. subtilis* can be successfully cultivated on inexpensive agricultural residues to produce appreciable levels of extracellular protease. The selection of rice bran, groundnut cake, and eggshell powder was based on their nutritional composition and local availability, and the results indicate that these substrates can reliably support microbial growth and enzyme synthesis.

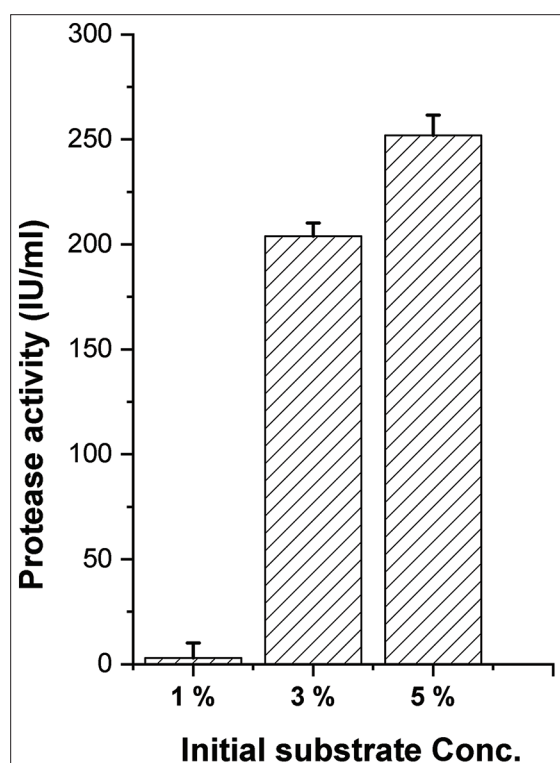


Figure 3: Impact of substrate concentration on enzyme production

Rice bran served as an effective carbon source, which agrees with earlier reports highlighting its usefulness in enzyme fermentation. The addition of eggshell powder may have provided essential minerals and buffering capacity, contributing to a more favorable environment for enzyme secretion.

Inoculum size played a decisive role in determining process efficiency. Too little inoculum led to delayed process growth and reduced enzyme titers, whereas excessive inoculum risked rapid nutrient depletion. The study identified 3% (v/v) as the most suitable concentration, producing a balanced microbial density that allowed steady substrate utilization and enzyme formation. This observation is consistent with general fermentation behavior, where an optimal inoculum ensures vigorous early growth without overwhelming the nutrient system.

Substrate concentration also had a clear influence on protease output. Increasing the substrate level resulted in a proportional rise in enzyme yield up to 5%, beyond which no further improvement was observed. This suggests that the organism could effectively metabolize the available nutrients up to this threshold, after which mass transfer limitations or inhibitory by-products may have come into play. The minimal enhancement observed with yeast extract supplementation indicates that the agro-residues used in this study already supplied sufficient nitrogen, vitamins, and growth factors for enzyme biosynthesis.

Purification of the enzyme using ammonium sulfate and cation exchange chromatography provided a preparation with increased specific activity, demonstrating that the enzyme is present predominantly in extracellular form and can be isolated with relatively simple downstream steps. The elution profile obtained from the CM-Sephadex column further confirmed that the protease responded predictably to changes in ionic strength, reflecting typical characteristics of alkaline and neutral proteases produced by *Bacillus* species.

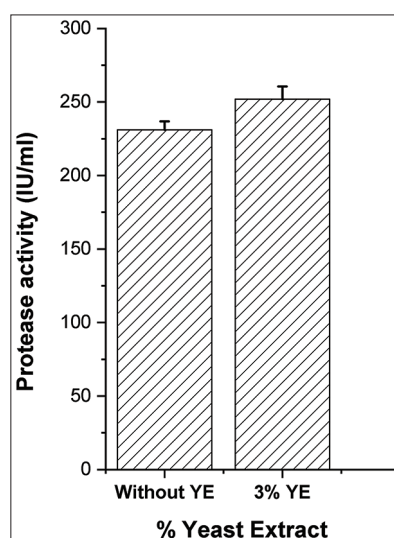


Figure 4: Effect of yeast extract on protease productivity

Overall, the findings support the potential of integrating agro-industrial by-products into enzyme fermentation systems as a practical step toward lowering production costs. Since these residues are abundant and often discarded as waste, their utilization also contributes to improved resource efficiency and environmental sustainability. The process conditions optimized in this study can be adapted to larger-scale operations, provided that aeration, mixing, and heat transfer are appropriately managed. The results reinforce the suitability of *B. subtilis* as an industrial enzyme producer and highlight the value of resource-efficient fermentation strategies in meeting growing enzyme demands.

## CONCLUSION

This study shows that *B. subtilis* can produce significant levels of protease using low-cost agro-industrial residues such as rice bran, groundnut cake, and eggshell powder. These substrates supported good microbial growth and consistent enzyme secretion, demonstrating that they can effectively replace more expensive conventional media components. The optimization experiments highlighted the importance of inoculum size and substrate concentration, with a 3% inoculum and 5% substrate level giving the best enzyme yield. Only a slight improvement was observed with yeast extract supplementation, indicating that the selected residues provide sufficient nutrients on their own.

The purification steps carried out in this work resulted in enzyme preparation with improved specific activity, confirming the presence of a predominant extracellular protease suitable for further biochemical or industrial evaluation. Overall, the findings emphasize that economically

viable protease production is achievable using readily available agricultural by-products combined with simple fermentation optimization. This approach not only reduces production costs but also supports waste valorization and contributes to sustainable bioprocessing. The optimized conditions outlined here provide a practical foundation for future scale-up and industrial applications.

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