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BVA and PTA conceived and designed the study. JIO and UEA did literature review. All the authors were involved in the writeup, and statistical analysis; FCO and MNI revised the paper.

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Optimization of Bioprotein Production by Native *Trichoderma harzianum* using *Terminalia superba* Sawdust as Substrate

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Abstract:
Locally, there is an abundance of sawdust from *Terminalia superba*, which is a waste product of wood processing industries and is difficult to biodegrade. However, it has great potential as a feedstock for bioconversion to various value-added products using enzyme-producing filamentous fungi. In this study, isolated fungi were identified using the conventional method. The sawdust was thermally pretreated at 121°C (15 psi) for one hour and inoculated with two plugs (6 mm cork borer) of a 5-day-old culture of *Trichoderma harzianum*. To determine the effect of pH (4–7), temperature (28°C–40°C), and incubation period (6–14 days) on total bioprotein and biomass production, the experiment was conducted in Solid State Fermentation (SSF). The study found that bioprotein and biomass production ranged from 149–346 mg/L and 0.08–0.91 g, respectively, and was significantly different ($P<0.05$). The production of optimum bioprotein (346 mg/L) and biomass (0.91 g) was achieved at pH 6.0. The highest bioprotein and biomass were produced at 35°C. The best incubation periods for bioprotein and biomass production were days 10 and 6, respectively. This study's findings could be invaluable in optimizing the production of fungal biomass and proteins for industrial applications.

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INTRODUCTION

Terminalia superba is a tropical wood species of deciduous tree of the family Combretaceae that is widely spread in Central and West Africa (Orwa *et al.*, 2009). The hardwood is processed into guitar bodies, millwork, furniture or cabinets, and other wood products, which accumulate sawdust in the environment (Omoniyi and Adeniran, 2019). The accumulated sawdust is usually dumped indiscriminately into water bodies or burned, thus constituting major environmental problems in Nigeria (Buraimoh *et al.*, 2015).

T. superba, being a lignocellulosic biomass, consists of cellulose, hemicelluloses, and lignin, which is recalcitrant to bio-degradation (Yadav *et al.*, 2022). However, wood-digesting fungi, such as *Trichoderma* spp., allow access to valuable wood components by producing lignocellulosic enzymes and other metabolites (Sánchez-Corzo *et al.*, 2021). Filamentous fungi are adapted to growth on solid surfaces, where they exhibit different physiological characteristics (Echevarría, 2019a; Iqbal *et al.*, 2019; Musoni *et al.*, 2015). Most fungi produce and secrete higher biomass and metabolites with improved characteristics at a lower cost in solid-state fermentation (SSF) than in submerged fermentation (SmF) (Higuchi, 2021). Furthermore, SSF has a low environmental impact and can directly be applied to agro-industrial wastes without pretreatments. Thus, SSF is a promising approach to increasing the availability of bioproteins while using plant biomass as a substrate.

Trichoderma spp. is known for the production of many lignocellulosic enzymes and other secondary metabolites in agro-industrial wastes (Hamrouni *et al.*, 2020). Some of these bioproteins include cellulase and hemicellulase, which are beneficial in the textile and detergent industries (Ejaz *et al.*, 2021; Imran *et al.*, 2019). The enzyme laccases are applied in the clarification of fruit juice, textile dye bleaching, pulp bleaching, effluent detoxification, and biosensors (El-Gendi *et al.*, 2021). Besides, *Trichoderma* sp. produces a self-assembled amphipathic membrane called hydrophobin, which is applied in medical and technical areas, and conidia, which serve as biofungicides. In addition, they stimulate plant growth as biofertilizers, significantly reduce cultivation costs, and accelerate composting and bioremediation (Sood *et al.*, 2020). For efficacy, it is required that fungal strains for bioprotein production be obtained from the regions in which they are to be applied (Blaszczyk *et al.*, 2014). Another study stated that fungal biomass protein from *Trichoderma harzianum* has good nutritional value for supplementing poultry feed (Ahmed *et al.*, 2017).

The production of fungal biomass and bioprotein is, however, affected by growth conditions such as pH, temperature, moisture content, nutrient content, and incubation period. Typically, *Trichoderma* spp. grows in a pH range of 2.0–6.0, with acidic ambient pH being a major factor that regulates biomass production (Singh *et al.*, 2014). The optimum temperature range for biomass production by *Trichoderma* spp. is 25 °C–30 °C, although the maximum production of bioprotein depends on the characteristics of the strain (Chimata *et al.*, 2010; Singh *et al.*, 2014). Other authors suggest that the type of lignocellulosic substrate also has the greatest effect on the bioprotein produced (Cai *et al.*, 2021; Ganash *et al.*, 2021). This requires that several agro-industrial wastes be screened for specific bioprotein production. In selection, the cost and availability of the substrate and its ability to provide solid support for fungal growth are paramount for the high production of biomass and bioprotein. Furthermore, *T. superba* sawdust has been used as a substrate for bioprotein such as laccase production using *Trametes* sp. (Ado *et al.*, 2022). This provided an alternative to waste disposal, promoted biorefining of bioproteins into value-added products, and enhanced the circular economy by converting wastes into wealth. Therefore, this study aimed to determine the optimum growth conditions of wild *T. harzianum* for bioprotein production using *T. superba* sawdust as a substrate.

MATERIALS AND METHODS

Sample collection and pretreatment

Wood shavings of *Terminalia superba* were collected from Bodija plank market, Ibadan North Local Government Area, Oyo State, Nigeria. The wood shavings were washed, sun-dried to reduce moisture content, and crushed using a locally fabricated motorized grinding machine. The ground sawdust was further oven-dried at 60 °C to a constant weight. It was then sieved through a 2.0 mm wire mesh. Twenty-five grams of the pulverized wood shavings were dispensed in 250 mL Erlenmeyer flasks and moistened with 75 mL distilled water (Adenipekun and Fasidi, 2005). The substrate was then autoclaved at 121°C (15 psi) for 1 hour, as described previously (Adesina *et al.*, 2013). The treated substrate was stored in airtight, clean plastic containers until use.

Isolation and identification of fungi

The fungi were isolated using the sawdust baiting method as described previously (Tuli *et al.*, 2015). Briefly, 200 g of soil from a dump site in the plank market was collected at a depth of 20 cm into a plastic bag. Approximately 20 g of the soil sample was weighed into a plastic container and baited with 50 g of sawdust. The mixture was moistened every 3 days with 10 mL of sterile water to avoid drying and incubated for 7 days. Approximately 10 g of the mixture was weighed into a 250 mL Erlenmeyer flask containing 100 mL of sterile water and shaken at 150 rpm for 2 hours to prepare the stock solution. The suspension was subjected to 10-fold serial dilution and 100 µL of 10^{-4} dilution plated on Potato Dextrose Agar (PDA Lab M) supplemented with streptomycin sulfate (0.4 mg/mL) in 9 cm Petri dishes. Incubation was carried out at 28 °C for 5–7 days (Echevarría, 2019b; Hewedy *et al.*, 2020). The isolates were subcultured by transferring actively growing mycelia at the edge of the culture onto fresh, sterile PDA plates using an inoculating needle. Pure cultures were stored on PDA slants at 4 °C for future use. Fungal cultures at 5–days old were observed for both cultural and morphological characteristics. Pure isolates of the fungus were identified based on the conventional method and compared with the compendium of soil fungi (Domsch *et al.*, 1980; Echevarría, 2022; Echevarría and Iqbal, 2021).

Preparation of growth medium

The nutrient medium for cellulase production as modified previously (Chahal, 1985) was used as the moistening medium. The nutrient medium contained 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 (Singh *et al.*, 2017). Thereafter, 25 g sawdust was moistened with the medium (15 mL) in 250 mL Erlenmeyer flasks and autoclaved at 121°C (15 psi) for 15 minutes.

Solid-state fermentation

Solid-state fermentation was carried out aerobically in Erlenmeyer flasks containing the sterile substrate. Each flask was inoculated with two agar plugs of a 5-day-old culture of *T. harzianum* using a 6 mm cork borer (Ado *et al.*, 2018). The isolate was selected as a starter for producing bioprotein based on a literature search (Ahmed *et al.*, 2017). The process of fermentation was observed for a period of six days at a temperature of 30°C, as per the adopted method. A 250 mL Erlenmeyer flask filled with sawdust was moistened with medium and incubated without inoculation to serve as the control.

Optimization of culture conditions for total biomass and bioprotein production

The optimization 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. Fifteen mL of each medium was dispensed into separate 250 mL Erlenmeyer flasks containing the substrate before sterilizing. 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 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.

Total 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. Bioproteins were determined by the method described previously (Lowry *et al.*, 1951). 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) (Olson and Markwell, 2007).

Determination of total fungal biomass

The total biomass produced was determined by direct dry weight. The biomass was carefully scraped and dried on pre-weighed 90 mm Whatman filter paper No. 1 in an oven at 60°C until constant weight (Laffey and Butler, 2005).

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 (Duncan, 1955).

RESULTS

The study aimed to determine the optimum growth conditions of pH, temperature, and incubation time for bioprotein production by *Trichoderma harzianum*. Fungi belonging to three genera were isolated; namely, *A. niger*, *T. harzianum*, and *Rhizopus* sp. Based on a literature search, *T. harzianum* was selected as a starter to produce the bioprotein (Ahmed *et al*., 2017). Plate 1a-c shows *A. niger*, *Rhizopus* sp. and *T. harzianum* on a PDA plate. Prior to optimizing the growth conditions, *T. harzianum* produced 118 mg/L of bioprotein at pH 3 and 30°C on day 6 (Figure 1). The total bioprotein yield ranged from 149 mg/L–346 mg/L, with the highest bioprotein produced at pH 6.0 on day 10 (Figure 2). In the same manner, the biomass yield ranged from 0.03 g–0.91 g, with the highest biomass produced at pH 6 on day 10 (Figure 3). There were significant differences in the bioprotein and biomass produced at pH 6 ($P< 0.05$).

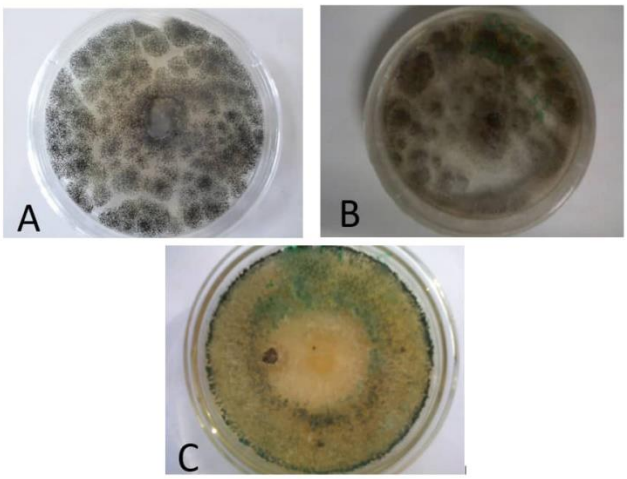

Plate 1 A-C: *Aspergillus niger*, *Rhizopus* sp., and *Trichoderma harzianum* isolated from sawdust using PDA.

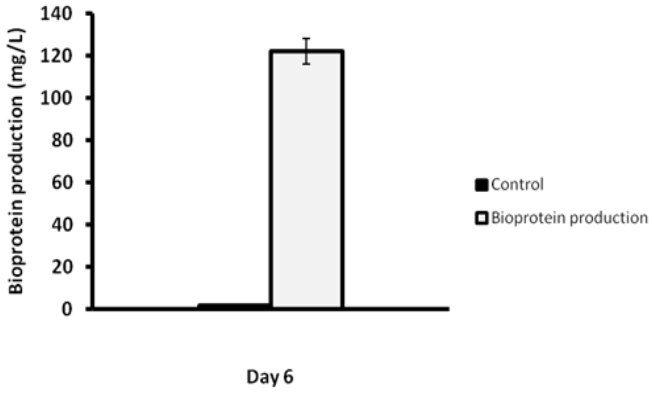

Fig. 1. Initial bioprotein produced by *T. harzianum* before optimization of fermentation conditions.
The bar represents the standard error of duplicate determination.

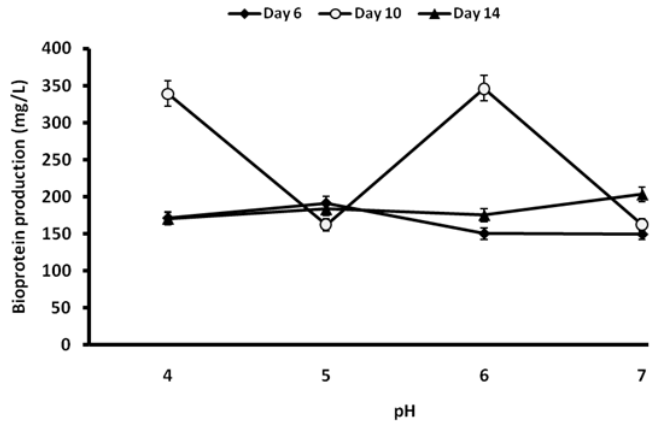

Fig. 2. Effect of initial pH on total bioprotein yield during *T. harzianum* fermentation of sawdust.
The bar represents the standard error of duplicate determination.

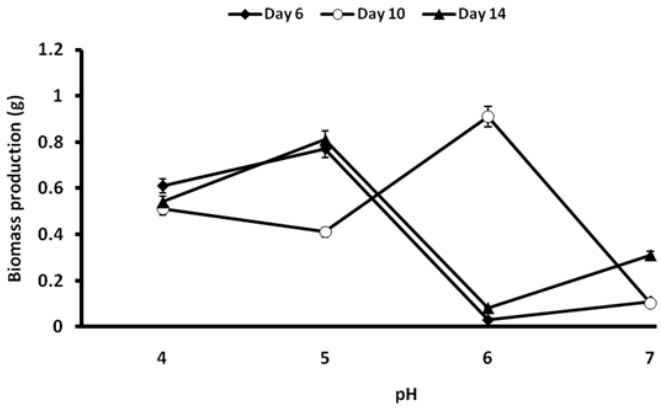

Fig. 3. Effect of initial pH on biomass yield during *T. harzianum* fermentation of sawdust.
The bar represents the standard error of duplicate determination.

Figure 4-5 presents the effect of temperature on bioprotein and biomass produced by *T. harzianum* on days 6 -14, respectively. The bioprotein yield ranged from 134 mg/L to 331 mg/L, with optimum production at 35 °C on day 10 (Figure 4). Total biomass produced ranged from 0.10 g-0.71 g, with an optimum yield at 35 °C on day 6. There were significant differences in the bioprotein and biomass produced at 35 °C ($P< 0.05$). The result showed that the maximum bioprotein produced on day 10 was preceded by optimum biomass production on day 6 (Figure 5). There were significant differences in the bioprotein and biomass produced at 35°C on the days of incubation ($P< 0.05$).

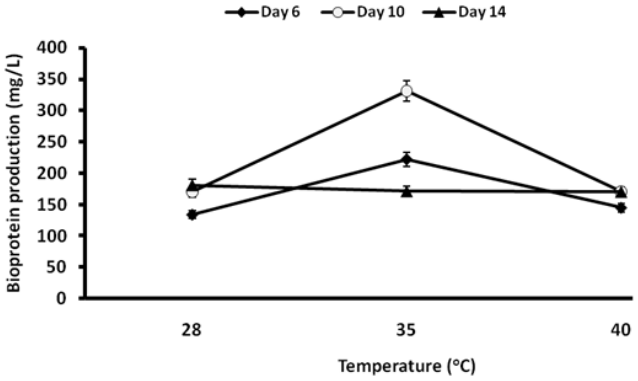

Fig. 4. Effect of initial temperature on bioprotein yield during *T. harzianum* fermentation of sawdust.
The bar represents the standard error of duplicate determination.

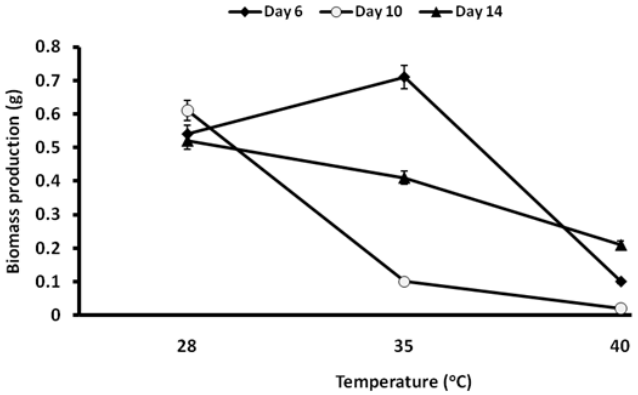

Fig. 5. Effect of initial temperature on biomass yield during *T. harzianum* fermentation of sawdust.
The bar represents the standard error of duplicate determination.

DISCUSSION

Trichoderma species are useful for producing bioactive secondary metabolites and other chemicals. The production of these products is regulated by growth conditions such as pH, temperature, incubation period, and substrate type. The main goal of this study was to find the best conditions for *Trichoderma harzianum* to produce bioproteins (BPs), with a focus on how pH, temperature, and incubation time affect this process. Our preliminary experiment established a baseline for bioprotein production, where *T. harzianum* was capable of generating 118 mg/L

of bioproteins at a pH of 3 and a temperature of 30 °C by the sixth day of incubation.

Through a systematic process of optimization, we were able to significantly increase bioprotein yield, reaching a peak at pH 6 on the tenth day. Similarly, the total biomass associated with this yield was measured at the same pH and day. This agrees with the report that *Trichoderma* spp. grow in a wide pH range of 2–6, with acidic ambient pH being a major factor that regulates biomass production (Singh *et al.*, 2014). In a separate study, the researchers found that the production of extracellular proteins increased as biomass production increased in the solid-state fermentation of *Aspergillus sojae* (Mora-Lugo *et al.,* 2015). Notably, these increments in bioprotein and biomass produced in this study were statistically significant ($p<0.05$) when compared with results obtained under other pH conditions.

The optimum temperature for biomass production by *Trichoderma* spp. is 25 °C –30 °C, although the maximum production of bioprotein depends on the characteristics of the strain (Chimata *et al.*, 2010; Singh *et al.*, 2014). In the current study, bioprotein and biomass yields were highest at 35 °C, probably due to the strain and type of substrate. In another study, the production of bioprotein by *T. viride* in SSF using cassava peels was highest at 30 °C (Ezekiel and Aworh, 2013), which is close to the peak of our study. Other authors suggest that the type of lignocellulosic substrate has the greatest effect on the bioprotein produced (Cai *et al.*, 2021; Ganash *et al.*, 2021; Immanuel *et al.*, 2007).

The optimization of temperature revealed that *T. harzianum* attained its maximal bioprotein yield at 35 °C, also on the tenth day of incubation. The peak of biomass production was observed slightly earlier, on day 6, at the same temperature. These findings were likewise statistically significant ($P<0.05$), underscoring the effect of temperature variations on both bioprotein and biomass production.

The observed increase in bioprotein and biomass outputs at a pH of 6.0 and a temperature of 35 °C imply that these parameters align more closely with the optimal conditions for *T. harzianum* growth and proliferation. This observation is in harmony with the existing body of literature that delineates a preference of *Trichoderma* species for a slightly acidic to neutral pH spectrum and mesophilic temperature ranges, which are known to foster their metabolic processes and biomass production (Andrzejak and Janowska, 2022; Singh *et al.*, 2015; Singh *et al.*, 2014).

The time gap between the highest production of biomass (day 6) and bioprotein (day 10) suggests that the fungus went through a developmental lag phase where it built up a critical mass of biomass before starting to synthesize bioproteins. This insight highlights the necessity for temporal synchronization of the growth and production phases to ensure optimized yields, a concept that is corroborated by other studies investigating the dynamics between microbial growth and metabolic production (Gonzalez and Aranda, 2023; Nev *et al.*, 2021).

There is a discernible interdependence between biomass accrual and subsequent bioprotein synthesis. The insight gleaned from this study not only broadens our comprehension of the metabolic characteristics of *T. harzianum* but also paves the way for potential applications of this fungus in biotechnological domains, encompassing bioprotein production and their deployment in sustainable agro-industrial practices.

CONCLUSION

Production of fungal biomass and bioprotein from *Terminalia superba* sawdust using wild-type *Trichoderma harzianum* was achieved. Optimum growth conditions of pH (6.0), temperature (35 °C), and incubation time (10 days) were found effective for maximum biomass and bioprotein production using *T. superba* as substrate. Furthermore, *T. superba* sawdust supports *T. harzianum*'s growth for bioprotein production in solid-state fermentation. The utilization of this substrate in bioprocessing will reduce environmental pollution associated with indiscriminate dumping or combusting of *T. superba* sawdust, and provide bioproteins for biotechnological applications.

CONFLICT OF INTEREST

Authors hereby declare that they have no conflict of interest.

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