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International
Journal
Of Modern Engineering Research (IJMER)

Study on fermentation conditions for pectinase and cellulase production by using filamentous fungi

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ABSTRACT: *Agricultural and food processing residues provide valuable substrates for the environmentally sustainable production of industrial enzymes using microbial fermentation. This study investigated the influence of fermentation conditions on pectinase and cellulase production by filamentous fungi using coffee husk and grapefruit peel as low-cost substrates under solid-state fermentation. Coffee husk contains considerable amounts of cellulose and pectin, while grapefruit peel is rich in pectin, making them suitable carbon sources for enzyme biosynthesis. The effects of substrate ratio, moisture content, and incubation time on enzyme production were evaluated. The fermentation medium consisted of wheat bran (75%) and ammonium sulfate (1%), while the combined proportion of coffee husk and grapefruit peel accounted for the remaining 24%. Results showed that the ratio of coffee husk to grapefruit peel significantly affected enzyme activities. Pectinase activity increased with increasing coffee husk content and reached a maximum value of 489.27 ± 4.18 U/mL at 9% coffee husk, whereas cellulase activity was highest at 6% coffee husk with a value of 5.22 ± 0.01 U/mL. Moisture content also played an important role in enzyme production, with the highest pectinase and cellulase activities observed at 60% moisture, reaching 462.05 ± 9.55 U/mL and 5.88 ± 0.04 U/mL, respectively. In addition, incubation time strongly influenced enzyme synthesis, with maximum activities obtained after 72 h of fermentation, followed by a decline due to nutrient depletion and reduced microbial metabolic activity. These findings demonstrate that coffee husk and grapefruit peel can serve as effective substrates for enzyme production and highlight the importance of suitable culture conditions for enhancing pectinase and cellulase yields from filamentous fungi.*
KEY WORDS: *cellulase; coffee husk; grapefruit peel; pectinase; solid-state fermentation.*

Date of Submission: 28-04-2026 Date of acceptance: 06-05-2026

INTRODUCTION

Enzymes are highly efficient biological catalysts that accelerate biochemical reactions without altering thermodynamic equilibrium, thereby playing a significant role in both cellular metabolism and industrial processes [1]. Their catalytic performance is controlled by highly specific three-dimensional structures, in which the active site selectively binds substrates and facilitates their conversion into products through mechanisms such as acid–base catalysis, covalent interactions, and metal ion coordination. Compared with conventional chemical catalysts, enzymes operate under milder conditions, exhibit superior substrate specificity, and generate fewer undesirable by-products, making them attractive for sustainable industrial applications. However, enzyme activity is strongly influenced by physicochemical factors such as temperature, pH, and substrate availability, and their large-scale application is often constrained by production costs and process optimization challenges.

Among industrial enzymes, hydrolases, particularly pectinases and cellulases, are of considerable importance due to their ability to degrade complex polysaccharides present in plant biomass [1, 2]. Pectinases catalyze the depolymerization of pectic substances in plant cell walls, facilitating cell wall disintegration and improving processes such as juice clarification and extraction yield in the food industry. In parallel, cellulases hydrolyze cellulose into soluble sugars such as glucose, which serve as key intermediates in the production of biofuels and other value-added products [3, 4]. Notably, the efficient degradation of lignocellulosic biomass requires the synergistic action of multiple enzyme components, including endoglucanases, exoglucanases, β-

glucosidases, and various pectinolytic enzymes. This enzymatic synergy is essential for overcoming the structural complexity and recalcitrance of plant cell walls.

The industrial production of enzymes is primarily based on the principle of enhancing enzyme expression in microbial systems to achieve yields that exceed the physiological requirements of the producing organisms [5]. Advances in microbial biotechnology have enabled the development of efficient expression systems and fermentation strategies that promote high-level enzyme production. In filamentous fungi, enzyme biosynthesis is often regulated by inducible mechanisms, in which the presence of specific substrates or structurally related compounds can stimulate the expression of genes encoding target enzymes [6]. In particular, complex polysaccharides such as cellulose and pectin can act as inducers that promote the production of corresponding hydrolytic enzymes. However, enzyme synthesis may be negatively affected by regulatory phenomena such as catabolite repression, where the presence of readily utilized carbon sources suppresses the expression of enzymes involved in the degradation of more complex substrates [6]. Therefore, the selection of appropriate substrates and the control of fermentation conditions are critical factors in directing enzyme production and improving overall process efficiency [5, 6].

Microbial fermentation has emerged as a dominant platform for enzyme production due to its scalability, economic feasibility, and adaptability to diverse substrates. Filamentous fungi are particularly suitable for this purpose because of their ability to secrete large quantities of extracellular enzymes and utilize a wide range of carbon sources [7, 8]. Among fermentation techniques, solid-state fermentation (SSF) has gained increasing attention as it closely mimics the natural habitat of filamentous fungi and enables efficient utilization of solid substrates. Compared with submerged fermentation, SSF offers several advantages, including higher product concentration, lower water and energy requirements, and reduced downstream processing costs [7, 9]. Nevertheless, SSF systems are inherently heterogeneous and present challenges related to heat and mass transfer, moisture distribution, and process scalability, which necessitate careful optimization of operational parameters.

In parallel with advances in fermentation technology, the utilization of agro-industrial residues as substrates has become an important strategy for sustainable enzyme production and waste valorization. Agricultural by-products such as cereal branes, fruit peels, and coffee processing wastes are abundant, renewable, and rich in lignocellulosic components, including cellulose, hemicellulose, and pectin, which serve as both carbon sources and enzyme inducers [9–11]. Coffee husk, a major by-product of the coffee industry, contains significant amounts of cellulose and hemicellulose, making it a suitable substrate for cellulase production [12, 13]. Similarly, citrus residues such as grapefruit peel are rich in pectin and have been widely reported as effective inducers for pectinase biosynthesis [14]. The valorization of these residues not only reduces environmental burdens associated with waste disposal but also contributes to the development of a circular bioeconomy.

Despite extensive research on enzyme production from lignocellulosic substrates, most studies have focused on single-substrate systems, thereby limiting the understanding of synergistic interactions between substrates with complementary compositions. The integration of multiple agro-residues represents a promising strategy to enhance enzyme production by simultaneously providing diverse carbon sources and inducing multiple enzymatic pathways. However, such systems are inherently complex, as enzyme biosynthesis is influenced not only by substrate-induced regulatory mechanisms but also by key process parameters, including substrate ratio, moisture content, and incubation time. These factors affect microbial growth, oxygen transfer, nutrient accessibility, and enzyme secretion dynamics, determining enzyme yield and process efficiency [7].

In this context, the combined use of coffee husk and grapefruit peel provides a model system to investigate substrate-driven enzyme production. Coffee husk serves primarily as a cellulose-rich inducer for cellulase synthesis, whereas grapefruit peel supplies pectin that promotes pectinase production. The gradual degradation of these lignocellulosic substrates may also help mitigate catabolite repression by ensuring a sustained release of sugars, thereby maintaining enzyme induction over time. However, the extent to which such substrate combinations lead to synergistic effects in enzyme co-production remains insufficiently understood, particularly under SSF conditions.

Based on these considerations, it is hypothesized that the integration of coffee husk and grapefruit peel, owing to their complementary compositions, can synergistically enhance the co-production of cellulase and pectinase by filamentous fungi. Furthermore, it is proposed that the interaction between substrate composition and key process parameters, specifically substrate ratio, moisture content, and incubation time, plays a critical role in regulating enzyme biosynthesis by modulating microbial metabolic activity and substrate accessibility. Conceptually, this study is based on a framework in which substrate-induced enzyme expression and process-driven environmental factors interact to determine overall enzyme yield and system performance.

Therefore, the present study aims to investigate the production of pectinase and cellulase by filamentous fungi using coffee husk and grapefruit peel as complementary agro-industrial substrates under solid-state fermentation. The effects of substrate ratio, moisture content, and incubation time were systematically

evaluated to identify optimal conditions for enzyme production and to provide mechanistic insights into substrate synergy in sustainable enzyme production systems.

MATERIALS AND METHODS

2.1 *Materials*

The strain *(Aspergillus niger)* used in this study was obtained from the Food Engineering Laboratory, International University – Vietnam National University, Ho Chi Minh City. The strain had been previously isolated and was maintained under controlled laboratory conditions, exhibiting stable growth characteristics and reproducibility. Rice bran was procured from manufacturing factories at Ho Chi Minh city, while coffee husk was sourced from industrial processing plants in Lam Dong Province. All substrates were carefully inspected and prepared to ensure they were free from insect infestation and other extraneous contaminants.
All chemicals and reagents used in this study were of analytical grade. Ammonium sulfate $(NH_4)_2SO_4$, 3,5-dinitrosalicylic acid (DNS), zinc sulfate $(ZnSO_4)$, and other analytical reagents were procured from reputable commercial suppliers. Pectin and carboxymethyl cellulose (CMC) were used as substrates for pectinase and cellulase activity assays, respectively. Anthrone reagent was employed for the determination of reducing sugars. All solutions were prepared using distilled water.

2.2 *Methods*

2.2.1 Study on fermentation conditions for pectinase and cellulase production

Fermentation conditions for enzyme production were optimized using solid-state fermentation with a total substrate weight of 100 g per sample. The basal medium consisted of wheat bran (75%, w/w) supplemented with 1% $(NH_4)_2SO_4$, while the remaining 24% (w/w) was composed of varying proportions of coffee husk and grapefruit peel powder. The medium was sterilized at 121 °C for 20 min prior to inoculation with *Aspergillus niger* at an initial cell concentration of approximately 10^7 cells/mL. Fermentation was then carried out at room temperature (22–30 °C).

The effect of substrate composition was evaluated by varying the proportion of coffee husk from 3% to 21% (w/w) at intervals of 3%, while the grapefruit peel powder content was adjusted accordingly to maintain a constant total of 24% (w/w). The initial moisture content was adjusted in the range of 48–68% with 4% intervals, and the incubation time was set at 24, 48, 72, and 76 h.

After fermentation, crude enzymes were extracted by mixing the fermented substrate with sterilized water at a ratio of 7 mL/g. The mixture was then centrifuged at 7000 rpm for 30 min and filtered through sterilized filter paper with a pore size of 0.2 µm to remove microorganisms.

2.2.2 Determination of pectinase activity

Pectinase activity was determined by anthrone assay [15]. A volume of 10 mL of 1% pectin solution was transferred into a test tube, followed by the addition of 5 mL of crude enzyme solution (1% pectinase). The mixture was thoroughly mixed and incubated at 30 °C for 60 min. Subsequently, 1 mL of 15% $ZnSO_4$ was added to terminate the reaction, and the mixture was filtered through filter paper. An aliquot of 0.5 mL of the filtrate was diluted to 100 mL with distilled water for analysis using the Anthrone method. For color development, 2 mL of the diluted solution was transferred into a test tube and mixed with 4 mL of Anthrone reagent. The mixture was then incubated at 90 °C for 7 min, allowed to cool to room temperature, and the optical density was measured at 584 nm.

2.2.3 Determination of cellulase activity

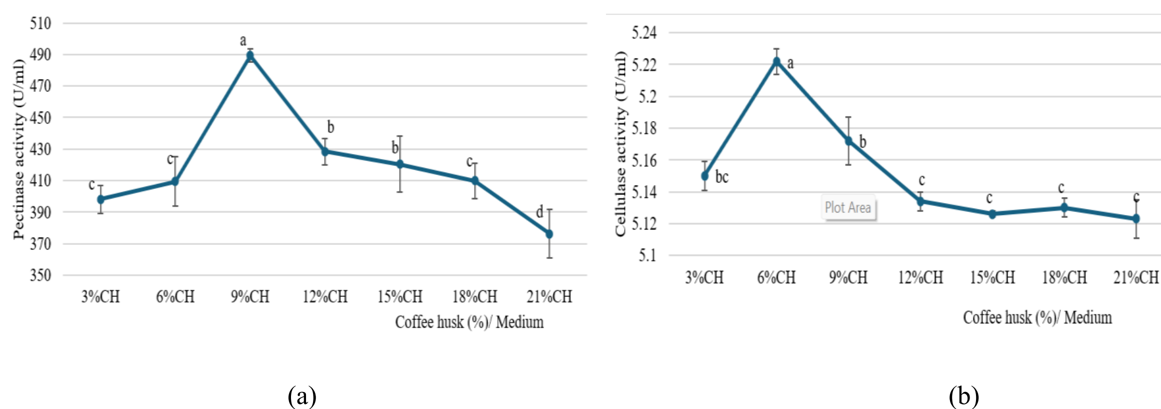
One unit of cellulase activity was defined as the amount (µmol) of glucose produced by the hydrolysis reaction of CMC 1% for 1 min. To measure, 1 ml of cellulase (sample) was reacted with 1 ml of CMC 1% for 10 mins. Then, 1 ml of 3,5-DNS was added, and the reaction was boiled for 5 mins. The absorbance (OD) of the reacted solution was measured by the spectrophotometer at wavelength $\lambda = 540$ nm. Finally, the concentration of glucose was calculated based on the standard curve of glucose. The blank sample was the sample without enzymatic reaction.

2.2.4 Statistical analysis

The SPSS statistical program with One-way ANOVA method and Duncan standard ($\alpha \leq 0.05$) was used to analyze means, standard deviation of replications and significance of samples.

RESULTS AND DISCUSSIONS

3.1. Effect of medium composition on enzymes biosynthesis



(a) (b)

Figure 3.1. Effect of ratio of coffee husk/ grapefruit peel powder on pectinase production (a) and cellulase production (b).

The study on the enzyme production conditions for high enzyme activity (pectinase and cellulase) involves investigating environmental conditions that allow microorganisms to grow and develop optimally. Concurrently, the goal is to obtain the highest enzyme yield. Substrates include pectin found in pomelo peel, where the pectin content accounts for over 30% (Nguyên et al., 2023), and cellulose and pectin in coffee husk, which were previously studied to constitute 35.6% and 20.5%, respectively [16]. These substrates are introduced into the medium at a specific, sufficient quantity to stimulate (environmental signal) the initiation of a new protein biosynthesis process, resulting in the desired enzymes.

In this study, with a fixed bran content of 75% and ammonium sulfate at 1%, the proportions of pomelo peel (a pectin-rich source) and coffee husk (a source rich in cellulose and partially in pectin) were varied such that their combined contribution accounted for the remaining 24%.

The proportions of pomelo peel (a pectin-rich source) and coffee husk (a source rich in cellulose and partially in pectin) have a significant effect on the enzymes produced. Based on Figure 3.1a, pectinase activity increased as the proportion of medium containing coffee husk, used as a complex substrate rich in cellulose and pectin, rose from 3% to 9% (3% CH, 9% CH). At 9% CH, this substrate level resulted in the highest enzyme activity, reaching 489.267 ± 4.179 U/mL. However, pectinase activity began to decrease or remain unchanged when the coffee husk content was further increased.

In addition to pectin, coffee husk also contains cellulose, which acts as a substrate for cellulase biosynthesis. Based on Figure 3.1b, cellulase activity gradually increased from media containing a low proportion of coffee husk to media containing 6% CH and reached its maximum at 6% CH. Specifically, at 3% CH and 6% CH, cellulase activities were 5.150 ± 0.009 U/mL and 5.222 ± 0.008 U/mL, respectively. It was also observed that in media containing 9% CH or more, cellulase activity was not high.

This can be explained by the fact that total enzyme activity depends not only on the structure of the enzymes but also on the amount of enzymes secreted by microorganisms. The quantity of enzyme produced by microorganisms depends on the proportion of the substrate present, whether partially or throughout the medium. Nevertheless, as long as the substrate is present, microorganisms continue to secrete enzymes. Therefore, total enzyme activity increases as the substrate gradually increases, but once all microorganisms are simultaneously producing the maximum amount of enzyme, the total activity reaches its peak. Further increases in substrate concentration do not lead to higher enzyme activity.

3.2. Effect of moisture content of medium on crude enzymes production

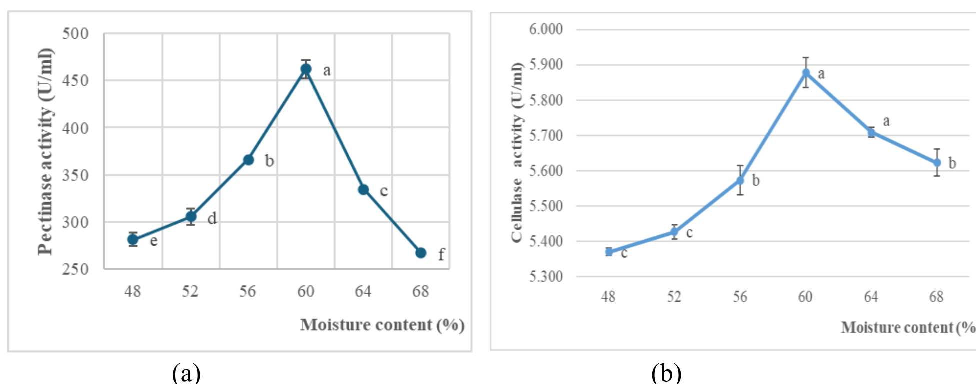


Figure 3.2. Effect of moisture content of medium on pectinase production (a) and cellulase production (b).

In this study, all medium components, including bran content (75%), ammonium sulfate (1%), and the optimized ratio of coffee husk to grapefruit peel powder, were maintained constantly, while only the moisture content was varied to evaluate its effect on enzyme production. Moisture is widely recognized as a critical factor in solid-state fermentation (SSF), as it directly influences microbial growth, substrate accessibility, and mass transfer processes within the fermentation matrix.

As illustrated in Figure 3.2, moisture content significantly affected both pectinase and cellulase production. The results demonstrated that enzyme activities increased with increasing moisture content up to 60%, at which point maximum activities of 462.053 ± 9.552 U/mL for pectinase and 5.878 ± 0.042 U/mL for cellulase were achieved. This optimal moisture level suggests that moderate water content provides a favorable microenvironment for filamentous fungal growth and metabolic activity. At this level, sufficient water availability enhances substrate swelling and solubilization of nutrients, thereby improving enzyme–substrate interactions and facilitating the diffusion of metabolites.

However, when the moisture content exceeded 60%, a significant decline in enzyme activities was observed. This decrease can be attributed to several interrelated factors. Excessive moisture reduces substrate porosity and limits oxygen transfer within the solid matrix, leading to suboptimal aerobic conditions for filamentous fungi. In addition, high moisture levels may result in particle agglomeration, which decreases the effective surface area available for microbial colonization and enzyme secretion. These effects collectively hinder fungal growth and enzyme biosynthesis.Conversely, at lower moisture levels, insufficient water availability can restrict microbial metabolic activity by limiting nutrient solubility and diffusion. Under such conditions, the reduced mobility of enzymes and substrates may lead to decreased catalytic efficiency. Therefore, both insufficient and excessive moisture levels negatively affect enzyme production, highlighting the importance of maintaining an optimal balance.

From a mechanistic perspective, moisture content governs the physicochemical properties of the fermentation system, including water activity, substrate structure, and oxygen diffusion, all of which are critical for fungal physiology and enzyme secretion. Filamentous fungi are particularly well adapted to moderately moist environments, where the interplay between solid substrate and limited free water promotes efficient enzyme production. Similar trends have been reported in previous studies, where optimal moisture levels were found to maximize enzyme yield, while deviations from this range resulted in reduced productivity due to mass transfer limitations and unfavorable microenvironmental conditions [17].

Overall, the results confirm that moisture content is a key parameter controlling enzyme biosynthesis in SSF systems. An optimal moisture level of 60% was identified as the most suitable condition for maximizing both pectinase and cellulase production in this study. These findings emphasize the importance of carefully controlling water availability to achieve efficient enzyme production and provide valuable insights for the optimization of SSF processes using lignocellulosic substrates.

3.3. Effect of incubation time on production of crude enzymes production

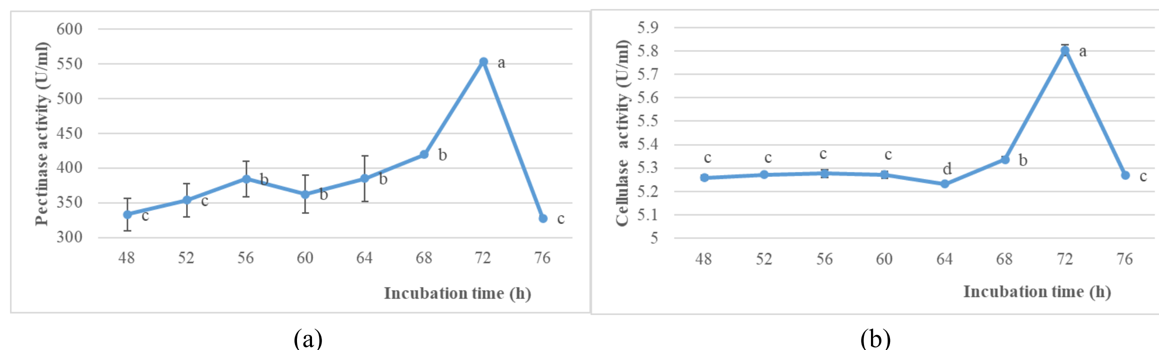


Figure 3.3. Effect of fermentation duration on pectinase production (a) and cellulase production (b).

In this research, all components of the medium, such as bran content (75%), ammonium sulfate (1%), and the ratio of coffee husk to grapefruit peel powder determined above, were fixed, as was the moisture content of the medium, with only the incubation time being varied.Under the optimized medium composition and moisture conditions, incubation time was found to exert a pronounced influence on both pectinase and cellulase production (Figure 3.3). As observed, enzyme activities remained relatively stable during the early fermentation phase (48–64 h), suggesting that the fungal cells were primarily in the adaptation and early exponential growth stages. During this period, metabolic activity is mainly directed toward biomass accumulation and establishment within the solid matrix rather than active enzyme secretion.

A marked increase in enzyme activity was observed between 64 and 72 h, with both pectinase and cellulase reaching their maximum levels at 72 h. This trend indicates a transition to the late exponential phase, during which enzyme biosynthesis is significantly enhanced. At this stage, the fungal system is fully adapted to the substrate environment, and the availability of inducers derived from partial hydrolysis of lignocellulosic components (cellulose and pectin) likely stimulates the expression of genes encoding hydrolytic enzymes. This observation is consistent with previous findings that maximum extracellular enzyme production in filamentous fungi typically occurs during the late exponential to early stationary phase, when metabolic activity and enzyme secretion are at their peak.Beyond 72 h, a sharp decline in enzyme activity was recorded. This reduction can be attributed to several factors. First, the depletion of essential nutrients in the fermentation medium limits microbial metabolic activity and enzyme synthesis. Second, the accumulation of metabolic by-products may exert inhibitory effects on enzyme production. Third, prolonged incubation can lead to enzyme denaturation or proteolytic degradation, reducing the measurable enzyme activity. Additionally, in solid-state fermentation systems, extended fermentation may result in reduced substrate porosity and oxygen transfer limitations, further constraining microbial activity.

From a mechanistic perspective, the observed trend reflects the dynamic balance between enzyme synthesis and degradation over time. According to previous studies, the timing of enzyme production is closely linked to microbial growth phases and environmental conditions, with optimal production occurring when metabolic processes are highly synchronized across the microbial population. As reported in recent work on solid-state fermentation systems, excessive incubation time can disrupt this balance due to nutrient exhaustion and unfavorable microenvironmental changes, ultimately leading to decreased enzyme yields (Shet et al., 2022).

Overall, these results highlight that incubation time is a critical parameter governing enzyme production efficiency. An optimal duration of 72 h was identified for maximizing both pectinase and cellulase activities under the studied conditions. This finding not only aligns with the general kinetics of enzyme production in filamentous fungi but also emphasizes the importance of precisely controlling fermentation time to avoid productivity losses associated with over-incubation.

CONCLUSIONS AND RECOMMENDATIONS

This study demonstrated that agro-industrial residues, specifically coffee husk and grapefruit peel, are effective and low-cost substrates for the production of pectinase and cellulase by filamentous fungi under solid-state fermentation. The results clearly indicate that fermentation conditions significantly influence enzyme biosynthesis.Among the parameters investigated, substrate composition played a critical role in determining enzyme yields. The best proportion for pectinase production was obtained at 9% coffee husk, achieving the highest activity (489.27 ± 4.18 U/mL), whereas cellulase activity reached its maximum at 6% coffee husk (5.22 ± 0.01 U/mL). Moisture content was also a key factor, with 60% identified as the optimal level for both

enzymes, ensuring favorable conditions for microbial growth and metabolic activity. In addition, incubation time significantly affected enzyme production, with peak activities observed after 72 h of fermentation, followed by a decline due to nutrient depletion and reduced microbial activity.

Overall, the findings highlight the importance of optimizing fermentation parameters to enhance enzyme production efficiency. The successful utilization of coffee husk and grapefruit peel not only improves enzyme yields but also contributes to sustainable waste valorization and cost-effective bioprocess development. These results provide a practical basis for further scale-up and industrial application of enzyme production using agro-industrial residues.

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