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Xylanase Production by *Aspergillus niger* Using Agro-Industrial Residues and Tween Surfactants: A Non-Parametric Analysis

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ABSTRACT

Xylanase plays a key role in hydrolyzing xylan, yet large-scale enzyme production remains limited by the high cost of purified xylan substrates. Although lignocellulosic agricultural residues offer a promising low-cost alternative, their effectiveness as substrates for Aspergillus niger and the influence of process additives on enzyme performance are not fully understood. This study addresses this gap by evaluating sugarcane bagasse and palm kernel cake as economical substrates and examining how substrate type, substrate concentration, and surfactant selection affect xylanase specific activity. Fermentation experiments were conducted using substrate concentrations of 1.5–3.0% (w/v) supplemented with Tween 20, Tween 60, or Tween 80, followed by statistical analysis using the Kruskal–Wallis test and Bonferroni-corrected Mann–Whitney U tests. Substrate concentration ($p = 0.016$) and surfactant type ($p < 0.001$) significantly influenced specific activity, whereas substrate type did not ($p = 0.224$). The highest activity (4.380 ± 0.052 IU/mg; median = 1.9113) was achieved using 3.0% palm kernel cake with Tween 20. These findings demonstrate that optimizing substrate load and surfactant choice is crucial for enhancing xylanase production from low-cost agro-industrial residues, providing practical insights for cost-efficient enzyme bioprocess development.

Keywords: *Aspergillus niger*, Kruskal-Wallis, palm kernel cake, sugarcane bagasse, surfactant, xylanase.

1. INTRODUCTION

Xylanase is an extracellular enzyme capable of hydrolyzing xylan (hemicellulose) into xylose and xylo-oligosaccharides as presented in Figure 1 [1]. The ability of xylanase to hydrolyze xylan into xylose can be applied to increase reducing sugars, which can subsequently be used as raw materials for bioethanol or bioenergy. Xylanase enzymes are also commonly used as substitutes for chemical compounds in waste management to degrade xylan into recyclable products, and they are employed in the food industry, as well as the paper and pulp industries, thus reducing the use of chemicals harmful to the environment.

Xylanase enzymes are typically produced by microorganisms. The use of microorganisms as enzyme producers offers several advantages, including relatively low production costs, short production times, high growth rates, and ease of control. The common microorganisms that produce xylanase are fungi and bacteria. Although xylanase produced by bacteria tends to be more thermostable than that produced by fungi, the xylanase activity of fungi is generally higher than that of bacterial xylanase [2]. According to research by Cristica, filamentous fungi are strong producers of xylanase and are easy to cultivate [3].

To optimize enzyme production, it is necessary to optimize various factors involved in the process, including the composition of the fermentation medium, temperature, and pH. The composition of the fermentation medium or enzyme production medium plays a crucial role. Media for xylanase production must contain carbon,

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nitrogen, and essential minerals. For large-scale production, a cost-effective and simple fermentation medium is required that can still yield the desired enzyme levels.

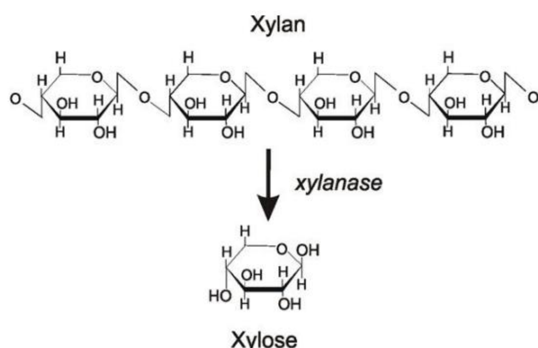

Figure 1. Hydrolysis of Xylan into Xylose [4].

Xylanase production by microorganisms requires an inducing substrate, typically xylan. Economically, the use of pure xylan on a large scale is too expensive, so research is needed to find a cheaper alternative inducing substrate. Agricultural waste, which primarily contains lignocellulose, is considered a suitable alternative. Utilizing agricultural waste can reduce enzyme production costs by 40-60% [5]. Adding surfactants to the fermentation medium is also an effective strategy to enhance xylanase activity. Research has shown that surfactants can accelerate the decomposition of cellulose and hemicellulose by increasing CMCase and xylanase activities [6].

Agricultural waste with potential as a medium for xylanase production includes sugarcane bagasse. Sugarcane (*Saccharum officinarum* L.) is a leading plantation commodity that plays a significant role in meeting food demand in Indonesia. Badan Pusat Statistika (BPS) reported that the sugarcane plantation area in Indonesia reached 488,900 hectares (ha) in 2022, producing approximately 2.49 million tons of sugarcane [7]. The utilization of sugarcane for sugar production generates a by-product, sugarcane bagasse. Sugarcane bagasse produced from sugar production accounts for about 35-40% of the total sugarcane used as raw material [8]. To date, sugarcane bagasse has been utilized only as livestock feed, for making briquettes, and for producing paving blocks. Sugarcane bagasse is a lignocellulosic material containing 40-50% cellulose, 20-30% hemicellulose or xylan, and 10-25% lignin [9].

In addition to sugarcane, oil palm is also one of the leading commodities in the agricultural sector. Indonesia has oil palm plantations covering 14.9 million hectares (ha) [10]. Palm kernel cake contained 21,7% high crude fiber, including hemicellulose (mannan and galactomannan) [11]. Processing palm kernels into oil generates palm kernel cake waste, which can cause environmental and health issues due to its rapid decomposition, odor production, and tendency to attract disease-vector insects [12]. Several studies have explored the utilization of palm kernel cake waste as animal feed, but its application has been limited due to its high crude fiber content. The relatively high levels of hemicellulose and xylan as its major components make sugarcane bagasse and palm kernel cake promising substrates for xylanase enzyme production.

To improve the quality of agricultural waste so that it can be used as a substrate in xylanase production, pretreatment is necessary to degrade the lignin, which acts as a binder for cellulose and hemicellulose. Lignin must be removed so that hemicellulose, primarily composed of xylan, can be converted or broken down into xylose and xylo-oligosaccharides.

Several studies have explored the use of agricultural waste to produce xylanase. For example, xylanase from *Aspergillus niger* was studied using submerged fermentation with a 2.5% (w/v) medium concentration of palm oil empty fruit bunches, yielding significant xylanase activity. The xylanase produced after a 9-day incubation period at 30°C reached 39.96±0.04 IU/mL [13]. In another study, xylanase production from *Aspergillus niger* was optimized using wheat bran as a medium, with variations in temperature, nitrogen sources, types and

amounts of surfactants. The results showed that optimal xylanase production occurred at 30°C, pH 7, after a 6-day incubation, with 1% peptone (w/v) as the nitrogen source, and the addition of 0.10% (v/v) Tween 20 surfactant. The maximum xylanase activity obtained was 1288.93 ± 22.30 IU/gds [14].

This study examines the use of sugarcane bagasse and palm kernel cake as substrates for xylanase production from *Aspergillus niger*, considering their potential and the limited application of these agro-industrial waste products.

Given these gaps, further investigation is needed to determine the suitability of these two agro-industrial residues as low-cost substrates and to understand how medium formulation factors influence enzyme productivity. Therefore, this study specifically aims to evaluate the effects of substrate type, substrate concentration, and surfactant type on the specific activity of xylanase produced by *Aspergillus niger*, thereby addressing the lack of comparative data and providing insights for optimizing cost-efficient xylanase production.

2. RESEARCH METHODS

2.1. Lignocellulose Content Assay of the Substrate

The determination of lignocellulose components, including hemicellulose, cellulose, and lignin, in this study was conducted using the Chesson method. For hemicellulose analysis, 1 gram of sugarcane bagasse were accurately weighed (weight a). To each sample, 150 mL of distilled water was added, and the mixture was refluxed at 100°C for 1 hour using a water bath. After refluxing, the samples were filtered through filter paper and washed with 300 mL of hot water. The resulting residue was oven-dried at 60°C until a constant weight was obtained (weight b).

Subsequently, 150 mL of 1N H_2SO_4 was added to the dried residue to solubilize the hemicellulose, and the mixture was refluxed again at 100°C for 1 hour using an oil bath. The residue was filtered and washed with water until neutral, then dried at 60°C in an oven until a constant weight was achieved (weight c), representing the hemicellulose-free fraction.

To remove cellulose, 10 mL of 72% (v/v) H_2SO_4 was added to the residue, and the samples were left to soak for 4 hours. Following this, 50 mL of 1N H_2SO_4 was added, and the mixture was refluxed at 100°C for 1 hour using an oil bath. The residue was then filtered, washed with water until neutral, and dried in an oven at 60°C to a constant weight (weight d), representing the cellulose-free fraction. Finally, the remaining residue was ashed, and the weight of the ash was measured (weight e). The percentage of hemicellulose, cellulose, and lignin was calculated using standard equations based on these weights.

2.2. Pre-Treatment Substrate

100 grams of sugarcane bagasse/palm kernel cake are weighed and transferred into a two-neck round-bottom flask. The substrates are then mixed with 1000 mL of 1% (w/v) NaOH solution. The mixture is heated at 80°C for 16 hours. After the heating process, the solution is filtered and rinsed with distilled water until the pH reaches 7. The substrates are then oven-dried at 60°C until they reach a constant weight.

2.3. Production of Enzyme Xylanase

The fungal strains *Aspergillus niger* were inoculated with a 6 mm diameter plug into Erlenmeyer flasks containing xylanase enzyme production media. The inoculated media were incubated in an incubator shaker at 30°C with a shaking speed of 150 rpm for 9 days. After incubation, the culture was centrifuged at 4000 rpm for 30 minutes at room temperature [14]. The supernatant was then separated from the mycelium and the media sediments, with the supernatant containing the enzyme produced in this study. The same steps were followed for samples with variable substrate concentrations of 1.5%, 2.0%, 2.5%, and 3.0% (w/v), as well as different surfactant variables.

2.4. Protein Content Assay

Protein content was determined using the Bradford method with Coomassie Brilliant Blue G-250 as the dye reagent, prepared according to the standard protocol described by Bradford [15]. For the protein assay, 1 mL of filtrate from the separation of media and product during xylanase enzyme production was transferred into a test tube. Next, 5 mL of Bradford reagent was added, and the mixture was homogenized using a vortex mixer. The solution was incubated at room temperature for 15 minutes. Finally, the absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 595 nm.

2.5. Xylanase Enzyme Activity Assay

The DNS (3,5-dinitrosalicylic acid) reagent was prepared by dissolving 16 grams of NaOH in 100 mL of distilled water. Subsequently, 10 grams of DNS was added to the NaOH solution. In a separate container, 30 grams of sodium potassium tartrate ($NaKC_4H_4O_6$) and 8 grams of sodium metabisulfite ($Na_2S_2O_5$) were dissolved in 500 mL of distilled water. The DNS-NaOH mixture was combined with the sodium potassium tartrate and sodium metabisulfite solution. Finally, the solution was diluted to a total volume of 1000 mL with distilled water and mixed until homogeneous.

Xylose concentration in the samples was analyzed twice, with the difference between the tests being the inclusion of 1% xylan incubation. The xylose content assay before correction included incubation with 1% xylan, while the corrected assay was performed without this incubation step.

The xylose assay was conducted by adding 0.2 mL of xylanase enzyme to a test tube, followed by the addition of 1.8 mL of 1% xylan solution. The mixture was incubated at 35°C for 10 minutes. After incubation, 3 mL of DNS reagent was added to the test tube, and the solution was homogenized by shaking. The mixture was then heated in boiling water for 10 minutes, followed by cooling in an ice bath for 10 minutes. The absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 540 nm. The same procedure was applied to samples with varying substrate concentrations of 1.5%, 2.0%, 2.5%, and 3.0% (w/v), as well as for samples with different surfactant types.

2.6. Experimental Design

The study employed a factorial design of 2×4×3 with the following factors:

- Factor A (Substrate type): Sugarcane bagasse and palm kernel cake
- Factor B (Substrate concentration): 1.5%, 2.0%, 2.5%, and 3.0% (w/v)
- Factor C (Surfactant type): Tween 20, Tween 60, and Tween 80

Each treatment combination was performed in duplicate, resulting in a total of 48 experimental units with 24 distinct treatment combinations.

2.7. Data Analysis

Enzyme activity data were analyzed using a non-parametric statistical approach, since the Shapiro–Wilk test indicated that the data were not normally distributed and Levene's test showed that the variances were not homogeneous, thereby violating the assumptions of parametric tests. The analyses included:

1. Descriptive Statistics: Calculation of median, mean, and standard deviation for each treatment combination
2. Kruskal–Wallis Test: To examine differences among treatment groups
3. Post-hoc Test: Mann–Whitney U test with Bonferroni correction to identify specific pairwise differences
4. Interaction Analysis: Evaluation of factor interactions using the Kruskal–Wallis test

The significance level was set at $\alpha = 0.05$. All analyses were conducted using Python with appropriate statistical libraries.

3. RESULTS AND DISCUSSION

3.1. Substrate and Pre-Treatment Process

The substrate used in this study was sugarcane bagasse. The pre-treatment process of the bagasse was carried out in

two stages: mechanical and chemical. The mechanical pre-treatment involved drying and size reduction through sieving, yielding a particle size between 100 and 120 mesh. The purpose of drying the substrate was to prevent spoilage, allowing for long-term storage. The size reduction process aimed to increase the contact area between the enzyme and the substrate, facilitating easier degradation of hemicellulose into xylose by the enzyme.

Following mechanical pre-treatment, chemical pre-treatment was performed in this study. The chemical pre-treatment consisted of a delignification process using 1% NaOH. The purpose of delignification was to remove lignin content. Lignin, present in sugarcane bagasse, has a strong and rigid structure that can hinder enzyme activity in degrading hemicellulose, thus affecting overall enzymatic efficiency. Lignin is a phenolic polymer found in the cell walls of plants, where it forms strong bonds with cellulose and hemicellulose [16]. The use of alkaline solutions can aid in the separation of lignin. The application of NaOH as a delignifying agent in this research helps to break down the lignin structure in both the crystalline and amorphous regions. The result observed was a reduction in the sample's weight, indicating the removal and release of lignin from the substrate. This suggests that the lignin content in the substrate has been successfully eliminated, yielding a substrate sample suitable for use as a medium for xylanase enzyme production.

After the delignification process, the cellulose, hemicellulose, and lignin contents were analyzed using the Chesson method. This method involves three stages of reflux with H_2O, 1N H_2SO_4, and 72% H_2SO_4. The results for hemicellulose, cellulose, and lignin content in both substrates used in this study are shown in Figures 2 and 3.

Based on Figures 2 and 3, it can be observed that the hemicellulose content, the primary substrate in this study, increased both before and after the delignification process.

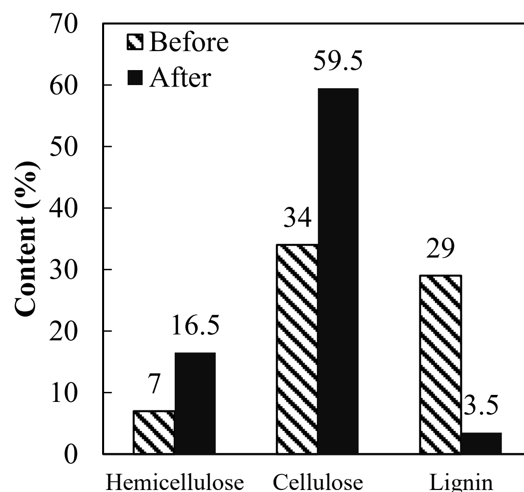

Figure 2. Hemicellulose, Cellulose, and Lignin Content of Sugarcane Bagasse Before and After Delignification.

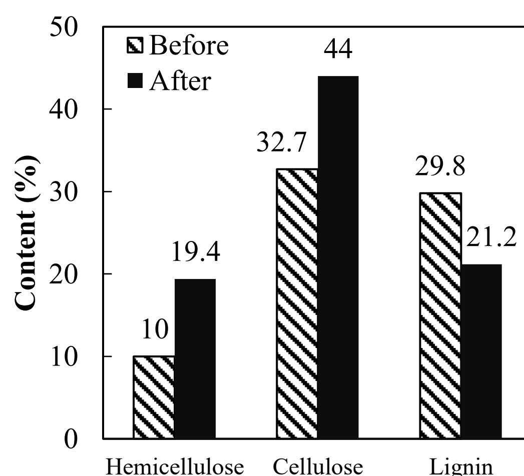

Figure 3. Hemicellulose, Cellulose, and Lignin Content of Palm Kernel Cake Before and After Delignification.

In the bagasse substrate, the hemicellulose content before the delignification process was 7%, while after the delignification process, the hemicellulose content reached 16.5%. In the palm kernel cake substrate, the hemicellulose content before the delignification process was 10%, while after the delignification process, the hemicellulose content reached 19.4%.

The increase in the measured percentage of hemicellulose after the delignification process is primarily due to the removal of lignin, which reduces the total mass of the substrate while leaving hemicellulose more exposed. Hemicellulose is located between

lignin and cellulose, where it is covalently linked to lignin and associated with cellulose through hydrogen bonds. During alkaline delignification, these lignin–hemicellulose linkages are disrupted, allowing lignin to dissolve and separate from the biomass [17]. As lignin is removed, the relative proportion of hemicellulose increases on a dry-weight basis, even though the absolute amount of hemicellulose may not change substantially. Thus, the apparent increase in hemicellulose content after treatment reflects a shift in composition due to mass loss from lignin removal rather than new hemicellulose formation.

3.2. Xylanase Enzyme Activity

In this study, the cultivation of *Aspergillus niger* was carried out on Potato Dextrose Agar (PDA) medium using the streak plate method. PDA is a commonly used medium for the growth of microorganism because it contains carbon sources and nutrients essential for microbial growth. Additionally, PDA has a pH that favorable for microorganism proliferation, allowing for optimal growth confitions.

In this research, protein content measurement and enzyme activity assays (IU/mL) were performed to obtain the specific activity of the enzyme (IU/mg protein). Enzyme activity is defined as the rate of substrate degradation or the rate of product formation under optimal conditions. One unit of xylanase enzyme activity is defined as the amount of enzyme that releases one micromole of reducing sugar (xylose) per minute.

The enzyme activity measurement in this study was conducted using the DNS (Dinitrosalicylic Acid) method, which is based on the amount of xylose produced because of xylan hydrolysis by the xylanase enzyme. Protein content was determined using the Bradford method, while enzyme activity was assayed using the DNS method. The specific activity of xylanase was calculated by dividing the enzyme activity by the protein concentration. The results of the specific xylanase activity determination in this study are presented in Figures 4 and 5.

The results of the study showed that the highest specific activity of crude xylanase enzyme was 4,380 ± 0.052 IU/mg protein found in the combination treatment of media with 3.0% (w/v) Palm Kernel Cake substrate and Tween 20 surfactant.

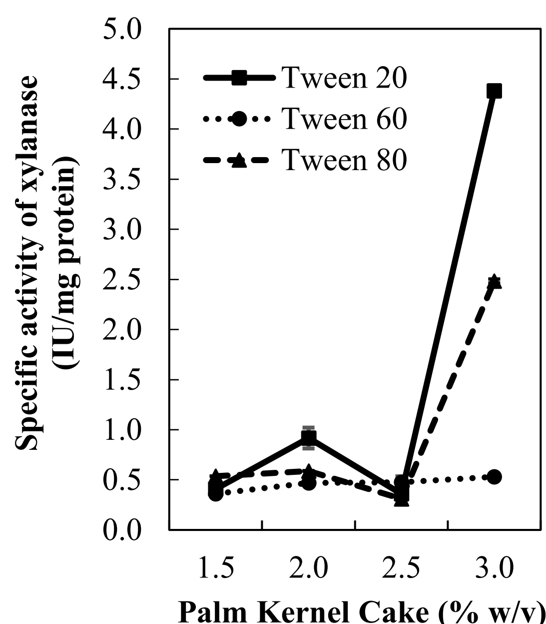

Figure 4. Specific Activity of Crude Xylanase (IU/mg) Using Palm Kernel Cake as Substrate.

From these results, it can be seen that the palm kernel cake substrate and bagasse with a concentration of 3.0% (w/v) are the best compositions capable of producing higher specific enzyme activity. This is because the palm kernel cake and bagasse substrates contain hemicellulose, with the main component being xylan. Xylan is an inducing substrate in the xylanase enzyme production media. Bagasse contains 16.5% hemicellulose, while palm kernel cake contains 19.4% hemicellulose.

Hemicellulose is an inducing substrate containing xylan, the main substrate for the xylanase enzyme. The higher the hemicellulose content, the more xylan is available, allowing for more xylanase production. This is related to the microorganism's response to the availability

of substrates required for enzyme synthesis and secretion. Research shows that increasing the hemicellulose content in the substrate increases the production of lignolytic enzymes, including xylanase [18].

In this study, surfactants (Tween 20, Tween 60, and Tween 80) were added to the xylanase production medium. Tweens possess both hydrophobic and hydrophilic regions, giving them amphiphilic properties that can influence biomass–enzyme interactions. The hydrophobic portion, composed of long hydrocarbon chains, can associate with residual lignin or other non-polar components, potentially reducing non-productive enzyme binding [19]. Meanwhile, the hydrophilic groups, such as hydroxyl and ester functionalities, form hydrogen bonds that enhance solubility and dispersion in aqueous media [20]. These hydrogen bonds enable the hydrophilic portion of Tween to interact with water, allowing it to dissolve or disperse easily in aqueous environments and interact with the xylanase enzyme. These combined effects may improve substrate accessibility, stabilize secreted enzymes, and facilitate more efficient hydrolysis, thereby contributing to differences in xylanase activity observed across treatments.

Figures 4 and 5 show that the xylanase production medium supplemented with Tween 20 yielded the highest specific enzyme activity, while Tween 60 and Tween 80 produced no significant improvement across the tested substrate concentrations. Tween 20 possesses a shorter fatty acid chain and fewer hydrophobic groups, giving it higher polarity than Tween 60 and Tween 80 and enhancing its ability to interact with and stabilize xylanase in aqueous conditions. This property may reduce non-productive adsorption and improve enzyme dispersion in the medium. Variations in substrate concentration (1.5%, 2.0%, and 2.5%) combined with Tween 60 or Tween 80 also showed no significant effect on activity. These results suggest that Tween 20 facilitates better enzyme–substrate interactions, likely due to its amphiphilic balance and higher polarity.

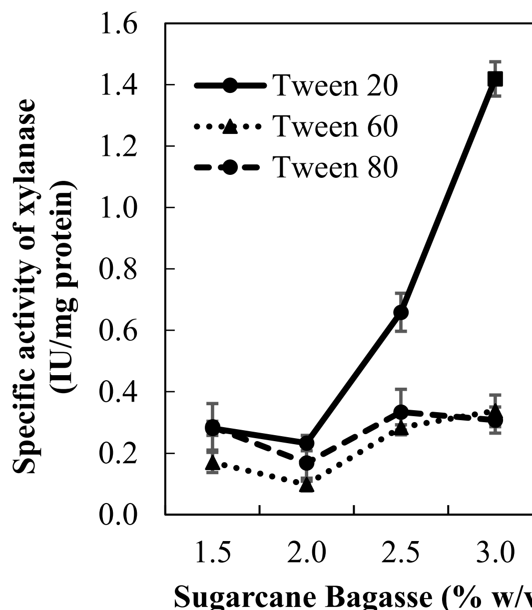

Figure 5. Specific Activity of Crude Xylanase (IU/mg) using Sugarcane Bagasse as Substrate.

3.3. Non-Parametric Statistical Analysis

From the 48 observations conducted, a wide variation in enzyme activity was observed among treatment combinations. The highest enzyme activity was recorded with palm kernel cake at 3.0% combined with Tween 20 (median = 1.9113), while the lowest activity was found with palm kernel cake at 2.0% combined with Tween 80 (median = 0.0703).

a. Effect of Single Factors

i) Substrate Type

The Kruskal–Wallis test showed that substrate type did not significantly affect enzyme activity (H = 1.4800, p = 0.224). Although palm kernel cake exhibited a slightly higher median enzyme activity (0.4474 IU/mg) compared to sugarcane bagasse (0.3065 IU/mg), the difference was not statistically significant.

ii) Substrate Concentration

Substrate concentration significantly influenced enzyme activity (H = 10.3852, p = 0.016). Post-hoc analysis identified a significant difference between 1.5% and 3.0% concentrations (p = 0.031). A trend of

increasing enzyme activity was observed with higher substrate concentrations, with 3.0% producing the highest median activity (0.4727 IU/mg).

iii) Surfactant Type

Surfactant type had the most significant effect ($H = 25.4923$, $p < 0.001$). All surfactant pairs showed significant differences: Tween 20 vs Tween 60 ($p = 0.026$), Tween 20 vs Tween 80 ($p < 0.001$), and Tween 60 vs Tween 80 ($p < 0.001$). Tween 20 resulted in the highest enzyme activity (median = 0.6286 IU/mg), followed by Tween 60 (0.4200 IU/mg) and Tween 80 (0.1183 IU/mg).

b. Interaction Analysis

Two-factor interaction analysis revealed:

- Substrate × Concentration: not significant ($p = 0.097$)
- Substrate × Surfactant: significant ($p < 0.001$)
- Concentration × Surfactant: significant ($p < 0.001$)

The significant interactions between substrate and surfactant as well as concentration and surfactant indicate that the effect of surfactants depends on both substrate type and concentration.

The five treatment combinations with the highest enzyme activity were:

1. Palm kernel cake 3.0% + Tween 20 (1.9113 IU/mg)
2. Sugarcane bagasse 3.0% + Tween 20 (1.4225 IU/mg)
3. Sugarcane bagasse 2.5% + Tween 20 (1.1146 IU/mg)
4. Palm kernel cake 2.0% + Tween 20 (0.6068 IU/mg)
5. Palm kernel cake 1.5% + Tween 20 (0.5818 IU/mg)

All top-performing combinations involved Tween 20, reinforcing the superiority of this surfactant in enhancing enzyme activity.

The lack of significant effect from substrate type may be attributed to the relatively similar nutritional composition of sugarcane bagasse and palm kernel cake as agro-industrial residues. Both contain cellulose and hemicellulose, which microorganisms can utilize for growth and enzyme production. The significant influence of substrate concentration aligns with enzyme kinetics theory, where increasing substrate concentration enhances enzymatic reaction rates until saturation is reached. The 3.0% concentration appears to approach optimal conditions in this study. The superiority of Tween 20 can be explained by its molecular structure, which is better suited for the fermentation system under investigation. Tween 20 has a shorter fatty acid chain compared to Tween 60 and Tween 80, enabling it to more effectively reduce surface tension and improve substrate bioavailability. The significant interactions among factors highlight the importance of simultaneously optimizing multiple parameters to achieve optimal fermentation conditions.

4. CONCLUSION

The concentration of substrate significantly influenced the specific activity of crude xylanase enzyme produced by *Aspergillus niger*, while the type of substrate alone had no significant effect. The highest specific activity of crude xylanase was achieved at a substrate concentration of 3% (w/v) palm kernel cake supplemented with Tween 20, resulting in a value of 4.380 ± 0.052 IU/mg protein. The type of surfactant also played a crucial role, with Tween 20 being the most effective in enhancing the specific activity of crude xylanase compared to Tween 60 and Tween 80.

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