
SCREENING AND IDENTIFICATION OF SIGNIFICANT PROCESS PARAMETERS FOR SOLID STATE FERMENTATION OF TRICHODERMA HARZIANUM

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ABSTRACT

Agro-industrial wastes have gained considerable attention as inexpensive and renewable lignocellulosic feedstock for the sustainable production of byproducts including bioherbicides, through solid-state fermentation (SSF), an efficient and environmentally friendly biotechnological process. However, maximizing fungal growth and sporulation requires the identification of the most influential process parameters prior to optimization. This study aimed to screen and identify the significant process parameters affecting the solid-state fermentation of *Trichoderma harzianum* cultivated on agro-industrial residues. Sugarcane bagasse and corncob were selected as the substrates due to their abundance, low cost, favourable physicochemical properties, and proven suitability for supporting the growth and sporulation of *Trichoderma harzianum* during solid state fermentation. A One-Factor-at-a-Time (OFAT) approach was employed to investigate the effects of substrate weight (5–25 g), moisture content (50–70%), particle size (75 μm –1.18 mm), initial pH (4–8), and inoculum concentration (10^{-3} – 10^{-8}) on conidial production. The fermentation was carried out under controlled incubation conditions, and conidial yield (CFU g⁻¹) was used as the response variable. The screening results demonstrated that all the investigated factors influenced fungal growth; however, moisture content, particle size, and initial pH exerted the greatest effects on conidial production. These three factors were therefore selected as the significant process parameters for solid-state fermentation of *Trichoderma harzianum*. The range of these parameters resulting in substantially higher conidial yields than the other treatment levels were determined to be approximately 55–60% moisture content, particle size around 150–300 μm , and pH 5.0–6.0. The findings provide a scientific basis for identification of and specification of range of values of parameters for optimization of SSF for large-scale production of *Trichoderma harzianum* as an environmentally friendly bioherbicide.

Keywords: Optimization; Solid State Fermentation; Agro-Industrial Waste; *Trichoderma harzianum*; Substrate; Sugarcane bagasse; Corncobs

1. INTRODUCTION

Weed infestation remains one of the major constraints to agricultural productivity worldwide and is particularly severe in Nigerian agriculture where it significantly reduces crop growth, yield and food production. Conventional weed management strategies have been reported to be unsustainable because of their extensive use, which has led to the development of herbicide-resistant weeds, adverse effects on human health, and environmental degradation, including biodiversity loss as well as soil and water pollution (Hasan *et al.*, 2022; Ortaça Portela *et al.*, 2024; Synowiec *et al.*, 2025). These concerns have prompted growing interest in exploring alternative weed management strategies based on natural products to promote environmental sustainability (Hasan *et al.*, 2022).

Bioherbicides are natural products or compounds derived from microorganisms or other natural sources that suppress weed growth with minimal adverse effects on the environment. They are commonly produced through either submerged fermentation (SmF) or solid-state fermentation (SSF). Among these, SSF has emerged as an efficient, environmentally friendly, and sustainable biotechnological process in which microorganisms such as fungi, bacteria, or yeasts grow on solid substrates in the absence or near absence of free water to produce valuable bioproducts. Compared with submerged fermentation, SSF offers several advantages, including lower water and energy requirements, higher product concentrations, and the efficient utilization of inexpensive agricultural

residues. The process generally involves substrate preparation, inoculation and fermentation, followed by the recovery and processing of the desired products (Yafetto 2022).

Agro-industrial wastes have gained considerable attention as inexpensive and renewable feedstock for the production of sustainable biproducts, primarily because of the lignocellulosic nature of many plant residues (Rasheed *et al.*, 2024). Among the most promising are rice husks, yam peels, cassava peels, sugarcane bagasse, banana *pseudostems*, coconut shells, corncobs, sawdust, among others. Their abundance, renewability, high carbohydrate content, and ease of preparation make them attractive substrates for supporting microbial growth during SSF (Naeimi *et al.*, 2020; Yafetto, 2022; C. Zhang *et al.*, 2022). These materials are predominantly composed of cellulose, hemicellulose, and lignin, which serve as carbon and energy sources for microorganisms and can be converted through fermentation into value-added products such as biofuels, bioethanol, biopesticides, protein-enriched products, antibiotics and other industrial bioproducts (Aruna, 2019; Awoyale *et al.*, 2021; Fadeyi *et al.*, 2020; Kaur *et al.*, 2025; Perwez & Al Asheh, 2025; Sadh *et al.*, 2018; C. Zhang *et al.*, 2022).

The suitability of agro-industrial waste for fungal growth and conidia production depends largely on their physicochemical and compositional properties. Consequently, comprehensive characterization of these materials is essential. Proximate analysis provides reliable essential information on the chemical composition of lignocellulosic biomass and is particularly valuable for the techno-economic assessment of biomass-based processes (Awoyale *et al.*, 2021). In addition to quantifying the major structural components; cellulose, hemicellulose, and lignin, it determines key parameters such as moisture content, ash content, dry matter, crude protein, crude fat, and pH, all of which significantly influence substrate performance, fungal growth, metabolite production, and conidia yield during solid-state fermentation. The cellulose contents of corncobs and sugarcane bagasse have been consistently reported to be comparable across studies. Awoyale *et al.*, (2021) reported cellulose contents of 38.6% for corncobs and 39.8% for sugarcane bagasse, while Rasheed *et al.*, (2024) reported slightly higher values of $40 \pm 0.32\%$ and $41 \pm 0.41\%$, respectively. Based on the screening of some selected agro-industrial residues, both studies concluded that corncobs and sugarcane bagasse are promising lignocellulosic feedstocks because of their favourable chemical composition, particularly their high cellulose content. Cellulose, the principal structural polysaccharide in lignocellulosic biomass, serves as an important carbon source for *Trichoderma harzianum*. Through the secretion of cellulolytic enzymes, the fungus hydrolyzes cellulose into fermentable sugars that support mycelial growth, sporulation (conidia production), and secondary metabolites synthesis during solid-state fermentation. Therefore, the high and comparable cellulose contents reported by Awoyale *et al.*, (2021) and Rasheed *et al.*, (2024) provide a scientific basis for selecting corncobs and sugarcane bagasse as suitable substrates for conidia production and optimizing other process parameters can further improve the effectiveness of SSF with this substrate mixture.

SSF, can be carried out using either a single substrate or a combination of two or more solid organic substrates to improve nutrient availability and fermentation performance (Ortaça Portela *et al.*, 2024). For instance, Mulatu *et al.*, (2021) demonstrated that a mixture of wheat bran and white rice (1:1, w/w) significantly enhanced the conidia production of *Trichoderma asperellum* and *Trichoderma longibrachiatum*. In addition to the choice of substrates, maximizing fungal growth and sporulation during solid state fermentation requires the identification of the most influential process parameters prior to optimization.

Although several studies have optimized SSF conditions for *Trichoderma harzianum* using individual lignocellulosic substrates, limited information is available on the combined use of corncobs and sugarcane bagasse as mixed substrates for enhancing conidia production and bioherbicidal metabolite synthesis. Therefore, this study aimed to screen corncobs and sugarcane bagasse as substrates and identify the significant process parameters affecting conidia production during the solid-state fermentation of *Trichoderma harzianum*.

2. METHODOLOGY

2.1 Materials

All chemicals used in this study were of analytical grade. The Sugarcane bagasse was obtained from a sugarcane juice vendor at Danfodio Hall, Ahmadu Bello University, Zaria, whereas

corncobs were sourced from a local farmer. The fungal strain used in this study was obtained from the Department of Crop Protection, Ahmadu Bello University, Zaria, Nigeria.

2.2 Pretreatment of Sugarcane Bagasse and Corncob

The substrates were thoroughly washed and sun-dried. They were subsequently air-dried before being chopped into smaller pieces and ground into coarse particles. The processed substrates were then oven-dried at 75°C for 2 hours to reduce their moisture content until a constant weight was attained, after which they were further milled using an electric grinder. Finally, following the procedure described by Narwade *et al.*, (2023), the ground materials were sieved using ASEW standard test sieves to obtain particle sizes of 75, 150, 250, 300, and 600 µm, as well as 1180 µm, and packaged in labelled Ziploc bags for subsequent use.

2.3 Spore Suspension Preparation and Serial Dilution

A spore suspension of *Trichoderma. harzianum* was prepared from a 7-day-old culture following the method described by Kaur *et al.* (2025) with slight adjustment. Conidia were harvested from the culture surface by gently scraping with a sterile spatula and transferring them into 10 mL of sterile distilled water. The resulting suspension was vortexed for 30 s to ensure uniform dispersion of the spores and subsequently filtered through sterile muslin cloth to remove mycelial fragments. Serial dilutions were then prepared and the spore concentration was determined using a hemocytometer (Neubauer chamber, Taufkirchen, Germany) under an Olympus BX41 phase-contrast microscope (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) at 40× magnification, as described by Pham *et al.* (2010).

For the serial dilution, eight sterile test tubes, each containing 9 ml of sterilized distilled water were labelled accordingly. One milliliter (1 mL) of the stock spore suspension was transferred aseptically into the first tube to obtain 10^{-1} dilution. Subsequently, 1 mL of the suspension from 10^{-1} dilution was transferred into the second tube to obtain 10^{-2} dilution. The same procedure was repeated subsequently to obtain 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , and 10^{-8} dilutions. The 10^{-5} dilution was used as the inoculum concentration for the subsequent experiments.

2.4 Solid-State Fermentation Procedure and Experimental Design

Processed corncob (CC) and sugarcane bagasse (SCB) were used individually and in combination as substrates for solid-state fermentation (SSF) of *Trichoderma. harzianum* to maximize conidia production. A summary of the experimental procedure is shown in Fig 1. Following incubation, conidia were harvested and quantified using a hemocytometer (Neubauer chamber, Taufkirchen, Germany) under an Olympus BX41 phase-contrast microscope (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) at 40× magnification, as described by Pham *et al.* (2010).

2.4.1 Substrate screening

To identify the most suitable substrate for enhanced conidia production by *Trichoderma*, different proportions of corncob (CC) and sugarcane bagasse (SCB) were evaluated. The substrate combinations tested were 100% SCB, 100% CC, 50:50 SCB: CC, 75:25 SCB: CC, and 25:75 SCB: CC. For each treatment, 20 g of a dry substrate was weighted into a 250 ml beaker and adjusted to 60% moisture content. The moistened substrates were autoclaved at 121°C for 15 min and allowed to cool to room temperature, before inoculation with 2 ml of spore suspension. The inoculated substrates were incubated at 25°C for 7 days. Following incubation, conidia were harvested and quantified. The substrate combination that produced the highest conidia yield was selected for subsequent screening of the critical growth factors using one-factor-at-time (OFAT) approach.

2.4.2 Identification of significant factors affecting spore production with their upper and lower levels using OFAT

OFAT experiments were conducted to evaluate the effects of key growth factors (GFs) on conidia production by *Trichoderma. Harzianum*. Five process variables were investigated: material

weight (MW, g: 5, 10, 15, 20, 25), pH (4 – 8), moisture content (MC, %: 50 – 70), particle size (PS, μm : 75 – 1180), and inoculum concentration (IC, dilution factors: 10^{-3} – 10^{-8}). Control conditions were set at 7-days of incubation at 25 °C and 60% MC. The selection of 60% moisture content and 25 °C as the initial control conditions was based on previously reported favourable conditions for the cultivation and conidia production of *Trichoderma harzianum* under solid-state fermentation (De Oliveira *et al.*, 2026; Heng & Hamzah, 2022). Spore counts were determined post-incubation. Based on the results, three factors with the highest impact on conidia yield were selected as SSF process parameters for further optimization via Response Surface Methodology (RSM), with experimental limits defined from the one-factor-at-a-time (OFAT) ranges.

The effect of inoculum concentration on conidial production was investigated using different inoculum concentrations prepared from the serially diluted spore suspension described in Section 2.3. The inoculum concentrations corresponding to serial dilution levels of 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , and 10^{-8} were evaluated. A dilution of 10^{-5} , was used as the standard inoculum concentration for the subsequent experiments. All other fermentation conditions were maintained constant during the assessment of inoculum concentration. Figure 1 shows the process flow of the methodology.

3. RESULTS AND DISCUSSION

3.1 Preparation of Inoculum

The microscopic morphology and pigmentation of *Trichoderma harzianum* conidia at different conidial concentrations are presented in Fig 2.

3.2 Effect of Different Agro-Waste Compositions on Spore Production

In this study, different compositions of agro-wastes, namely 100% sugarcane bagasse (SCB), 100% corncob (CC), 50:50 SCB:CC, 75:25 SCB:CC, and 25:75 SCB:CC, were evaluated for their effects on the spore production of *Trichoderma harzianum* under solid-state fermentation (SSF). The results showed variations in spore production among the individual substrates and their different combinations Fig 3 and Fig 4.

Trichoderma harzianum produced the highest spore yield on 100% corncob, followed by the 25:75 SCB:CC combination, whereas 100% SCB supported the lowest spore production. The spore yield obtained using pure corncob was approximately 3×10^6 spores/g on the seventh day of fermentation.

The superior performance of corncob may be attributed to its favourable physicochemical characteristics and nutrient composition, which may have provided a more suitable matrix for fungal growth and sporulation than sugarcane bagasse. The improved performance observed with the SCB:CC combinations also suggest that blending the agro-wastes may enhance the availability of nutrients and improve the physical properties of the fermentation substrate. These findings indicate that corncob is a more suitable substrate than sugarcane bagasse for the production of *Trichoderma harzianum* spores under the conditions investigated.

However, SCB, either alone or in combination with CC, was found to be less suitable for *Trichoderma harzianum* spore production, as indicated by the relatively low spore yields obtained. Although SCB was adjusted to the same particle size as CC, it still did not support high spore production within the relatively short fermentation period used in this study. This finding is consistent with Zhang *et al.* (2022), who reported lower spore yields and longer fermentation periods when sugarcane bagasse was used as a substrate. Similarly, Sachdev *et al.*, (2018) recorded a spore yield of 2.0×10^8 spores/g from SCB after 20 days of fermentation. In another study, the maximum growth of *T. asperellum* on rice bran reached 10.8×10^8 CFU/g, whereas only 3.73×10^8 CFU/g was obtained on SCB after 20 days of incubation. These findings suggest that SCB may require a longer fermentation period or additional substrate conditioning to achieve higher conidia production. More broadly, the variation in spore production among substrates demonstrates that *Trichoderma* species and strains have specific nutritional and environmental requirements for optimal conidiation. Consequently, no single culture medium or substrate can be considered universally optimal for conidia production, as the final yield depends on the interaction among the fungal strain, culture conditions, and substrate characteristics (Mulatu *et al.*, 2021).

3.3 Effect of Single Factors on Spore Production

According to Teo *et al.*, (2025), fungal growth and sporulation are strongly influenced by physicochemical factors. In this study, the effects of individual process parameters, namely inoculum concentration, pH, particle size, initial moisture content, and material weight, were investigated to determine their effects on the spore production of *Trichoderma harzianum* using corncob as the substrate. The experiments were conducted at an incubation temperature of 25 °C for 7 days. The effects of inoculum concentration, pH, particle size, initial moisture content, and material weight on spore production are presented in Figures 5 a–e, respectively.

The effects of inoculum concentration and material weight on spore production were investigated using a single-factor experimental approach. All treatments were conducted in duplicate ($n = 2$), and the results are presented as the mean of the duplicate measurements. The results showed some variation in spore production across the tested inoculum concentrations and material weights; however, the differences were relatively small compared with the variations observed for the other process parameters. Therefore, inoculum concentration and material weight were considered to have comparatively limited effects on spore production within the experimental ranges investigated and were not selected as factors for subsequent response surface methodology (RSM) optimization.

However, a previous study by (Mulatu *et al.*, 2021) reported that inoculum concentration could significantly affect fungal sporulation when a wider range of inoculum concentrations was investigated.

3.3.1 Effect of initial moisture content

Moisture content is an important factor in SSF because it influences substrate swelling, nutrient solubility, fungal growth, and metabolic activity. Adequate moisture facilitates nutrient uptake by the fungus, whereas insufficient moisture may reduce nutrient solubility and increase the surface tension of the water layer surrounding the substrate, thereby restricting fungal growth (Zhang *et al.*, 2022; Teo *et al.*, 2025). Conversely, excessive moisture can reduce substrate porosity and gas exchange, thereby limiting oxygen transfer and reducing the efficiency of SSF (Mulatu *et al.*, 2021).

This trend was observed in the present study. Spore production increased from 4.7×10^5 spores/g at an initial moisture content of 50% to 1.22×10^6 spores/g at 55%, reaching a maximum of 1.33×10^6 spores/g at 60%. However, further increases in moisture content resulted in a progressive decline in spore production. At 70% moisture content, spore production decreased to 4.4×10^5 spores/g, indicating that excessive moisture adversely affected the sporulation of *Trichoderma harzianum* Figure 5 (a). The reduction in spore production at higher moisture levels may be attributed to reduced substrate porosity and aeration, which can restrict oxygen transfer and create conditions less favourable for fungal growth and sporulation. Therefore, an initial moisture content range of 55–60% was considered suitable for spore production under the conditions investigated.

The maximum spore production observed at 60% moisture content agrees with the findings of Sala *et al.*, (2020) who reported that moisture content was one of the major factors influencing spore production of *Trichoderma harzianum* during solid-state fermentation of rice husk, with an optimum moisture range of 55–60%. Similarly, Zhang *et al.*, (2022) reported an optimum moisture content of 55.13% for sporulation of *Trichoderma spp.* using cassava peels as the substrate. These findings demonstrate that moisture content is an important factor influencing fungal sporulation, although the optimum level may vary depending on the fungal species, substrate characteristics, and other fermentation conditions.

3.3.2 Effect of pH

The effect of pH on conidia production was investigated by adjusting the pH of the moistening agent, sterile distilled water, using a mineral acid solution over a pH range of 4–8 Figure 7 (b). Conidia production was favoured under acidic conditions, particularly between pH 5 and 6, with the highest yield of 2.33×10^6 CFU/g obtained at pH 5.0. This finding agrees with previous reports indicating that pH can significantly affect fungal growth and sporulation during fermentation (Steffen *et al.*, 2026; Sargin *et al.*, 2013; J. D. Zhang & Yang, 2015). Changes in the pH of the fermentation medium can influence fungal morphology and several physiological and biochemical processes, including nutrient transport, enzymatic activity, and spore formation (Zhang *et al.*, 2022; Maurya *et al.*

al., 2012). Perwez and Al Asheh (2025) reported that the optimum initial pH for SSF media used for most fungi generally falls within the acidic range of approximately 4.5 – 5.5. The high conidia production observed at pH 5.0 in the present study therefore falls within the range reported in the literature.

The observed effect of pH indicates that it is an important process parameter for *Trichoderma harzianum* cultivation. Since pH can influence fungal growth, enzymatic activity, nutrient availability, and secondary metabolite production, its optimization is essential for maximizing conidia production. Consequently, pH was selected as one of the process parameters for subsequent optimization of the SSF process.

3.3.3 Effect of particle size

Particle size is another important factor affecting conidia production during SSF. It influences the physical structure of the substrate and consequently affects microbial growth, heat transfer, mass transfer, aeration, and oxygen availability (Perwez & Al Asheh, 2025). The particle size also determines the formation of void spaces within the substrate matrix, which can influence the movement of air and moisture through the fermentation bed.

Smaller substrate particles generally provide a larger specific surface area for fungal attachment and interaction with nutrients, potentially facilitating substrate degradation and fungal metabolism (Teo *et al.*, 2025). However, excessively fine particles may compact the substrate and restrict aeration, whereas very large particles may provide insufficient surface area for effective fungal colonization. Thus, an appropriate particle size is required to achieve a balance between surface area and substrate porosity. {Citation}

Particle size had a pronounced effect on conidial production by *Trichoderma harzianum*. The conidial yield varied across the particle size range investigated (75 μm – 1.18 mm), with yields of approximately 1.81×10^6 , 1.87×10^6 , 1.72×10^6 , 2.47×10^6 , 1.82×10^6 , and 1.70×10^6 CFU/g at 75, 150, 250, 300, 600, and 1180 μm , respectively. The highest conidial production was observed at 300 μm (2.47×10^6 CFU/g), while the lowest was recorded at 1.18 mm (1.70×10^6 CFU/g). This substantial variation in conidial production across the tested particle sizes indicates that substrate particle size influenced fungal sporulation and therefore justified its selection as a significant parameter for subsequent optimization

The results obtained in this study demonstrated that particle size had a pronounced effect on conidia production. Together with initial moisture content and pH, particle size was identified as one of the three major process parameters influencing the response. These three factors were therefore selected for subsequent statistical optimization of the SSF process.

Overall, the one-factor-at-a-time experiments indicated that initial moisture content, pH, and particle size exerted stronger effects on conidia production than inoculum concentration and material weight under the experimental conditions employed. Accordingly, moisture content, particle size, and pH were selected as the independent variables that have significant impacts on the solid-state fermentation of *Trichoderma harzianum*. These variables can be used as key parameters for the optimization of solid-state fermentation of *Trichoderma harzianum*, which is a subject of another study.

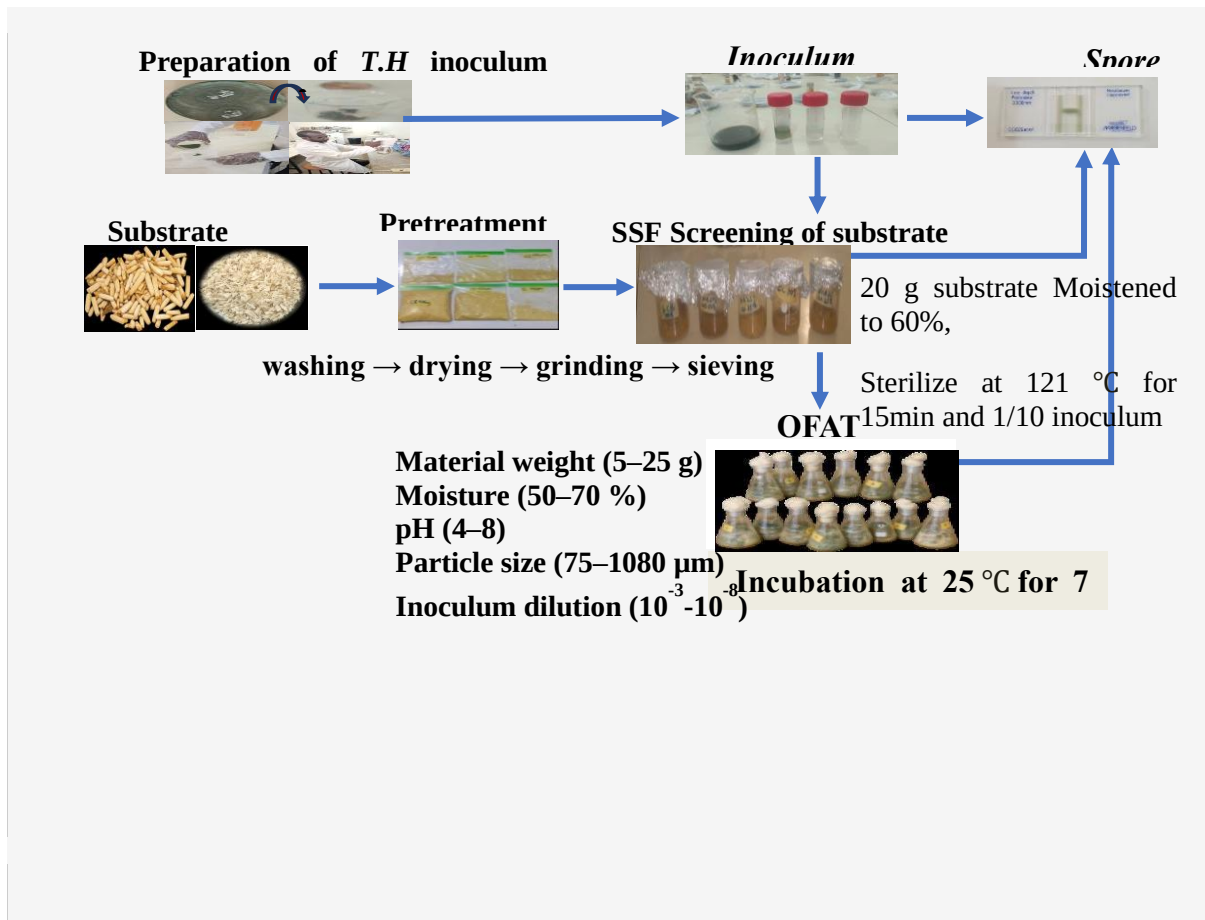


Figure 1: Process flow of the methodology

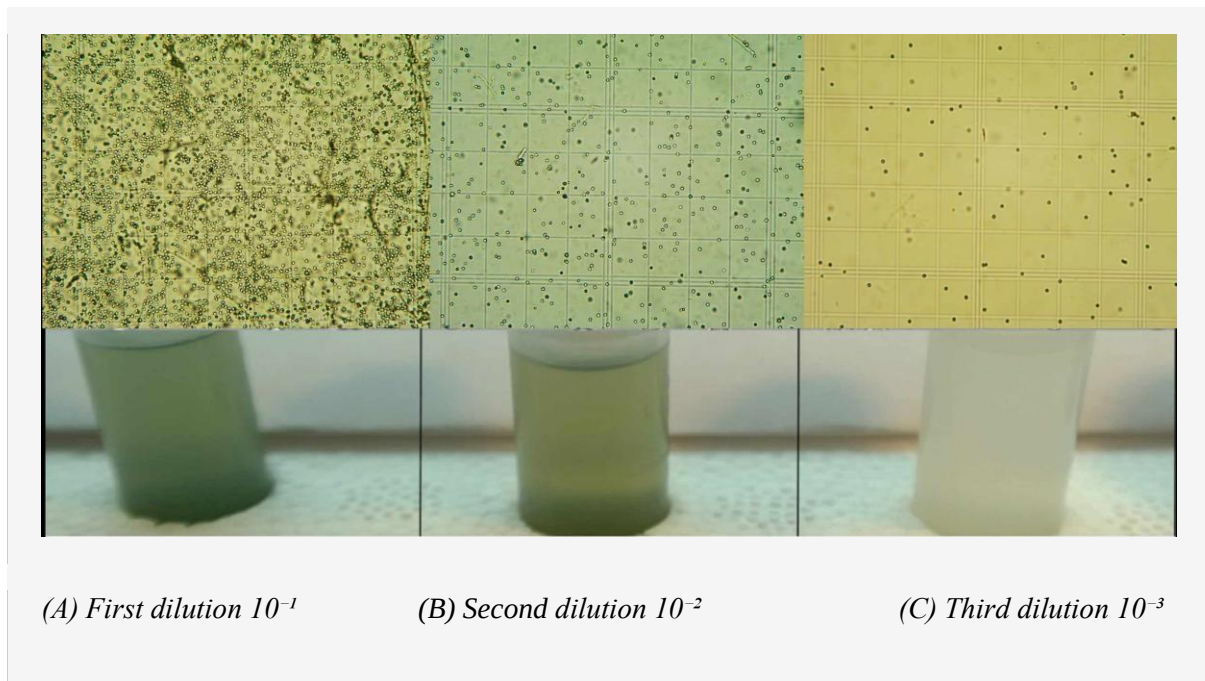


Figure 2: Microscopic morphology and pigmentation of *Trichoderma harzianum* conidia at different dilution factors



Figure 3: Colonization and Sporulation of *Trichoderma harzianum*

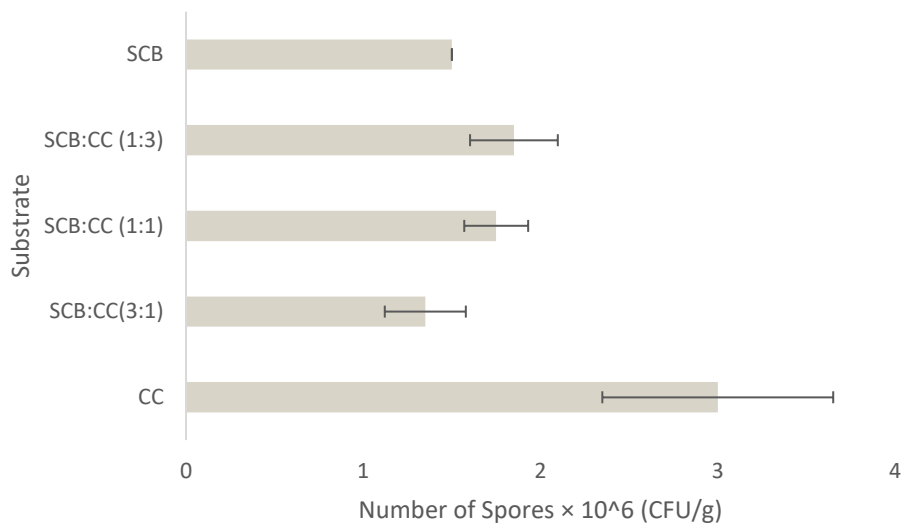
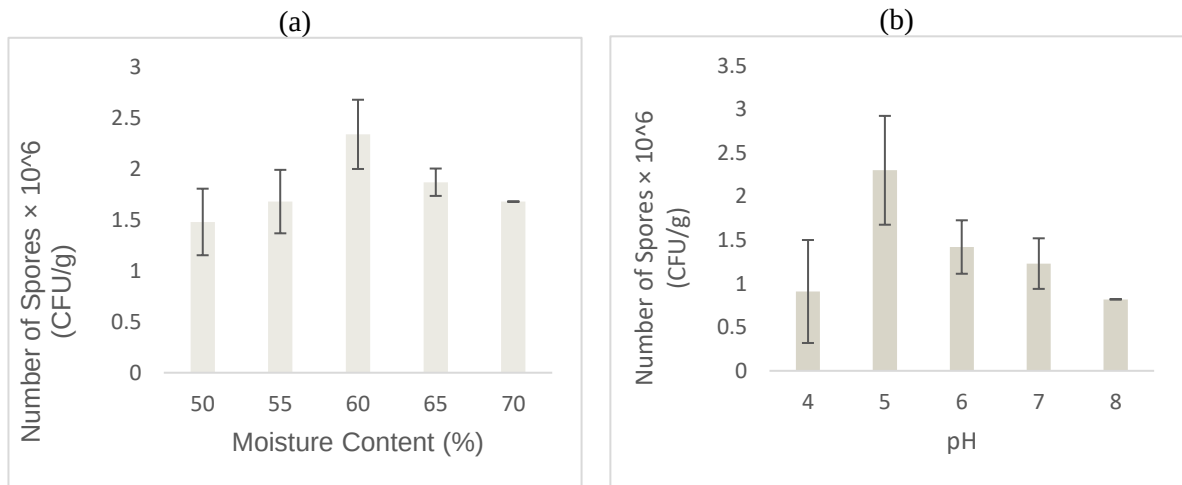


Figure 4: Spore production of *Trichoderma harzianum* on the two different agro-industrial wastes examined separately and in combination after 7 days of incubation at 25°C



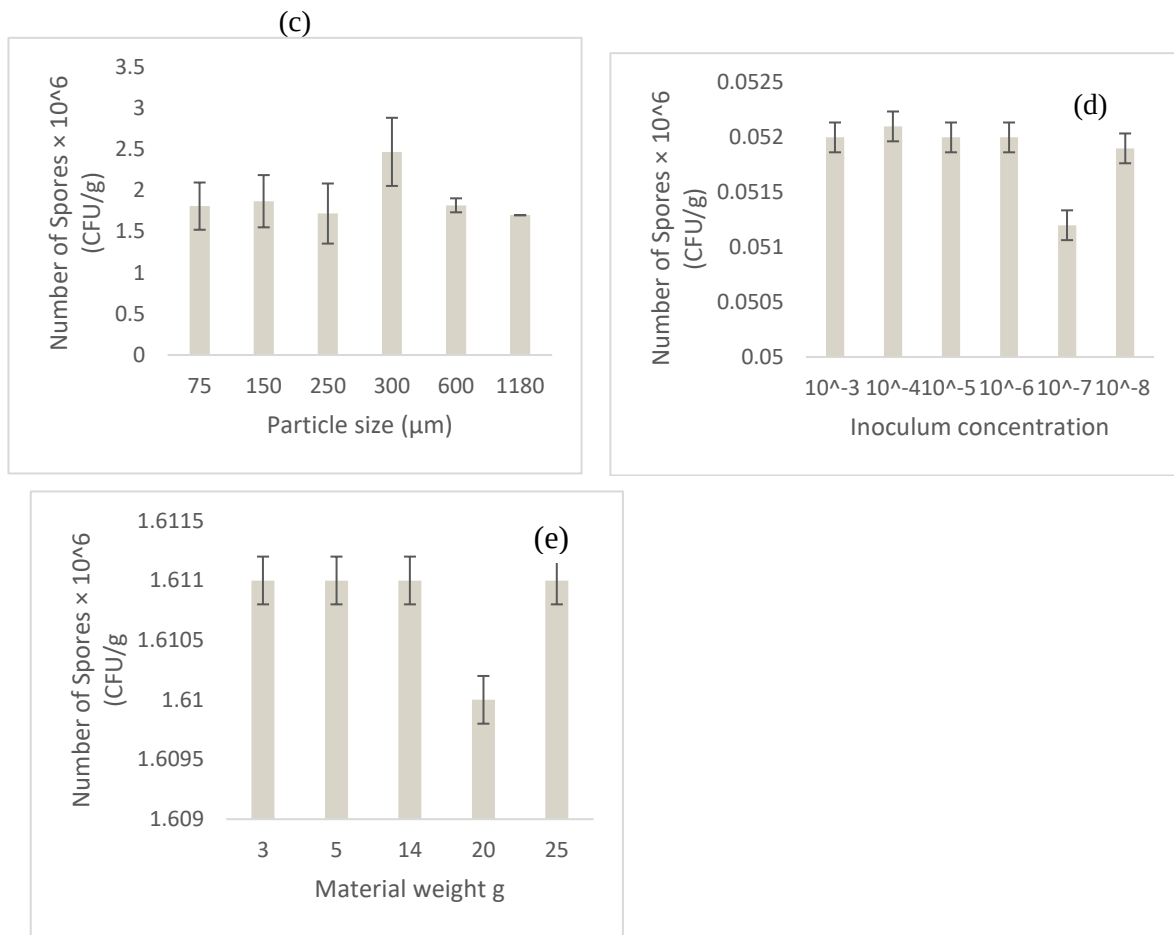


Figure 5: Effects of different parameters on the production of spores by *Trichoderma harzianum*, at incubation temperature of 25°C and fermentation period of 7 days. a) moisture content, b) pH, c) particle size d) inoculum concentration and e) material weight

4. CONCLUSION

The study successfully screened and identified moisture content, particle size, and pH as important process parameters influencing the solid-state fermentation of *Trichoderma harzianum* for conidia production. The screening results indicated that higher conidial yields were obtained at approximately 55–60% moisture content, 300–600 μm particle size, and pH 5.0–6.0. However, the one-factor-at-a-time (OFAT) approach evaluates the effects of individual parameters independently and does not account for possible interactions among process variables. Therefore, response surface methodology (RSM) was subsequently employed to evaluate these interaction effects and determine the optimal combination of the selected parameters. These findings provide a scientific basis for defining suitable parameter ranges for optimization of solid-state fermentation and potential large-scale production of *T. harzianum* as an environmentally friendly bioherbicide.

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