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Optimization of Ph, Temperature, and Temporal Dynamics for Sustainable Bio-Protein Production from Lignocellulosic Sawdust Using *Aspergillus Niger*

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Abstract

Aspergillus niger serves as a bio factory of numerous active secondary metabolites with valuable applications in industry and biotechnology. This study focuses on isolating active strains for bio-protein production using low-cost sawdust substrates. Twenty-one fungi were isolated from the sawdust of four wood types: *Gmelina arborea, Sacoglottis gabonensis, Anogeissus leiocarpus*, and *Terminalia superba, comprising Aspergillus niger* (69%), *Trichoderma harzianum* (14%), *Fusarium oxysporum* (9.5%), and *Rhizopus stolonifer* (9.5%). The isolates were identified through conventional methods. The sawdust was thermally pretreated at 121°C (15 psi) for 1 hour and inoculated with two agar plugs (6 mm) from a 5-day-old culture of *A. niger*. Optimization studies were conducted to assess the effects of pH (4.0-7.0), temperature (28-40°C), and incubation period (6-14 days) in solid-state bioprocessing (SSB). Results indicated that bio-protein production ranged from 103-590 mg/L, with significant differences ($P<0.05$). Optimal bio-protein production occurred at pH 5.0, 35°C, and on day 10 of incubation. Among the substrates, sawdust from *G. arborea* yielded the highest bio-protein, followed by *A. leiocarpus*. These results highlight the potential of utilizing sawdust, an abundant lignocellulosic waste in Nigeria, for sustainable bio-protein production via SSB with *A. niger*, offering a practical approach to waste management and industrial biotechnology.

Keywords: Aspergillus Niger; Bio-Protein Production; Optimization; Sawdust Substrates; Solid-State Bioprocessing (SSB)

Abbreviations: SSB: Solid-State Bioprocessing; SSF: Solid-State Fermentation; GRAS: Generally Recognized as Safe; PDA: Potato Dextrose Agar; TSP: Total Soluble Protein

Introduction

Fungi, including *Aspergillus niger*, *Trichoderma harzianum*, *Fusarium oxysporum*, and *Rhizopus stolonifer*, are extensively studied for their ecological significance and industrial potential. Understanding the distribution, metabolic versatility, and growth characteristics of these fungi is essential for leveraging their biotechnological applications. Among them, *A. niger* is particularly notable for its ability to produce a wide range of bioactive secondary metabolites (SMs) with significant roles in agriculture, food, and pharmaceuticals. Filamentous fungi, such as *A. niger*, are widely used in industrial applications due to their ability to produce high yields of enzymes and other valuable compounds. *A. niger* is a key microorganism in solid-state fermentation (SSF), particularly for its production of industrially relevant enzymes like amylases. Its ability to penetrate solid substrates and tolerate low water availability makes it well-suited for SSF [2,3]. While traditionally, α-amylase production relied on submerged fermentation (SmF), recent advancements favor SSF due to its advantages, including low energy requirements, higher volumetric productivity, and minimal waste generation [2-4]. Over the decades, *A. niger* has been employed for the large-scale production of compounds such as citric acid, proteases, vitamins, and bioactive molecules used in various industries [5,1,6].

As a species generally recognized as safe (GRAS) by the U.S. Food and Drug Administration, *A. niger* offers a sustainable approach to industrial bioprocessing. It can utilize low-cost agro-industrial waste, such as rice bran, corn stalks, potato peels, cassava peels, and sawdust, thereby reducing production costs and mitigating environmental challenges [7,8]. The adaptive nature of *A. niger* allows it to thrive in diverse environments-soil, oceans, plants, and animal habitats due to its ability to metabolize various carbon sources and adjust to different growth conditions (Toghueo et al., 2018). Key environmental parameters such as pH, temperature, fermentation duration, and substrate composition significantly influence fungal growth and metabolite production. Research shows that *A. niger* grows optimally in acidic conditions, with a pH range of 2.5-3.5, and at temperatures of 30-35°C [9]. In addition, the duration of fermentation is critical for maximizing the yield and quality of bioactive compounds [10]. Reports of declining discoveries of novel bioactive compounds from *A. niger* emphasize the importance of optimizing fermentation conditions and exploring new strains [1].

Sawdust, a lignocellulosic biomass abundant in countries like Nigeria, offers a promising substrate for sustainable bioprocessing. Derived from the timber industry, sawdust is rich in cellulose and lignin but resistant to microbial degradation. Through hydrolytic enzymes such as cellulase and ligninase, *A. niger* can break down these complex polymers into simpler, valuable bioactive products [11]. This approach addresses dual challenges: reducing environmental pollution from improper waste disposal and providing a low-cost material for bioprocessing. Solid-state bioprocessing (SSB) is particularly effective for utilizing lignocellulosic substrates, as it mimics the natural environment of filamentous fungi, enhancing microbial-substrate interactions and enzyme productivity while minimizing energy requirements compared to liquid fermentation methods [12]. Using agro-industrial residues like sawdust aligns with environmentally friendly practices and offers economic advantages by producing industrial enzymes and bio-proteins sustainably.

Given the rising global demand for cost-effective bio-products, microbial fermentation, especially with fungi, has emerged as a promising solution. Fungal enzymes now dominate the industrial enzyme market, with filamentous fungi like *A. niger* excelling in production efficiency and downstream processing [13]. This research aims to optimize key parameters-pH, temperature, fermentation time, and substrate type for producing bio-proteins using *A. niger* and sawdust from four tropical wood species. The findings will contribute to scalable, sustainable solutions for industrial bioprocessing, addressing environmental and economic challenges posed by agro-industrial waste while meeting the growing demand for high-quality bio-products.

Materials and Methods

Sample collection and pretreatment

The wood shavings of *Gmelina arborea*, Sacoglottis gabonensis, *Anogeissus leiocarpus*, and Terminalia superba were obtained from Bodija plank market, Ibadan North Municipality Area, Oyo State, Nigeria. The samples were sun-dried to minimise moisture content and crushed to sawdust using a motorised grinding machine [14]. The sawdust was oven-dried at 60°C to a constant weight and sieved through a 2.0 mm wire mesh to create particles of uniform size for oxygen diffusion, nutritional absorption, and assimilation by fungal mycelia [15]. The sawdust samples were stored in airtight, clean plastic containers until use. Twenty-five grams of each sample were poured into duplicate Erlenmeyer flasks (250 mL) and wet with 75 mL of distilled water [16]. Samples were thermally processed by autoclaving at 121°C (15 psi) for 1 hour to make the wood components more susceptible to hydrolysis by the fungal enzyme [17].

Isolation and identification of fungi

The fungi were isolated using the sawdust baiting approach, as reported by [18]. Initially, 200 g of soil was collected from a dump site in the wood market at a depth of 20 cm and placed into a plastic container. Subsequently, 20 g of the soil sample was mixed with 50 g of sawdust in a plastic container and watered every 3 days with 10 mL of sterile water to prevent dryness. The mixture was then incubated for 7 days. Afterward, 10 g of the mixture was transferred to a 250-mL Erlenmeyer flask with 100 mL of sterile water and agitated at 150 rpm for 2 hours to create a stock solution. The resulting suspension was subjected to 10-fold consecutive dilutions and 100 µL of the 10^{-4} dilution was inoculated on Potato Dextrose Agar (PDA Lab M) supplemented with streptomycin sulphate (0.4 mg/mL) in 9 cm Petri plates. Incubation was carried out at 28°C for 5-7 days [19]. The isolates were then sub-cultured by transferring actively growing mycelia near the edge of the culture onto fresh, sterile PDA plates using an inoculating needle. Pure cultures were maintained on PDA slants at 4°C for future use. Furthermore, the fungal cultures at 5 days old were thoroughly examined for both cultural and morphological features. The pure isolates of the fungus were identified using the standard approach and compared with the compendium of soil fungi [20].

Preparation of growth medium

The nutritional media for cellulase synthesis, as modified by [21], was used as the moistening medium. The nutritional medium included the following (g/L): KH_2PO_4 2.0; $(NH_4)_2SO_4$ 1.4; Urea 0.3; $MnSO_4.7H_2O$ 0.0016; $ZnSO_4.7H_2O$ 0.0014; $CaCl_2$ 0.3; $FeSO_4.7H_2O$ 0.005; and yeast extract 0.1 [22]. Thereafter, 25 g of sawdust was soaked with 15 mL of the medium in 250 mL Erlenmeyer flasks and sterilised at 121°C (15 psi) for 15 minutes.

Solid-state bioprocessing (SSB)

Solid-state bioprocessing was carried out aerobically in sterile Erlenmeyer flasks. The substrates were placed in the flasks, and two agar plugs of 5-day-old cultures of *Aspergillus niger* were inserted into each flask using a 6 mm cork borer. The selection of this isolate as a starter for producing total soluble protein (TSP) was based on the frequency of its isolation from the substrates. The fermentation process was studied for six days at 30°C. To

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serve as a control, a 250-mL Erlenmeyer flask filled with sawdust was wet with medium and incubated without inoculation.

Optimization of culture conditions for total soluble protein production

The optimization process was carried out using a one-factor-at-a-time approach. The pH of the moistening medium was adjusted with 0.1 M HCl to various pH values of 4.0, 5.0, 6.0, and 7.0. Each medium was dispensed into separate 250 mL Erlenmeyer flasks containing the substrate before being sterilized. The sample was set at 60% moisture content and then incubated at 30°C. The effect of temperature was determined by adjusting the medium to the optimum pH and incubating the contents of 250 mL Erlenmeyer flasks at 28°C, 35°C, and 40°C. The effect of incubation time was determined by varying the period of incubation for 6, 10, and 14 days. All experiments were conducted using duplicate Erlenmeyer flasks to ensure accuracy [17].

Total soluble protein determination

Fifty mL of cold 0.05M sodium phosphate buffer (pH 7.0) was added to each fermentation flask (1:2) and agitated vigorously for 10 minutes. The broth was then filtered with 90-mm Whatman filter paper No. 1, and the filtrate was stored at 4°C. Total soluble proteins were determined by the method described by [23]. The reaction mixture consisted of 0.1 mL of soluble protein, 0.5 mL of sterile distilled water, and 3 mL (2% Na_2CO_3 in 0.1 M NaOH and 1.0 mL of 0.5% $CuSO_4.5H_2O$ in 1% sodium potassium tartrate) incubated for 10 minutes at room temperature. Folin-Ciocalteu reagent (BDH) (0.3 mL) was added and incubated for 30 minutes. The optical density (O.D.) was determined at 670 nm using a spectrophotometer (Uniscope 23D), and the protein content was extrapolated from a standard curve using egg albumin (BDH) [24].

Statistical analysis

Results obtained from the study were subjected to analysis of variance using one-way ANOVA, and differences between means of test samples were separated by the Duncan Multiple Range Test [25].

Results

In the study, twenty-one fungi comprising of four genera, namely, *Aspergillus niger*, *Trichoderma harzianum, Fusarium oxysporum*, and *Rhizopus stolonifer,* were isolated from the sawdust of the different woods. *Aspergillus niger* had the highest percentage occurrence of 69%, followed by *Trichoderma harzianum* with 14%, while *Fusarium oxysporum* and *Rhizopus stolonifer* both had 9.5% (Figure 1). The frequency of isolates probably reflected their ability to colonise and metabolise the substrates. This informed the decision to choose *A. niger* as a starter in solid-state bioprocessing for bioprotien production. (Plate 1A-D) presents *T. harzianum*, *A. niger*, F. oxysporum and R. stolonifer respectively on PDA. The distribution of fungal isolates from the different sawdust is presented in Figure 2. The highest isolation of *A. niger* were from *G. arborea* and *A. leiocarpus* with 86% and 78%, respectively (Figure 2).

(Figure 3) displays the results of the initial bio-protein production prior to optimizing fermentation conditions. The amount of bio-protein produced varied between 102 and 131 mg/L. (Figure 4-6) presents the effect of pH on bio-protein production by *A. niger* on different days of incubation. The result shows that production of bio-protein was high on *A. leiocarpus* and *S. gabonensis* at pH 7.0, with 321 mg/L and 269 mg/L, respectively on day 6. The highest bio-protein production in the study was obtained at pH 5.0 from *G. arborea* followed by *A. leiocarpus* with peaks of 590 mg/L and 365 mg/L, respectively on day 10. The production of bio-protein on day 14 was low and ranged from 131 mg/L-217 mg/L. There was significant difference in bio-protein production at the different pH ($P<0.05$).

(Figure 7-9) presents effect of variation of temperature on bio-protein production by *A. niger* on different days of incubation. The production of bio-protein ranged from 102 mg/L-357 mg/L. Bio-protein yield was high on *A. leiocarpus* at 35°C with 314 mg/L on day 6. The best bio-protein yield was obtained at 35°C from *G. arborea* and *A. leiocarpus* with 357 mg/L and 350 mg/L, respectively on day 10. The production of bio-protein declined on day 14. There was significant difference in bio-protein production at the different temperatures ($P<0.05$). (Figure 10) illustrates the impact of the incubation period on bio-protein production, with the highest production occurring on day 10.

Discussion

This study emphasizes the versatility of *Aspergillus niger* as a bio factory for producing valuable secondary metabolites, particularly bio-proteins, using low-cost, sustainable substrates like sawdust. The isolation of 21 fungal species from the sawdust of four wood types: *Gmelina arborea*, Sacoglottis gabonensis, *Anogeissus leiocarpus*, and Terminalia superba-reveals the diverse mycobiota inherent in these substrates. Notably, *A. niger* dominated the fungal population with a 69% occurrence rate, demonstrating its robust adaptability and effectiveness in colonizing various substrates. The dominance of *A. niger* in *G. arborea* and *A. leiocarpus* likely results from specific nutrients in these substrates that promote its growth. This observation aligns with existing literature highlighting *A. niger*'s effectiveness in industrial applications, particularly solid-state bioprocessing (SSB) [8,26].

Preliminary experiments established a baseline for bio-protein production, where *A. niger* produced 102-131 mg/L of bio-proteins at pH 3.0 and 30°C on the sixth day of incubation. Subsequent optimization significantly improved production, revealing that pH, temperature, and incubation time are critical factors. *A. leiocarpus* and *S. gabonensis* sawdust supported high bio-protein yields at pH 7.0, corresponding with optimal

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growth conditions for many fungi. However, maximum yields were observed at pH 5.0, with *G. arborea* producing 590 mg/L and *A. leiocarpus* producing 365 mg/L, suggesting slightly acidic conditions enhance metabolic activity and bio-protein synthesis. This agrees with earlier studies reporting optimal pH of 4.0-5.0 for crude protein production in solid-state fermentation [27,3,28]. Furthermore, the acidic pH range (2-6) has been proven to support *A. niger*'s enzymatic activity and biomass production, as confirmed by [29]. and others. This supports the general notion that fermentation alters substrate pH, moisture, and other parameters, making wood components more susceptible to enzymatic breakdown of more fermentable components into degradation products [30,31].

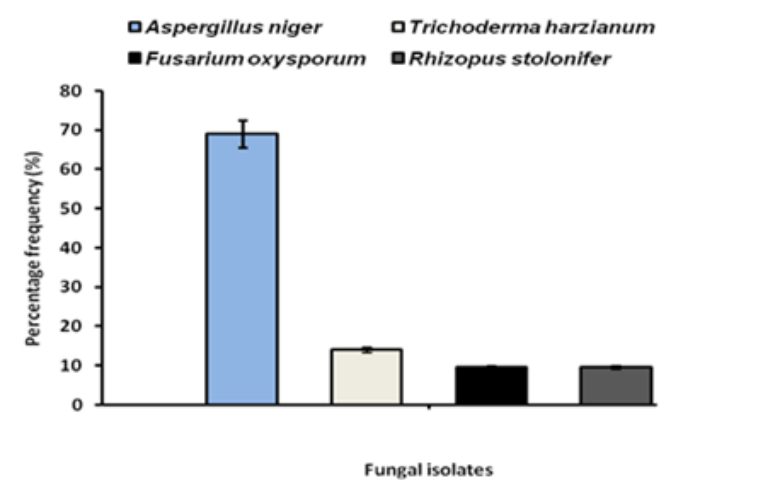

Figure 1: Frequency of occurrence of fungal isolates from the sawdust samples.

Temperature also played a pivotal role, with the highest bio-protein yields recorded at 35°C on the 10th day for *G. arborea* (357 mg/L) and *A. leiocarpus* (350 mg/L). This finding corroborates [32], who identified 35°C as optimal for *A. niger* fermentation of rice bran and attributed it to enhanced enzymatic activities. Although earlier studies reported optimal temperatures ranging from 30-35°C [33,3], variations may result from differences in substrate composition and fungal strains. These results suggest moderate temperatures favor enzymatic efficiency, metabolic rates, and protein synthesis [34]. Temporal dynamics further influenced bio-protein production, peaking on the 10th day before declining on day 14. This pattern aligns with [32], suggesting nutrient uptake kinetics and developmental phases influence production [35]. also stated that differing nutrient levels can trigger diverse physiological responses that alter uptake kinetics and growth. Thus, during the exponential growth phase, *A. niger* synthesized extracellular enzymes, increasing nutrient accessibility and bio-protein production [13,30]. The developmental lag phase preceding peak production likely reflects fungal adaptation and biomass buildup before optimal synthesis begins [35,36].

Variations in bio-protein yield across substrates indicate the significance of lignocellulosic composition and fermentation parameters in optimizing production. The high yields from *G. arborea* and *A. leiocarpus* sawdust suggest these substrates possess favorable lignin and cellulose contents that enhance enzymatic breakdown and protein synthesis. These findings are consistent with studies highlighting the influence of substrate type on fungal metabolism and secondary metabolite production [37,38]. Furthermore, the results highlight the potential for *G. arborea* and *A. leiocarpus* as superior substrates for bio-protein production under the tested conditions.

The use of sawdust as a substrate provides a cost-effective medium for bio-protein production and also addresses environmental concerns by utilizing lignocellulosic waste, which is abundantly available in Nigeria. The significant bio-protein yields from *G. arborea* and *A. leiocarpus* under optimized conditions demonstrate the feasibility of this approach for large-scale applications. This is likely due to their specific lignin and cellulose content, which may be more readily broken down by *A. niger* enzymes, thus enhancing protein synthesis. The significant differences ($P<0.05$) in bio-protein yields across different conditions also suggest the importance of optimization in maximizing production. This study provides a framework for integrating biotechnological innovation with waste management to enhance food security, industrial efficiency, and economic growth.

Future Research Directions

Future studies should explore the scalability of this process, focusing on the economic feasibility of large-scale bio-protein production using sawdust. Additionally, further research could investigate the genetic manipulation of *A. niger* to enhance its bio-protein production capabilities, potentially leading to even higher yields. The use of other lignocellulosic wastes and

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the exploration of different fungal strains could also provide insights into optimizing the bioprocess for broader applications in industrial biotechnology. Finally, an in-depth analysis of the nutritional content of the produced bio-protein would be valuable for assessing its suitability for various applications, including as an animal feed supplement.

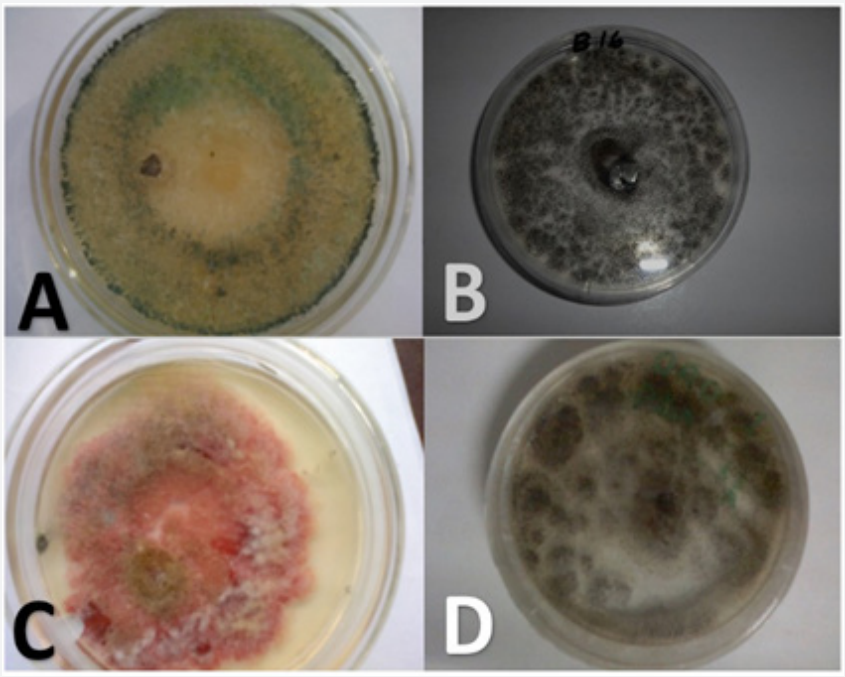

Plate 1A-D: Shows *Trichoderma harzianum, Aspergillus niger, Fusarium oxysporum and Rhizopus stolonifer* respectively, isolated from sawdust using PDA.

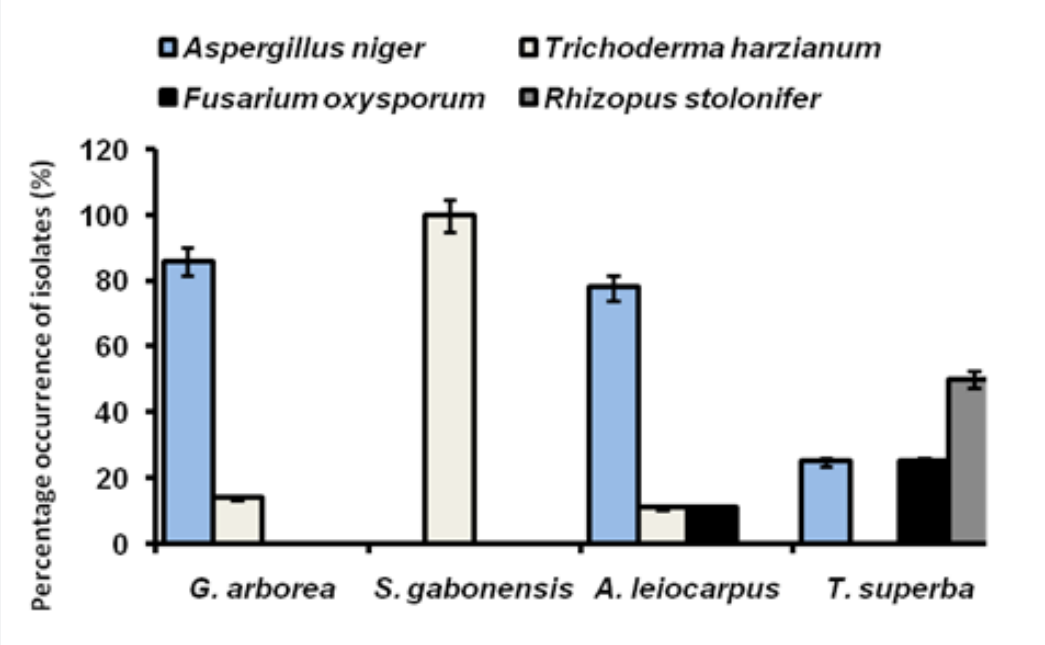

Figure 2: Diversity and distribution of fungal isolates from the different sawdust samples.

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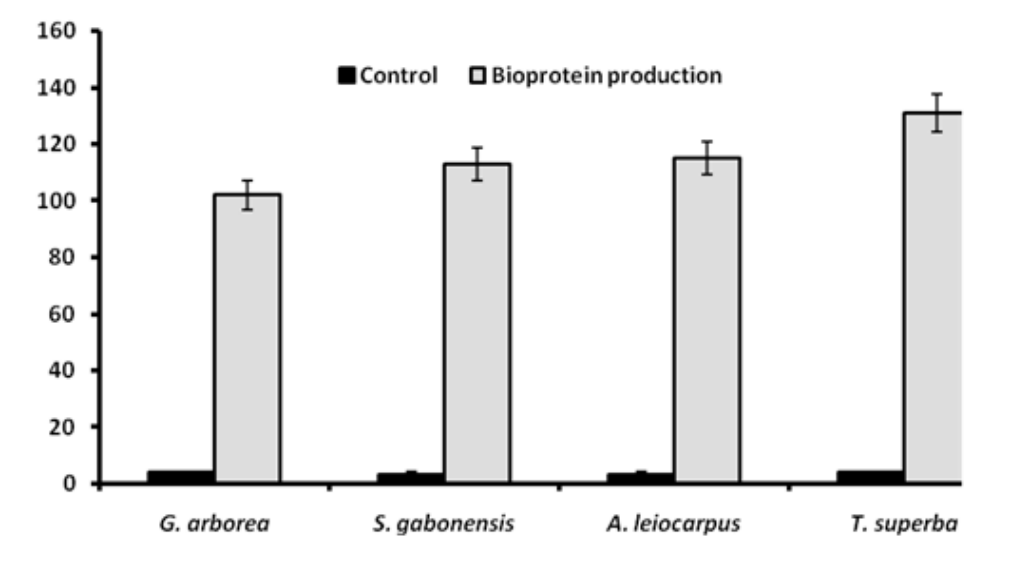

Figure 3: Initial bio-protein produced by *A. niger* before optimization of fermentation conditions the bar represents the standard error of duplicate determination.

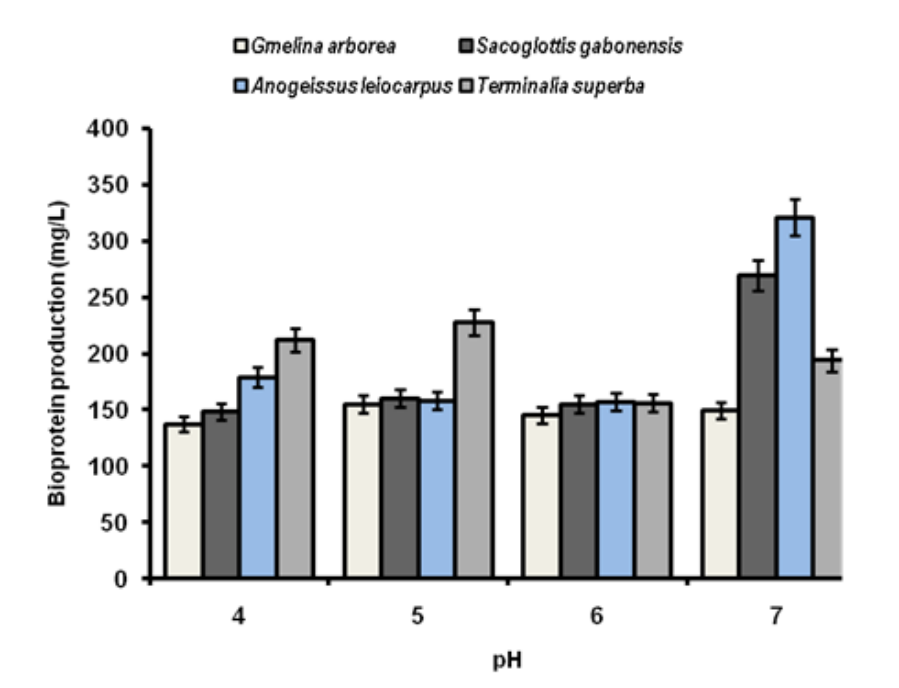

Figure 4: Effect of pH variation on bio-protein production by *A. niger* on different sawdust at day 6. The bar represents the standard error of duplicate determination.

Conclusion

This study demonstrates the potential of *Aspergillus niger* as an efficient bio factory for bio-protein production using low-cost sawdust substrates from tropical wood species. Among the 21 fungi isolated, *A. niger* dominated the fungal population, showcasing its adaptability and effectiveness in utilizing lignocellulosic biomass. Optimization experiments revealed that bio-protein production was significantly influenced by pH, temperature, and incubation time, with the highest yield of 590 mg/L achieved under optimal conditions: pH 5.0, 35°C, and a 10-day incubation period.

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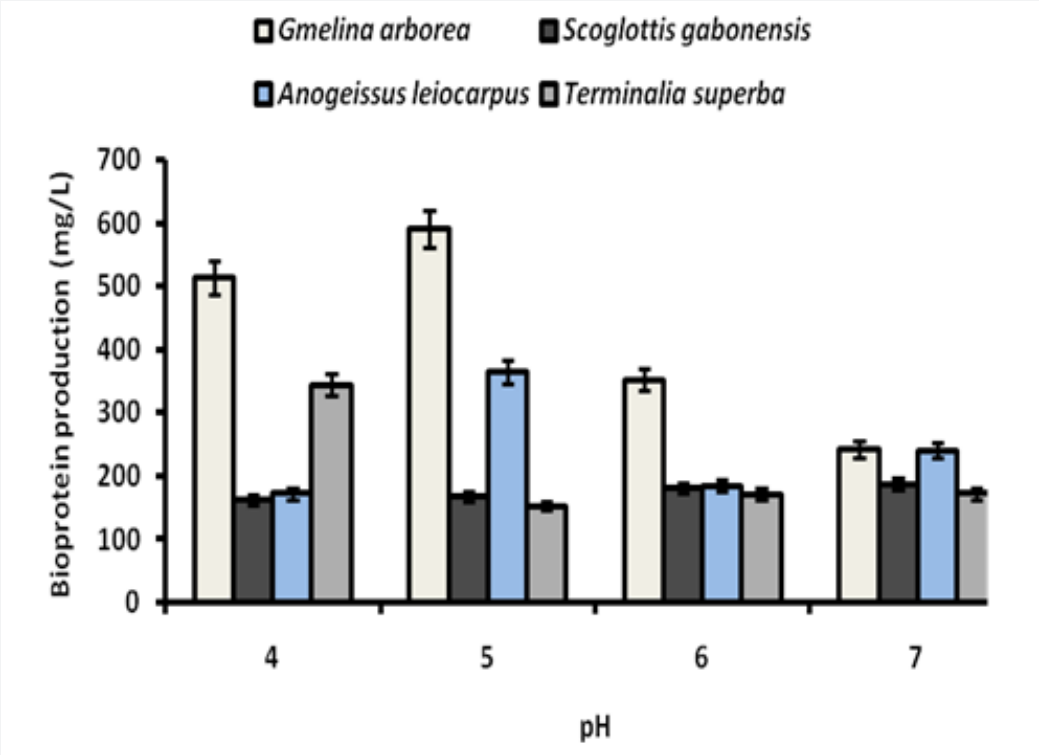

Figure 5: Effect of pH variation on bio-protein production by *A. niger* on different sawdust at day 10 The bar represents the standard error of duplicate determination.

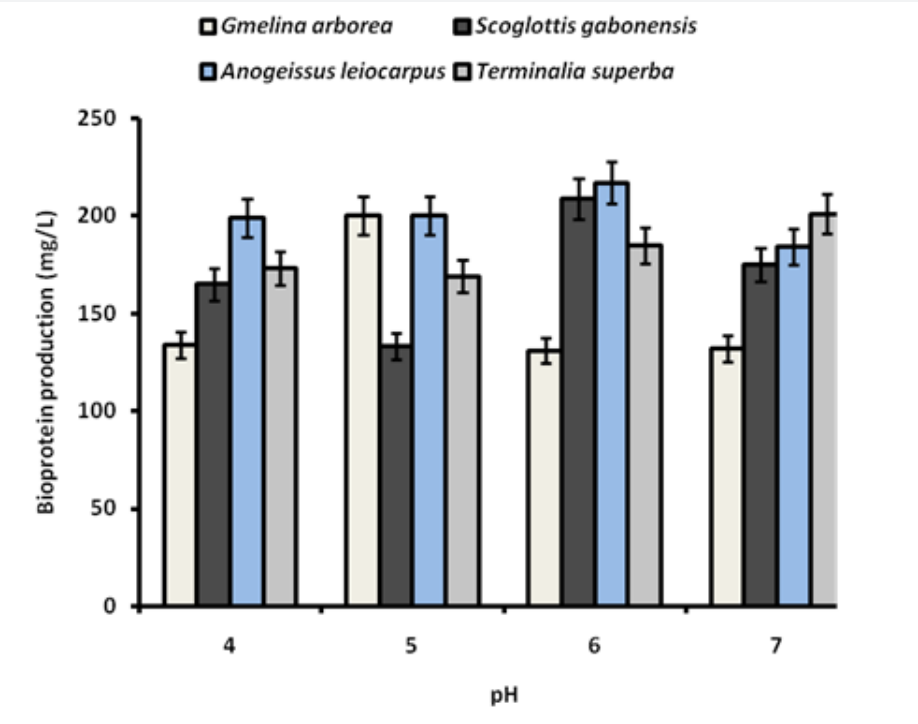

Figure 6: Effect of pH variation on bio-protein production by *A. niger* on different sawdust at day 14. The bar represents the standard error of duplicate determination.

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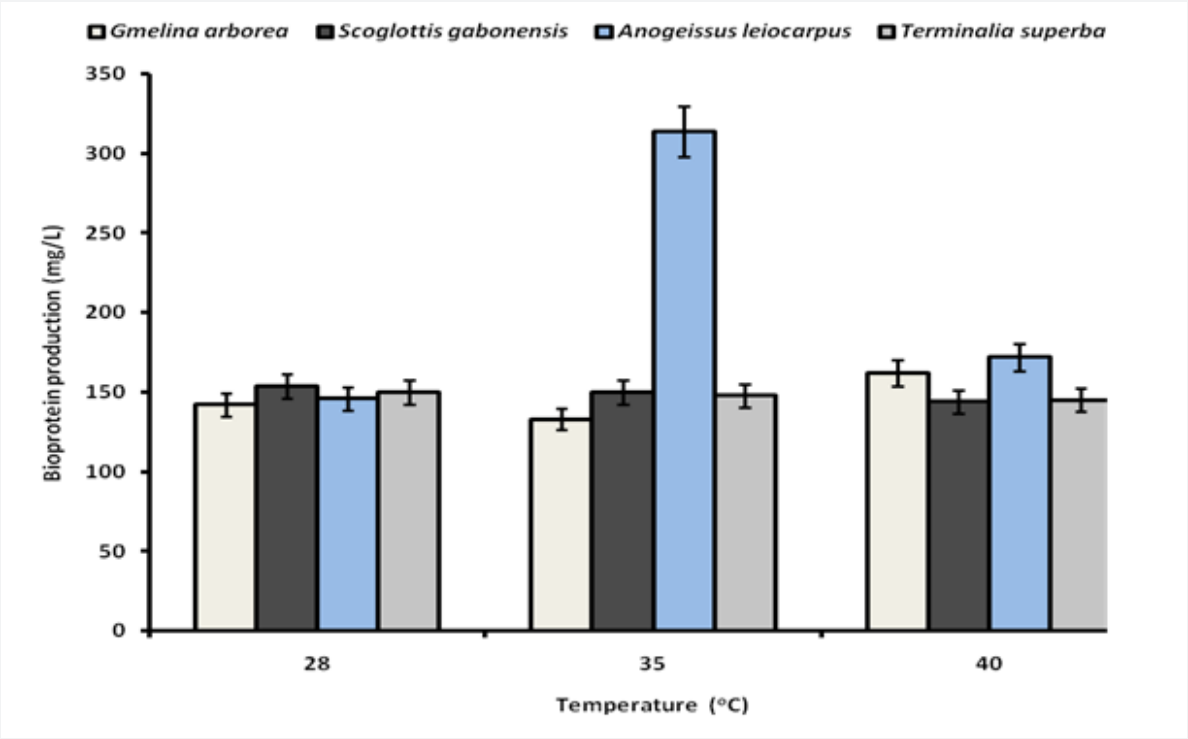

Figure 7: Effect of temperature variation on bio-protein production by *A. niger* on different sawdust at day 6. The bar represents the standard error of duplicate determination.

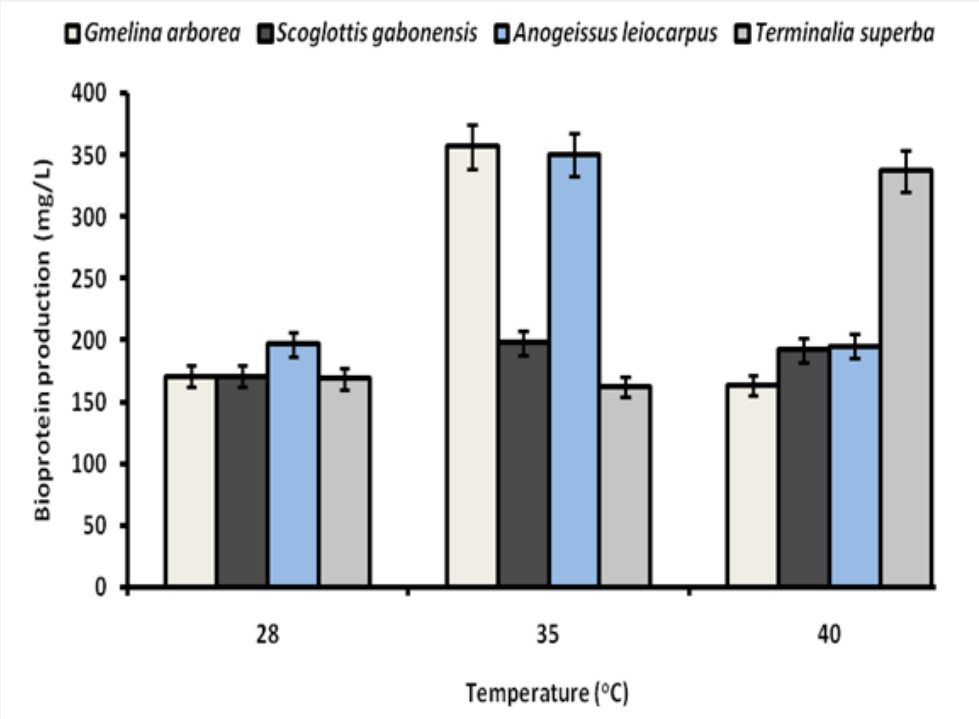

Figure 8: Effect of temperature variation on bio-protein production by *A. niger* on different sawdust at day 10. The bar represents the standard error of duplicate determination.

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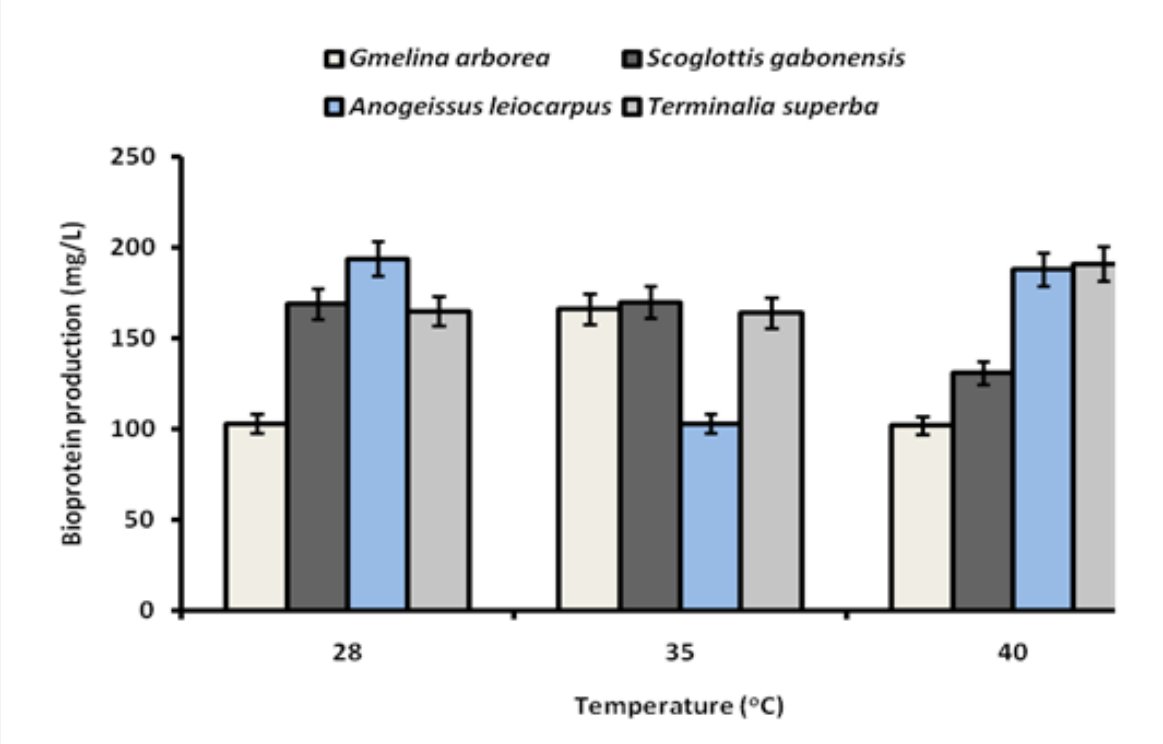

Figure 9: Effect of temperature variation on bio-protein production by *A. niger* on different sawdust at day 14. The bar represents the standard error of duplicate determination.

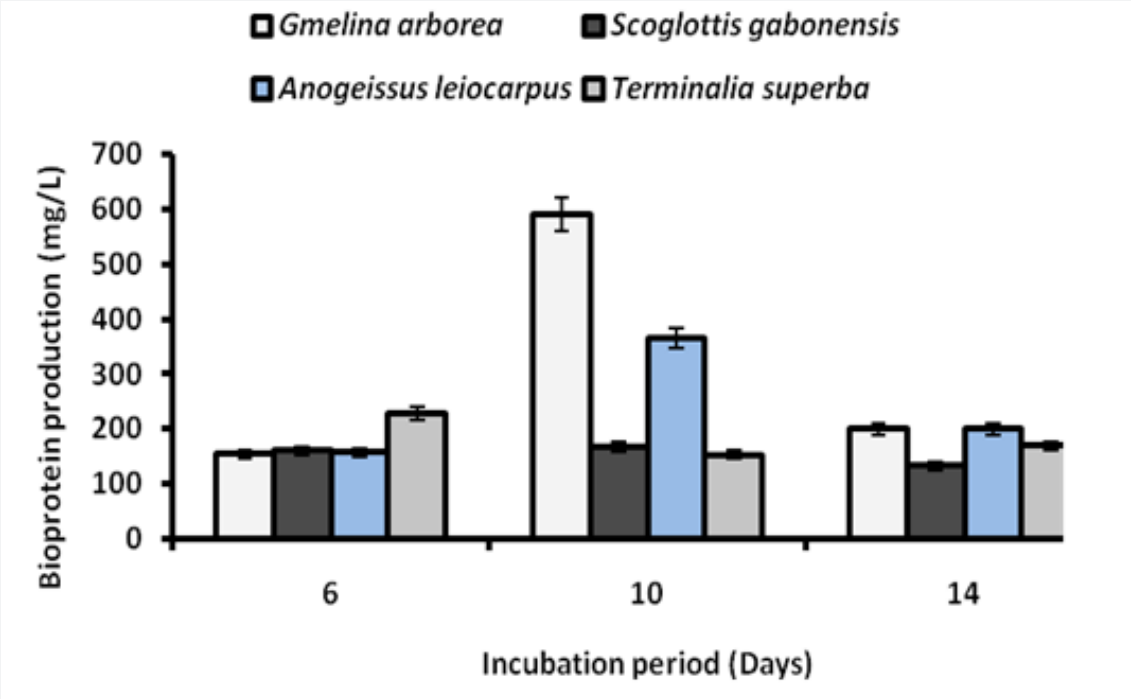

Figure 10: Effect of incubation period on bio-protein production by *A. niger* on the different days

The bar represents the standard error of duplicate determination.

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The use of *Gmelina arborea* and *Anogeissus leiocarpus* sawdust as substrates highlights their potential as sustainable and abundant resources for solid-state bioprocessing. This approach not only offers a cost-effective method for industrial bio-protein production but also contributes to environmental sustainability by repurposing lignocellulosic waste. The findings demonstrates the feasibility of integrating *A. niger*-based solid-state fermentation into scalable biotechnological applications, providing innovative solutions to waste management and the demand for sustainable bio-products in developing regions like Nigeria.

Conflict of Interest

The authors report there are no competing interests to declare.

Authors' Contributions

BVA conceived and designed the study. DDM did literature review. All the authors were involved in the write up, laboratory experiments, and statistical analysis; JIO revised the paper.

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