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Production of the Cellulase Enzyme System by Locally Isolated *Trichoderma* and *Aspergillus* Species Cultivated on Banana Pseudostem during Solid-State Fermentation

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Abstract: The production cost of cellulases is regarded as a limiting factor in the cellulosic ethanol production chain. *Trichoderma* and *Aspergillus* species were used to produce cellulases through solid-state fermentation (SSF) utilizing banana pseudostem (BPS) as a carbon source. The production of cellulases was investigated at various substrate moisture contents (65–80%), incubation temperatures (30–40 °C), substrate pre-treatment methods (3% *w*/*v* NaOH, 5% *v*/*v* H_2SO_4, and water), and with different co-culturing of microorganisms. *Trichoderma longibrachiatum* LMLSAUL 14-1 produced the maximum total cellulase (75 FPU/g d.s), endoglucanase (11.35 U/g d.s), and β-glucosidase (235.83 U/g d.s) activities at a 75% moisture content of the untreated BPS at 30 °C in static culture. Pre-treatment of BPS improved the production of specific enzymes. *Aspergillus fumigatus* LMLPS 13-4 produced more β-glucosidase (259.8 U/g d.s) when grown on acid-pre-treated BPS, whereas *T. harzianum* LMLBP07 13-5 produced the highest β-glucosidase activity (319.5 U/g d.s) on alkali-pre-treated BPS. Co-culturing of *T. harzianum* LMLBP07 13-5 and *A. fumigatus* LMLPS 13-4 improved the production of endoglucanase. These results suggest that banana pseudostem, a waste product of the banana industry, could be a potentially cheaper and abundant substrate for the production of the cellulase enzymes.

Keywords: solid-state fermentation; co-culture; banana pseudostem; cellulases; pre-treatment

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1. Introduction

Generally, fungi produce a cellulase enzyme system, which includes endoglucanase (EC 3.2.1.4 endo-1,4-β-glucanase), exoglucanase (EC 3.2.1.91 1,4-β-cellobiohydrolase), and β-glucosidase (EC 3.2.1.21) [1,2]. Cellulases are complex enzymes that facilitate the complete solubilization of cellulose and hemicellulose into simple sugar monomers [3]. The current demand for cellulases is mostly fulfilled through submerged fermentations (SmF) with genetically modified strains of *Trichoderma*. However, the production costs of these enzymes in SmF are high [4,5]. The cost of cellulases is a limiting factor for the feasible production of ethanol from fibrous biomass. [6]. In efforts to reduce the cost of producing cellulases, solid-state fermentation (SSF) can serve as an alternative to SmF. Cost estimations indicated that unit costs might be lower using SSF compared to SmF [6]. The estimates of the enzyme cost differ amongst the analyses carried out. Enzyme producers such as Dyadic, Novozymes, and DuPont have reduced the cost of cellulases per gallon (i.e., approximately 3.785 L) from USD 2 in 2010 to USD 0.30 in 2014 using SSF [7]. Through a process simulation of cellulolytic enzyme production from coffee husk, the production cost was USD 42/kg [8]. The type of substrate material used in the production of enzymes might also directly impact the final cost. The enzyme cost was estimated to be as low as USD 2–4/kg [9]. The reduction in enzyme cost is seen as a breakthrough toward the commercialization of large-scale biomass-to-ethanol production [10,11]. Remarkable progress has been made to reduce the

cost of cellulases; however, cellulases still present a significant part of the operational cost in the cellulosic bioethanol production chain [9].

Solid-state fermentation mimics natural microbiological processes, such as composting and ensiling [12]. SSF has evolved to include other applications, such as the production of bioactive compounds, organic acids, bioethanol and biodiesel as a source of alternative energy, bio-surfactant molecules, and biological controls for environmental purposes [13,14]. This type of fermentation process offers a high volumetric productivity with a highly concentrated product [4]. Various microorganisms such as bacteria, fungi, and actinomycetes, which possess different enzyme induction systems, have been used in the production of various hydrolytic enzymes [15]. The most commonly isolated species of cellulolytic fungi in composting materials are *Aspergillus*, *Penicillium*, *Rhizopus*, *Trichoderma*, *Fusarium*, *Chaetomonium,* and *Cladosporium* [16]. The critical factors to consider in SSF are the choice of microorganisms, substrate support, and bioreactor [17,18]. In SSF, the growth of microorganism(s) in the absence of free liquid can occur on the surface of a solid substrate (or nutrient-impregnated solid material) or within the whole substrate, depending on the porosity of the substrate [19,20]. The production of the enzyme system is influenced by the nature of the solid substrate, microorganism used, and environmental conditions, such as pH, temperature, and moisture [4,12,15]

The pre-treatment (e.g., chemical, mechanical, biological, and physico-chemical) of lignocellulosic biomass enables the biomass to be porous and easily accessible for both microbial growth in SSF and susceptible to enzymatic hydrolysis. The hydrolysis reaction increases the production of fermentable sugars that can be converted through biochemical activities of fermenting microorganisms to ethanol [21]. The high cost of cellulases hampers the commercialization of biomass processing. As it stands, large amounts of cellulases are still required for biomass saccharification [11]. For instance, approximately 100 g of enzyme is needed per gallon (i.e., 3.785 L) of cellulosic ethanol production [2]. The amount of cellulases required for lignocellulosic biomass during saccharification is one-fold higher than the amount of enzyme needed for the saccharification of starch [22]. To overcome the high cost of cellulases, the production strategies should increase enzyme volumetric activity, produce enzymes using cheaper substrates, produce enzyme preparations with greater stability for specific processes, and produce cellulases with a higher specific activity on the solid substrate [2]. Furthermore, on-site enzyme production can also reduce the cost by approximately 30–70% [7,23,24].

SSF offers the advantage of utilizing agro-industrial waste as substrate support. The utilization of renewable resources contributes towards sustainable development by minimizing the generation of pollutants and harmful wastes [13,18]. Recent developments in SSF include the use of the on-site production of cellulolytic enzymes (i.e., whole medium-containing enzymes, fungal cells, and residual substrate) to hydrolyze lignocellulosic biomass in an effort to minimize the waste stream. It has been suggested that during hydrolysis biomass, the mycelium-bound enzymes also contribute significantly to the final yield [25].

Bananas are tropical plants cultivated under sub-optimal, sub-tropical conditions in South Africa and are available throughout the year [26]. Bananas and plantains are staple diets in other parts of Africa. The banana plant is a herbaceous plant of the genus *Musa* and cultivated for mainly fruit production. There are about 25–80 species of the genus *Musa* [27]. South Africa is a relatively small banana grower in terms of global hectares. Banana and other sub-tropical fruits are cultivated mainly in three provinces: Mpumalanga, Limpopo, and Kwa-Zulu Natal. Based on the annual average quantity produced during the period 2015/2016, the country production decreased from 401,400 tons of bananas to 287,912 in the period 2016/2017 [28,29]. The main banana cultivar grown in Limpopo province is "Pisang Awak", ABB genome (70%), followed by "Cavendish", AAA genome (30%), of *Musa acuminate* [30].

The use of agricultural biomass such as banana waste as feedstock for ethanol production can potentially reduce the amount of food crops such as corn (maize) and wheat used

for the production of ethanol [31]. Banana waste, including leaves, rachis, and pseudostem, contain considerable amounts of cellulose [32,33]. Approximately 80% of the banana biomass generated is made up of banana pseudostem [27]. These cellulose-rich materials are discarded after the harvest of banana fruit and left to decay either in the plantation site or at a dumping site [33]. The utilization of low-cost agricultural residues, such as banana waste, in SSF to produce enzymes could help mitigate waste disposal, which contributes to environmental pollution [12], and it is a sustainable cost-effective approach [34]. In a previous study [35], *Trichoderma harzianum* LMLBP07 13-5 (6 FPU/mL), *T. longibrachiatum* LMLSAUL 14-1 (8 FPU/mL), and *Aspergillus fumigatus* LMLPS 13-4 (3.1 FPU/mL) produced a substantial amount of cellulases in submerged cultivation using microcrystalline cellulose (Avicel) as a substrate. Given the costs associated with Avicel, it became necessary to opt for a low-cost alternative substrate to produce and improve the cellulases activity in SSF. The influence and effect of SSF on cellulases using banana fruit stalk waste was reported by Krishna [36]. The results showed a 12-fold increase in total enzyme (cellulases) production compared to submerged fermentation (0.9 U/L) [36]. It has been reported that banana pseudostem induces cellulase synthesis and secretion in *Talaromyces amestolkiae* CMIAT055 better than microcrystalline cellulose [3]. Hence, this study was aimed at using banana pseudostem as an inducing substrate for cellulase production by locally isolated *Trichoderma* and *Aspergillus* species in the SSF process.

2. Materials and Methods

2.1. Microorganisms and Inoculum Preparation

We used the fungal strains obtained by Legodi et al. [35], namely *Trichoderma harzianum* LMLBP07 13-5, *Trichoderma longibrachiatum* LMLSAUL 14-1, and *Aspergillus fumigatus* LMLPS 13-4. The strains were stored at 4–8 °C with periodic sub-culturing onto malt extract agar (MEA, Merck (biolab), Midrand, South Africa).

For inoculum preparation, the fungi were grown on MEA until spore formation at 30 °C. Spores were harvested by modifying the method described by Ramanchandran et al. [37]. Spores from *A. fumigatus* LMLPS 13-4 were harvested by adding 10 mL of 0.05% sterile Tween 80 solution (Merck, Midrand, South Africa) onto the culture plates and sterile swabs were then used to dislodge the spores from the agar surface under aseptic conditions. For *Trichoderma* species, spores were harvested by cutting the overlaid agar into pieces by using a sterile metal spatula and added into 20 mL 0.05% sterile Tween 80 solution. The spore suspensions were shaken vigorously to dislodge the spores. All the spore suspensions were filtered through a folded gauze cloth to remove agar debris and mycelia. The filtrates were centrifuged at 3834× *g* (Beckman Coulter Allegra X 22R refrigerated benchtop centrifuge; Model: CAT No 392188, Schönwalde Glien, Germany) for 10 min. The supernatant was discarded and the pellet of spores were re-suspended in 5 mL 0.05% sterile Tween 80 solution. A standard spore count procedure was carried out using the Neubauer bright line (1/10 mm) counting chamber. Spore suspensions were adjusted to contain 1×10^8 spores/mL.

2.2. Collection and Preparation of Banana Pseudostem

Fresh banana pseudostems (BPSs) that remained after banana fruit harvest were collected from Allesbeste farm, Tzaneen (23.800943° S 30.123264° E 799 m M1 R71 Tzaneen 0850), Limpopo province, South Africa. The BPS was washed to remove soil and other debris using tap water. The washed BPS was cut into small pieces (approximately 20–25 cm in diameter and 10 cm in height) and the outer and core bark (pith) were separated into several blocks (Figure 1).

The blocks and piths were dried at 65–70 °C (Scientific, Digital Model no. 276; Effective Laboratory Supplies, Johannesburg, South Africa) until a constant mass was obtained and then subjected to grinding using a milling and crushing machine (Zhuans, Electric model, Cape Town, South Africa). The ground material was sieved through a Universal Test Sieve

with aperture 500 µm [38]. The sieved ground particles (<500 µm) were stored at room temperature (20–25 °C) in a sealed container until needed.

Figure 1. Banana plant (**A**), banana pseudostem (**B**), and cut banana pseudostem, pith and outer portion of pseudostem (**C**).

2.3. Pre-Treatment of Banana Pseudostem

Three different pre-treatment procedures were evaluated. One hundred and fifty grams of ground BPS was suspended in each of the pre-treatment solutions, i.e., 3% *w*/*v* NaOH [39], 5% *v*/*v* H_2SO_4 [40–42], and hot water, at a ratio of 1:10 (solid: liquid). The slurries were autoclaved at 121 °C, 15 psi for 1 h (HL-340 Vertical Type Steam Sterilizer, Taipei, Taiwan) and cooled prior to washing with tap water until pH 7.0 was reached. The solid material was dried at 65–70 °C (Scientific, Digital Model no. 276; Effective Laboratory Supplies, Johannesburg, South Africa) until a constant mass and ground using a Waring commercial blender (Model 32BL8, New York, NY, USA). The ground material was stored at room temperature in a sealed container until required for use.

2.4. Chemical Composition of Banana Pseudostem

The chemical composition of the untreated, thermo-alkali-, thermo-acid-, and liquid hot water (or hydrothermal)-pre-treated BPS samples was determined. The cellulose and lignin contents were analyzed by a reaction with sulphuric acid according to a standard method by TAPPI-T222 om-88, and the hemicellulose content was obtained as described in TAPPI1T19m-54 standards [43].

2.5. Solid-State Fermentation of Untreated Banana Pseudostem

Three milligrams of untreated BPS was moistened to 75% with a synthetic medium as described by Peixoto [44] in 250 mL Erlenmeyer flasks. The synthetic medium consisted of 2 g/L K_2HPO_4, 0.5 g/L KCl, 0.01 g/L $FeSO_4{\cdot}7H_2O$, 0.15 g/L $MgSO_4{\cdot}7H_2O$, 7 g/L g KH_2PO_4, 1 g/L $(NH_4)SO_4$, and 1.2 g/L Yeast Extract. The pH of the medium was adjusted to 6.5 using 1 M NaOH or 1 M HCl prior to sterilization at 121 °C, and 15 psi for 1 h. One mL spore suspension (1×10^8 spores/mL) of either *T. longibrachiatum* LMLSAUL 14-1, *A. fumigatus* LMLPS 13-4, or *T. harzianum* LMLBP07 13-5 was inoculated into separate 250 mL Erlenmeyer flasks in triplicate and each contained 3 g of untreated BPS. The flasks were incubated at 30 °C (Incubator Shaker Series, New Brunswick, EXCELLA E25R; Eppendorf, Germany) for 9 days without shaking. The whole content of each flask was sampled from day 3 up to day 9.

2.6. Enzyme Extraction

Enzyme extraction was carried out by modifying the method described by El-Shishtawy et al. [45]. Crude enzyme was extracted by adding 50 mL of 0.05 M sodium citrate buffer pH 4.8 to the fermented contents in flasks with intermittent shaking for 1 h at room temperature (20–25 °C). The mixture was filtered through a folded gauze cloth and centrifuged at 3834× *g* (Beckman Coulter Allegra X 22R refrigerated benchtop centrifuge; Model: CAT

No 392188, Beckman Coulter Schönwalde Glien, Germany) for 10 min and the supernatant was used for enzyme assays.

2.7. Effect of Moisture Level on the Production of the Cellulase Enzyme System

The effect of initial substrate moisture content was investigated in the range of 65–80% (*v*/*w*) at 30 °C under static conditions. Three grams (3 g) of untreated BPS was inoculated as indicated above for all fungal species. The fermentation progressed for a period of 9 days under static conditions. The whole flask content was used for enzyme extraction as described earlier.

2.8. Effect of Temperature on the Production of the Cellulase Enzyme System

The effect of temperature on cellulase production was investigated in the range of 30–40 °C under static conditions and 75% initial moisture content of the substrate. Three grams of untreated BPS was moistened with synthetic medium, inoculated as described previously. The culture was incubated at the above temperature range. The fermentation continued for 9 days under static conditions. The enzymes were extracted as described earlier and the enzyme activity determined.

2.9. Effect of Different Pre-Treatment Methods on the Production of the Cellulase Enzyme System

Three grams of each pre-treated BPS substrate was moistened to 75% initial moisture content with synthetic medium, inoculated, and incubated at 30 °C. The culturing conditions were maintained as described previously.

2.10. Cellulolytic Activity Assays

2.10.1. Cellulase

The cellulase (FPase) activity was determined using the filter paper assay with a Whatman No.1 filter paper strip of 1 × 6.0 cm, equivalent to 50 mg of substrate according to Ghose [46]. At least two dilutions were made, one dilution that released slightly less than 2.0 mg and another dilution releasing more than 2.0 mg. The reaction mixture contained 1.0 mL of 0.05 M Na-citrate, pH 5.0, the filter paper strip, and 0.5 mL of crude enzyme diluted accordingly. The mixture was incubated at 50 °C for 1 h. The reducing sugar released was estimated by the addition of 3,5-dinitrosalicylic acid (DNS) with glucose as standard. The absorbance was read at 540 nm using a Beckman Coulter DU® 720 UV/Vis spectrophotometer (Model no. 1267202, Brea, CA, USA). The assay was performed in triplicate including controls. Filter paper activity (FPU) was defined as 0.37 divided by the concentration of enzyme required to liberate 2.0 mg of glucose from the filter paper strip (≈50 mg) in 1 h.

2.10.2. Endoglucanase Assay

Endoglucanase activity in the culture supernatant was determined according to the method described by Ghose [46]. The reaction mixture contained 0.5 mL of 1% CMC in 0.05 M Na-acetate buffer, pH 5.0 and 0.5 mL of appropriately diluted crude enzyme. The mixture was incubated at 50 °C for 30 min and the released reducing sugar was estimated as indicated in the FPase assay earlier. One unit of endoglucanase activity was defined as the amount of enzyme liberating one µmole of reducing sugar from CMC under the assay conditions.

2.10.3. β-Glucosidase Assay

β-glucosidase activity was determined according to the method described by Herr [47]. The reaction mixture contained 0.2 mL of 0.01 M *ρ*-nitrophenyl β-d-glucopyranoside (pNPG, Sigma) in 0.05 M citrate buffer, pH 4.8 and 0.2 mL of appropriately diluted enzyme solution. The substrate control contained 0.4 mL of 0.01 M pNPG prepared in 0.05 M citrate buffer at pH 4.8. The mixtures were incubated at 50 °C for 30 min. The reaction was stopped by adding 4 mL of a 50 mM NaOH-Glycine buffer, pH 10.6. The activity of the enzyme,

indicated by the release of ρ-nitrophenol, was determined at 420 nm using a Beckman Coulter DU® 720 UV/Vis spectrophotometer [48,49]. One unit of β-glucosidase activity was defined as the amount of enzyme liberating one µmole of ρ-nitrophenol under the assay conditions.

2.11. Calculations of Enzyme Activities

2.11.1. Cellulase (Filter Paper Activity)

Filter paper activity was used to determine total cellulase activity of the crude enzyme using Equation (1):

$$\text{FPA}\left(\frac{\text{FPU}}{\text{mL}}\right) = \frac{0.37}{[\text{Enz}]} \quad (1)$$

where FPU is filter paper activity unit and [Enz] is the concentration of enzyme that releases 2.0 mg of glucose from the 50 mg filter paper in 60 min under the conditions of the assay [45]. For cellulase activity in SSF, FPU/mL was converted to FPU/g d.s (d.s refers to dry substrate) using Equation (2) [50]:

$$\text{FPA}\left(\frac{\text{FPU}}{\text{g d.s}}\right) = \frac{\frac{\text{FPU}}{\text{mL}} \times \text{total volume of fungal extract (mL)}}{\text{Dry weight of the substrate used in SSF (g)}} \quad (2)$$

2.11.2. Endoglucanase (CMCase) and β-Glucosidase Activities

To estimate the activities of either endoglucanase or β-glucosidase, the following Equation (3) was used [51].

$$\beta - \text{glucosidase or CMCase}\left(\frac{\text{U}}{\text{mL}}\right) = \frac{\Delta\text{E} \times \text{Vf} \times \text{Df}}{\varepsilon \times \Delta\text{t} \times \text{Venz}} \quad (3)$$

where ΔE is absorbance at 540 nm, Vf is the final volume of the reaction mixture including the amount of DNS added, Df is the dilution factor, ε is the extinction coefficient of glucose, Δt is the reaction time, and Venz is the volume of crude enzyme added. To convert the U/mL to u/g d.s, the above equation becomes

$$\text{CMCase or }\beta\text{glucosidase}\left(\frac{\text{U}}{\text{mL}}\right) = \frac{\frac{\text{U}}{\text{mL}} \times \text{total volume of fungal extract (mL)}}{\text{Dry weight of the substrate used in SSF (g)}} \quad (4)$$

2.12. Statistical Analysis

All the experiments were carried out in triplicates. The data generated were statistically analyzed by a two-way analysis of variance (ANOVA) test using MS Excel 2010. Differences were considered significant when the probability value (p) was <0.05. The error bars in the graphs represent the standard deviation (SD).

3. Results

3.1. Chemical Composition of Banana Pseudostem

The amounts of the cellulose, hemicellulose, and lignin found in untreated (24.47 ± 0.84; 22.56 ± 1.66; and 14.12 ± 1.59) and pre-treated BPS (alkaline: 52.32 ± 2.88, 10.84 ± 1.59, and 8.68 ± 0.46; acid: 48.17 ± 0.35, 9.88 ± 1.64, and 8.31 ± 1.69; hot water: 25.44 ± 0.31, 15.02 ± 1.19 and 9.25 ± 0.07) were determined. The pre-treatment results showed an increase in the percentage of cellulose and a loss of hemicellulose as well as lignin. The loss of lignin is desirable, since it has been shown that lignin binds and limits the accessibility of cellulases to cellulose [52].

3.2. The Production of the Cellulase Enzyme System

Solid-state fermentation (SSF) was used to produce the cellulase enzyme system. Three filamentous fungi, *T. harzianum* LMLBP07 13-5, *T. longibrachiatum* LMLSAUL 14-1, and *A. fumigatus* LMLPS 13-4, were investigated for the ability to produce the cellulase enzyme

system in SSF of untreated BPS as the inducing substrate. The enzyme activities of cellulase (FPase), endoglucanse (CMCase), and β-glucosidase are shown in Table 1.

Table 1. Production of cellulases by fungal species cultivated on solid-state fermentation of untreated banana pseudostem as a substrate.

Fungi	Total Cellulose Activity (FPU/g d.s)	Endoglucanase Activity (U/g d.s)	β-Glucosidase Activity (U/g d.s)
T. longibrachiatum LMLSAUL 14-1	75.04 ± 3.2 (168 h)	111.35 ± 2.2 (192 h)	235.83 ± 12.3 (192 h)
T. harzianum LMLBP07 13-5	21.75 ± 3.9 (120 h)	9.46 ± 0.51 (144 h)	30.87 ± 8.1 (144 h)
A. fumigatus LMLPS 13-4	41.33 ± 5.0 (216 h)	4.39 ± 4.1 (120 h)	116.68 ± 17.8 (72 h)

NB: The ± values are standard deviation of independent triplicates (n = 3); $p < 0.05$.

The amount of enzymes produced varied amongst the different fungal species investigated. *Trichoderma longibrachiatum* LMLSAUL 14-1 produced the highest activities for all enzymes investigated, followed by *A. fumigatus* LMLPS 13-4, and *T. harzianum* LMLBP07 13-5 produced the lowest enzyme activity, especially for FPase and β-glucosidase. *Aspergillus* species are known to produce β-glucosidase with high activity more than the *Trichoderma* species. However, in the current study, *T. longibrachiatum* LMLSAUL 14-1 produced β-glucosidase with an activity of 234 U/g d.s, almost twice as high as the activity of *A. fumigatus* LMLPS 13-4 and higher than endoglucanase activity.

3.3. Effect of Temperature on the Production of the Cellulase Enzyme System

The effect of temperature on the production of the cellulase enzyme system was investigated between 30 and 40 °C (Figure 2). In general, the temperature had a direct influence on the production of the cellulase enzyme system by all fungal strains. Cellulase (FPase) production by *T. longibrachiatum* LMLSAUL 14-1 showed a 0.47-fold decrease in activity at 35 and 40 °C (i.e., from 75 to 35.3 U/g d.s). The optimum temperature for FPase production was attained at 30 °C. Endoglucanase activity increased by 3.25-fold at 40 °C to a maximum of 363 U/g d.s. No significant change was observed in the production of β-glucosidase between 30 and 40 °C (Figure 2a).

Trichoderma harzianum LMLBP07 13-5 production of all cellulases increased proportionally by 1.8-fold with an increase in temperature from 22 FPU/g d.s at 30 °C to 39.9 FPU/g ds. at 40 °C. The production of endoglucanase and β-glucosidase also increased 4.2-fold to 40 U/g d.s and 4.8-fold to 164 U/g d.s, respectively, as the temperature increased to 40 °C (Figure 2b). The cellulase enzyme system of *A. fumigatus* LMLPS 13-4 exhibited a different trend. The production of cellulase decreased by 0.8-fold from 41 FPU/g d.s at 30 °C to 33 FPU/g d.s at 40 °C. Unlike the decreasing cellulase, the endoglucanase and β-glucosidase increased by 15.6-fold to 69 U/g d.s and by 2.4-fold to 276 U/g d.s, respectively (Figure 2c).

3.4. Effect of Initial Substrate Moisture Content on the Production of the Cellulase Enzyme System

The moisture content of the substrate is a critical factor for growth support and enzyme production under SSF [53]. The effect of initial substrate moisture content on the production of the cellulase enzyme system was assessed in the range of 65 to 80% moisture content. The production of the cellulase enzyme system by *T. longibrachiatum* LMLSAUL 14-1 during SSF is strongly dependent on initial moisture level of the substrate (Figure 3a). The production of the cellulase enzyme system was optimal at 75% moisture content.

Trichoderma harzianum LMLBP07 13-5 has exhibited different moisture optima conducive for the production of the cellulase enzyme system (Figure 3b). Each enzyme showed a specific moisture optimum for maximum production, unlike the trend exhibited by *T. longibrachiatum* LMLSAUL 14-1. High FPase activity was observed at a moisture content of 65 to 75%. At 80% of the initial moisture content of the substrate, the FPase was lower by 0.6-fold. The endoglucanase production during SSF remained low, irrespective of the initial

moisture content when compared with the endoglucanase levels attained by *T. longibrachiatum* LMLSAUL 14-1 and *A. fumigatus* LMLPS 13-4. Maximum β-glucosidase activity was produced at an initial substrate moisture content of 70% (294.3 U/g d.s) and reduced to 0.7-fold at 80% moisture content.

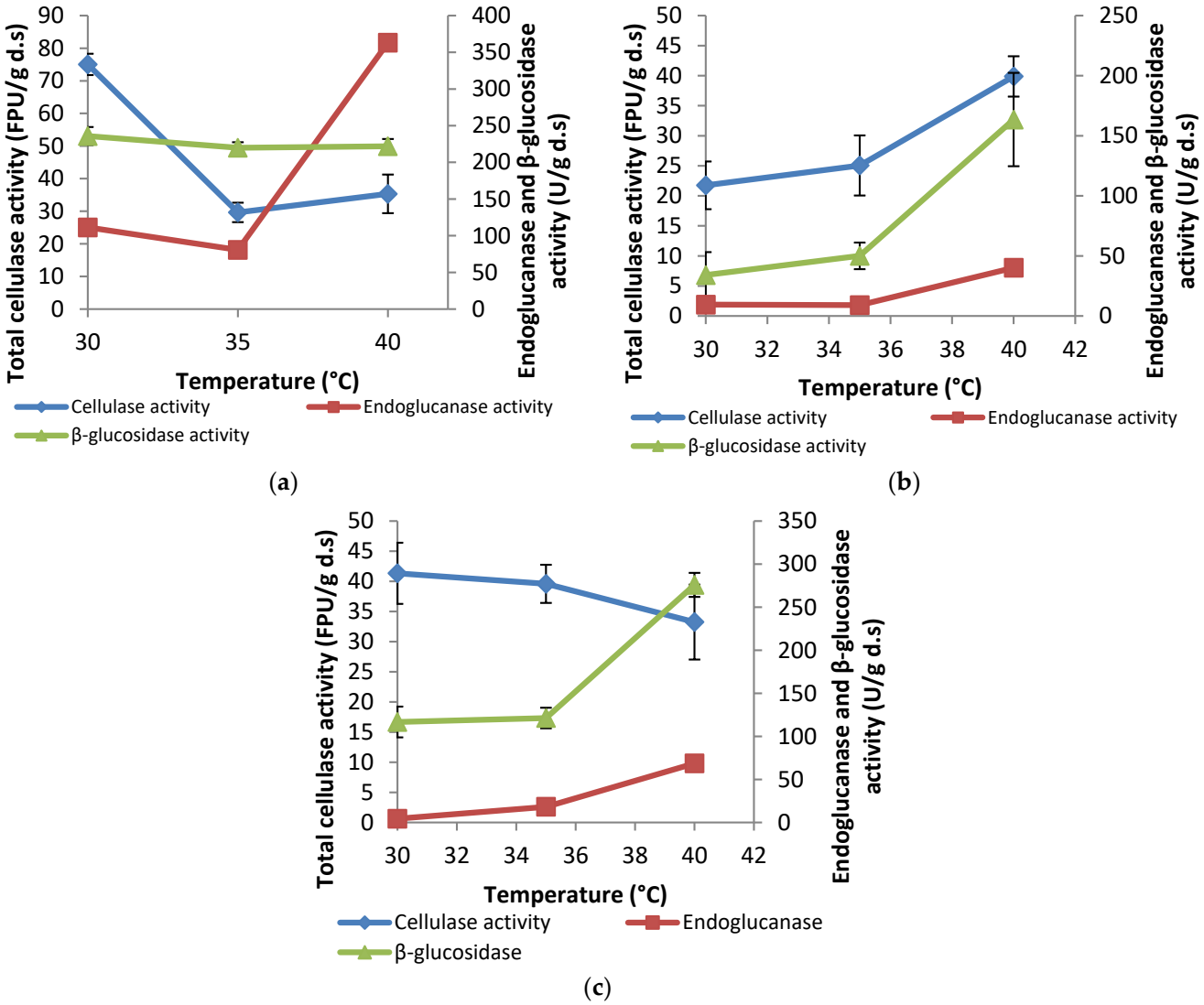

Figure 2. Effect of temperature on the production of cellulase enzyme system during solid-state fermentation of untreated banana pseudostem: (**a**) *T. longibrachiatum* LMLSAUL 14-1, (**b**) *T. harzianum* LMLBP07 13-5, (**c**) *A. fumigatus* LMLPS 13-4. Data represent mean ± SD (n = 3); $p < 0.05$.

The production of the cellulase enzyme system by *A. fumigatus* LMLPS 13-4 was also attained at different moisture contents, as illustrated in Figure 3c. The maximum FPase activity of 41.3 FPU/d.s was produced at 75% of the initial substrate moisture content. At 65% of the initial moisture content of the substrate, the production of the endoglucanases reached 193 U/g d.s but drastically decreased to 8.5 U/g d.s at 80% initial moisture content of the substrate. *Aspergillus fumigatus* LMLPS 13-4 produced high levels of β-glucosidase (>100 U/g d.s) from 65 to 80% initial moisture content of the substrate.

3.5. Effect of Pre-Treated Banana Pseudostem on the Production of the Cellulase Enzyme System

Three pre-treatment methods, namely thermo-diluted acid, thermo-alkaline, and hydrothermal (or hot water), were applied to BPS. The productions of the cellulase enzyme system were influenced by the nature of the BPS substrate (Figure 4a–c). The pre-treated BPS was not a strong inducer for the synthesis and secretion of cellulase (FPase) and

endoglucanase by *T. longibrachiatum* LMLSAUL 14-1, when compared to the untreated BPS substrate. The cellulase activity decreased from 70 FPU/g d.s on untreated BPS to 21 FPU/g d.s in 3% NaOH pre-treated BPS and to 16.6 FPU/g ds.s in both 5% H_2SO_4 and hot water-pre-treated BPS. Similarly, endoglucanse activity decreased during SSF on the pre-treated substrate. There is no significant change in β-glucosidases production between hot water and untreated BPS (Figure 4a).

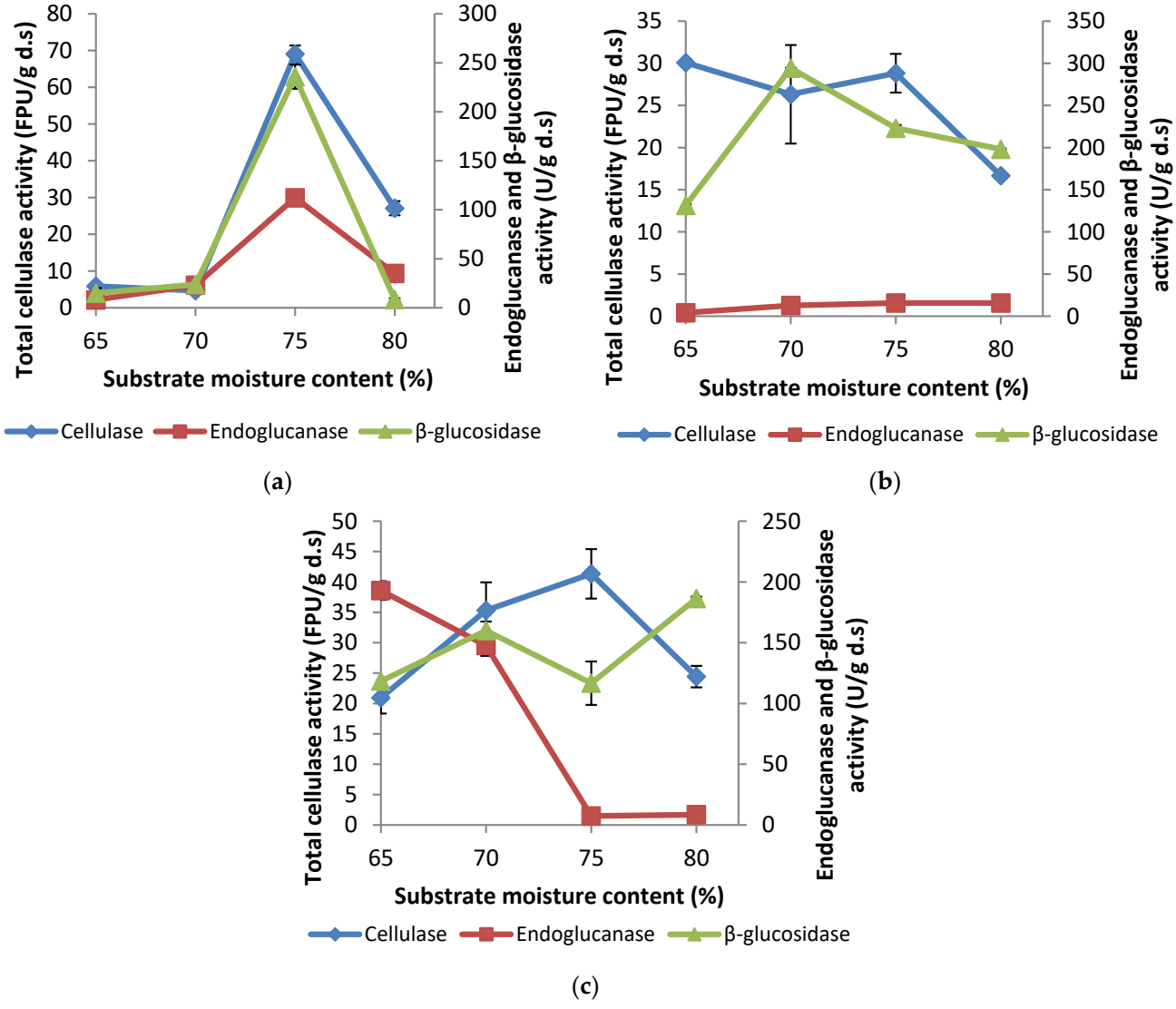

Figure 3. The effect of initial moisture content of banana pseudostem on the production of the cellulase enzyme system during solid-state fermentation of untreated banana pseudostem. (**a**) *T. longibrachiatum* LMLSAUL 14-1, (**b**) *T. harzianum* LMLBP07 13-5, (**c**) *A. fumigatus* LMLPS 13-4. Data represent mean ± SD (n = 3); $p < 0.05$.

The production of the cellulase enzyme system by *T. harzianum* LMLBP07 showed that untreated BPS was more suitable for cellulase (FPase) production (Figure 4b). Hot water-pre-treated BPS led to a significant decrease in FPase activity with only 6.2 FPU/g d.s compared to 28.8 FPU/g d.s on untreated BPS. The production of endoglucanase was similar to untreated BPS. The β-glucosidase was the highest during SSF on 3% NaOH BPS. The highest cellulase activity produced for *A. fumigatus* LMLPS 13-4 was detected during SSF on untreated BPS (41 FPU/g d.s) followed by hot water-pre-treated BPS (24 FPU/g d.s). SSF on 5% H_2SO_4 pre-treated BPS yielded the highest levels of endoglucanases (45 U/g d.s) and β-glucosidases (260 U/g d.s) (Figure 4c).

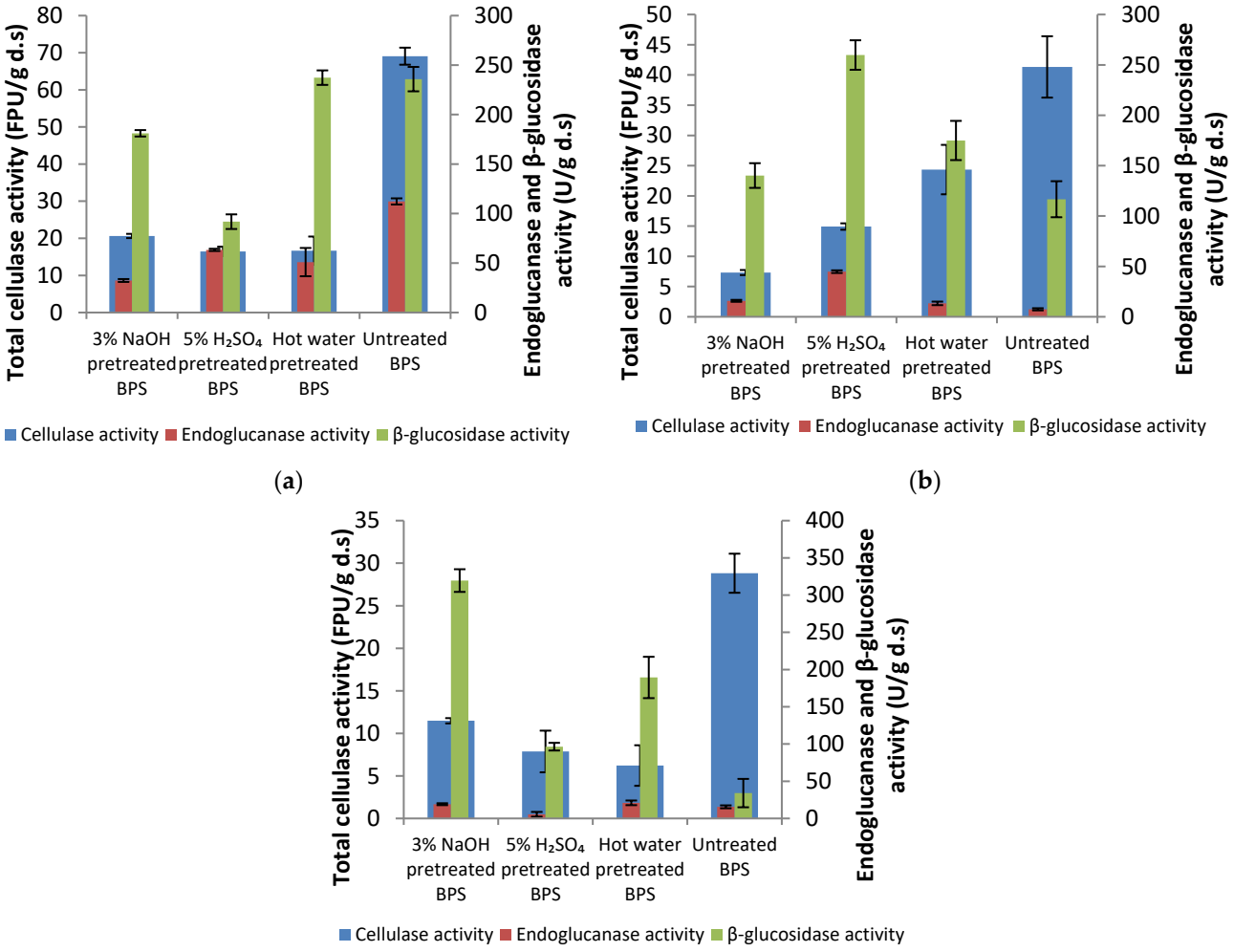

Figure 4. The effect of different pre-treatment methods on the production of the cellulase enzyme system during solid-state fermentation of pre-treated banana pseudostem. (**a**) *T. longibrachiatum* LMLSAUL 14-1, (**b**) *T. harzianum* LMLBP07 13-5, (**c**) *A. fumigatus* LMLPS 13-4. Data represent mean ± SD (n = 3); $p < 0.05$.

3.6. Effect of Co-Cultivation of Trichoderma and Aspergillus Species on the Production of the Cellulase Enzyme System

Co-cultivation of *Trichoderma* and *Aspergillus* species from this study was assessed in SSF using untreated BPS as a substrate in an attempt to increase the hydrolytic activity of the cellulase enzyme system. Co-culturing of *T. longibrachiatum* LMLSAUL 14-1 and *A. fumigatus* LMLPS 13-4 produced 61 FPU/g d.s, which is a 0.8-fold decrease in FPase activity when compared to 75 FPU/g d.s obtained in the monoculture of *T. longibrachiatum* LMLSAUL 14-1 (Figure 5). There was also a 0.47-fold reduction in the β-glucosidase activity. In general, the co-cultivation of the above fungi did not yield an improvement in the production of the cellulase enzyme system. On the contrary, co-cultivation of *T. harzianum* LMLBP07 13-5 and *A. fumigatus* LMLPS 13-4 yielded a 2.23-fold improvement in cellulase activity and a 10-fold increase in endoglucanase production compared to the monoculture of *T. harzianum* LMLBP07 13-5. However, the production of β-glucosidases decreased by 0.48-fold compared to the monocultures (Figure 5).

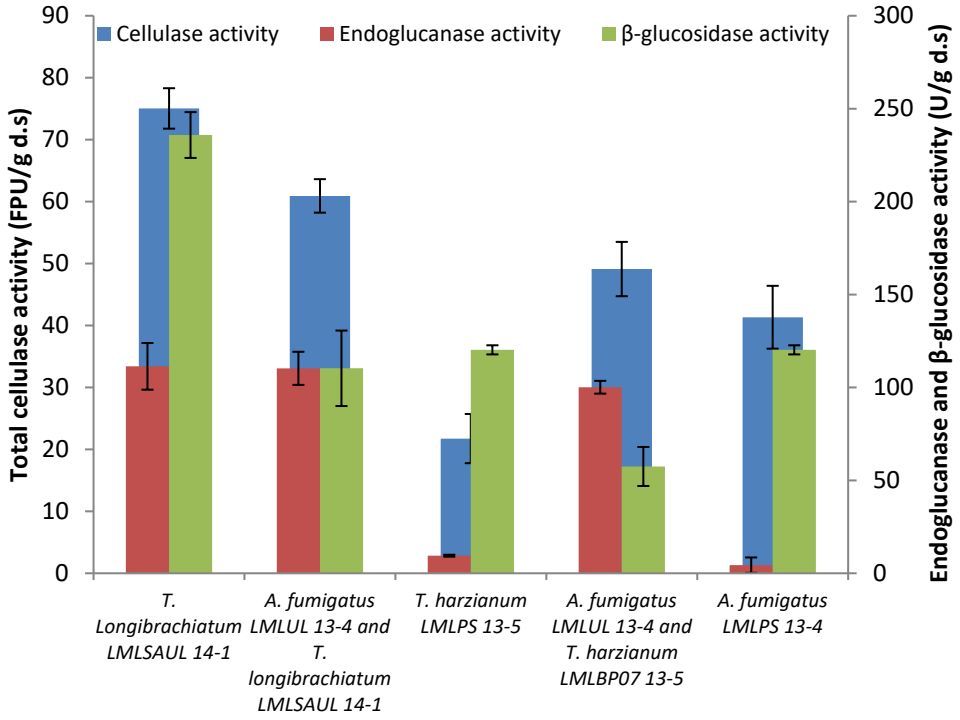

Figure 5. The effect of co-culturing the *Trichoderma* and *Aspergillus* strains on the production of the cellulase enzyme system during solid-state fermentation of untreated banana pseudostem. Data represent mean ± SD (n = 3); $p < 0.05$.

4. Discussion

Most microbes, including fungi that inhabit and decompose plant materials, require the production and secretion of cell wall-degrading enzymes acting synergistically to deconstruct the cell wall, thereby releasing the sugar monomers for energy. The production of enzymes involved in the degradation of lignocellulosic biomass is regulated at the transcriptional level [54] and is therefore subjected to the substrate used [55,56]. Cellulase enzyme production is dependent on the nature of the substrate and cellulosic content. Several carbon sources including lactose, sophorose, L-sorbose, etc., have been shown to induce the formation and secretion of the cellulase enzyme system, namely cellobiohydrolases, endoglucanases, and β-glucosidases [55,56]. However, industrial fermentation using the above carbon sources to produce cellulose-degrading enzymes remains expensive. This study utilized banana pseudostem (BPS), an inexpensive agricultural waste product, to produce the cellulase enzyme system in solid-state fermentation (SSF). The potential of banana wastes (banana pseudostem and leaf biomass) as a substrate for the production of cellulase enzymes was demonstrated using *Pleurotus ostreatus* and *P. sajor-caju* under solid-state fermentation [57,58]. Our findings noted that BPS has the ability to induce the production of these enzymes in all *Trichoderma* and *Aspergillus* species studied (Table 1). *Aspergillus fumigatus* LMLPS 13-4 produced higher cellulase activity than *Trichoderma harzianum* LMLBP07 13-5. A similar observation was reported by [59], whereby *A. niger* produced more cellulase activity than *T. viride* using sawdust in SSF. In another study, a high cellulase yield was produced by *Trichoderma* spp. using a corn cob as the carbon source [60], suggesting that different fungi respond differently to their environment, particularly the type of substrate. To further demonstrate this effect of the substrate, various agro-wastes such as wheat bran, rice straw, bagasse, banana peels, Bengal gram husk, and corn husk were evaluated for suitability in the production of cellulase by *A. niger* and *T. viride*. Banana peels induced the highest cellulase activity in SSF by *T. viride*, whereas rice straw induced more cellulase activity than other agro-wastes in SSF by *A. niger* [61]. In SSF using *T. viride*, the partially purified cellulase enzyme exhibited activity of 104.8 FPU/g d.s, which was an 8-fold increase compared to the crude enzyme [61]. Sun et al. [62] reported

maximum cellulase activities (i.e., FPase, 5.56 FPU/g d.s; endoglucanase, 10.31 U/g d.s; and β-glucosidase, 3.01 U/g d.s) in SSF using banana peel. Banana peel was ineffective in inducing cellulase enzyme production by *A. niger* in SSF. The highest reported activities for cellulase (PFase), endoglucanse, and β-glucosidase were 1.96 FPU/g d.s, 2.0 U/g d.s, and 1.22 U/g d.s, respectively [63]. Banana leaves were also found to be a poor inducer of cellulase enzyme production. *Aspergillus* sp. B11 produced cellulase activity of 0.94 FPU/g d.s and endoglucanase activity of 2.2 U/g d.s [64]. The poor inducing ability of the banana peel and leaves could be attributed to the low cellulose (11%), hemicellulose (9%), and lignin (3%) content [63]. *Trichoderma harzianum* TF2, which was isolated from banana rhizosphere, had produced a high cellulase activity of 38.5 U/g.ds using wheat bran as the substrate [65]. Partial purification or full purification of the crude enzyme could enhance or improve their activities [61]. Other factors that influence the cellulase enzyme production or the degradability of the lignocellulose biomass include the substrate moisture content, the initial pH, the fermentation temperature, and microorganism(s) used [66], including the type of nitrogen sources that supplement the solid substrate material and the inoculum size of the culture [61].

4.1. Effect of Temperature on the Production of the Cellulase Enzyme System

Temperature is an important physiological factor that affects the growth and metabolic activities of microorganisms [67–69]. The production of the cellulase enzyme system appears to be dependent on a particular temperature for specific enzymes that were evaluated. For instance, the maximum cellulase (FPase), endoglucanases, and β-glucosidases of *T. longibrachiatum* LMLSAUL 14-1 were produced at different temperatures (Figure 2a). This trend shows that different incubation temperatures activate the different and specific regulatory enzyme systems. The optimum temperature for the production of cellulases and β-glucosidases by *T. longibrachiatum* LMLSAUL 14-1 was 30 °C. *Trichoderma longibrachiatum* LMLSAUL 14-1 produced β-glucosidases with activity of 234 U/g d.s, almost twice as high as the activity of *A. fumigatus* LMLPS 13-4 and higher than the endoglucanase activity it produced. A cellulose enzyme production system with higher β-glucosidase than endoglucanase activity in *Trichoderma* species has been previously reported. Damaso et al. [70] reported different genera of fungi that were able to produce a high amount of β-glucosidase. *Trichoderma* (isolate 4), *Penicillium* (isolate 7), and *Aspergillus* (isolate 11) produced endoglucanase activity of 23.3 U/g d.s, 31.0 U/g d.s, and 29.3 U/g d.s, whereas the higher β-glucosidase activities for the fungi were 155 U/g d.s, 168 U/g d.s, and 158 U/g d.s, respectively [70]. Therefore, very few fungi producing good quantities of β-glucosidase are available; however, with continuing screening of the best β-glucosidase producers, the cost of cellulosic ethanol can be reduced.

At a higher temperature, there was a decrease in the synthesis and secretion of these enzymes. According to Jecu [71], the decrease in enzyme production at elevated temperatures could be due to the deactivation of a particular regulatory enzyme system. Conversely, the optimum temperature for the production of endoglucanases by *T. longibrachiatum* LMLSAUL 14-1 was found to be 40 °C. On the contrary, Leghlimi et al. [72] reported the maximum FPase and endoglucanase activities produced by both *T. longibrachiatum* (GHL) and *T. reesei* Rut C-30 to be at 35 °C. Such differences are exacerbated by the chemical composition of the medium and other factors, such as the pH. *Trichoderma harzianum* LMLBP07 13-5 was able to produce the maximum activity of the cellulase enzyme system at one specific temperature. The optimum temperature for production of cellulase, endoglucanase, and β-glucosidase by the fungus was 40 °C (Figure 2b). *Aspergillus fumigatus* LMLPS 13-4 also produced higher endoglucanase and β-glucosidase activity at 40 °C, while its cellulase activity was higher at 30 °C (Figure 2c). Although the optimum temperature for β-glucosidase production by *A. niger* NCIM 1207 was 30 °C, sequential incubation at an increased temperature (36 °C) enhanced the production of the enzyme [73]. Another study reported the optimum temperature for the production of the cellulase enzyme system by

A. fumigatus to be 40 °C [74]; this result is incongruent with the current findings that a higher growth temperature favored the production of the enzyme.

The differences in optimal temperatures for the production of cellulase, endoglucanases, and β-glucosidase, as observed in Figure 2a,c, were also reported in other studies. Pirota et al. [75] noted a significant influence of temperature on the production of both endoglucanases and β-glucosidases. These authors reported a 2.3-fold increase in endoglucanase activity at 28 °C and a higher β-glucosidase activity between 35 and 37 °C under static aeration and optimum moisture content of 70%. Liu et al. [76] reported a maximum production of cellulase and endoglucanase at 50 °C by *A. fumigatus* Z5 in SSF using corn stover. Another fungus, *A. fumigatus* fresenius (AMA), produced the highest cellulase activity at 45 °C when cultivated on rice straw [77]. It has been reported that *A. fumigatus* has the ability to grow in high temperatures, a feature that distinguish it from other mesophiles [78]. The ability of the fungal species to grow and produce enzymes at temperatures between 45 and 55 °C indicates the acquired thermotolerance by the organism [79,80].

Generally, *Trichoderma* and *Aspergillus* species are mesophilic and these organisms exhibited different optimum temperatures for the production of the cellulase enzyme system. The differences in optimum production temperature observed in this study when compared to other findings [77,78] might be influenced by the natural habitat from which these fungal species were isolated and the type of substrate used for cultivation. It is also known that when an organism is subjected to certain environmental conditions, it responds differently by activating regulatory mechanisms that trigger growth, conidiation, and biosynthesis of secondary metabolites [81]. This study revealed that temperature influences growth as well as conidia formation and color (i.e., green at 30 °C to white conidia at 40 °C; data not shown) and the synthesis of enzymes.

4.2. Effect of Initial Moisture Content of the Banana Pseudostem on the Production of the Cellulase Enzyme System by the Selected Fungal Species

Another important factor that affects the metabolic activities of fungi for efficient enzyme synthesis and secretion in SSF is the moisture content of the solid substrate [82]. The results obtained in this study showed that the production of the cellulase enzyme system in SSF of untreated BPS was strongly influenced by the moisture content of the substrate. The optimum initial moisture content of BPS that supported maximum production of the cellulase enzyme system by *T. longibrachiatum* LMLSAUL 14-1 was 75% (Figure 3a). Similarly, findings by Sherief et al. [74] reported that *A. fumigatus* growing on a mixture of rice straw and wheat bran (1:1) produced the highest cellulase activity at an initial moisture content of 75%.

According to Lee [83], too little moisture prevents fungal growth and too much moisture clogs inter-particle spaces, thereby inhibiting oxygen circulation resulting in compaction of the substrate or possibly contamination by bacteria. Lower substrate moisture content also decreases the solubility of nutrients leaching out of the substrate [84]. Depending on the type of substrate, some microorganisms respond positively to high/low initial moisture of the substrate during synthesis of the enzymes. For instance, a thermophilic fungi, *M. thermophile* M77, produced 10.6 FPU/g d.s of cellulase activity when cultivated on a mixed substrate of soybean and sugarcane (10:90) with the moisture content adjusted to 80% and at a temperature of 45 °C [85]. *Trichoderma harzianum* TF2 produced the most cellulase using wheat bran as a substrate with an initial moisture content of 60% [65]. A different production trend was observed for *T. harzianum* LMLBP07 13-5 with the cellulase enzyme system being produced over a fairly wide initial moisture content (Figure 3b).

The endoglucanase production was highest at an initial moisture content of between 75% and 80%. The initial moisture content of BPS higher than 75% has reduced FPase and β-glucosidases production by 0.53 and 0.67, respectively. *Aspergilus fumigatus* LMLPS 13-4 also responded differently under varying moisture contents of BPS for the production of the cellulase enzymes system. The optimum initial moisture content conducive for maximum FPase and endoglucanase production was 75% and 65%, respectively. The optimum initial

moisture content of BPS for the production of β-glucosidase by *A. fumigatus* LMLPS 13-4 was 80% (Figure 3c). Similarly, higher initial moisture content of substrates was found to improve cellulase activity in *Aspergillus*. For instance, Delabona et al. [86] observed that an initial moisture content of 80% in untreated sugarcane bagasse enhanced the production of endoglucanase by both *A. niger* P47C3 and *A. fumigatus* P40M2. Ang et al. [87] also reported cellulase activity of 3.36 FPU/g d.s, endoglucanase of 54.27 U/g d.s, and β-glucosidase of 4.54 U/g d.s produced by *A. fumigatus* SK1 grown on untreated palm oil trunk with optimum initial moisture content of 80% at room temperature. Furthermore, it has been reported that *A. fumigatus* Z5 produces FPase activity of 139.9 FPU/g d.s and endoglucanase of 325 U/g d.s when cultivated on corn stover as the substrate with an initial moisture content of 75 or 80% [76].

Pirota et al. [75] reported that the optimum initial moisture content for the production of cellulases on wheat bran by *A. oryzae* P27C3 was 80%. In this case, the maximum cellulase activities produced were 0.48 FPU/g d.s and 0.14 FPU/g d.s under forced aeration and static aeration, respectively. Despite this improvement in the production of cellulase at 80% initial moisture content, Yoon et al. [88] found that moisture content lower than 60% and higher than 80% was unfavorable for both fungal growth and cellulase production in SSF. When non-treated sugarcane bagasse is used, moisture content of 70% resulted in better production of enzymes [64]. The results indicated that each fungus has a specific requirement for optimum moisture content of the substrate to fully activate the regulatory enzyme involved in the synthesis and secretion of the cellulase enzyme system. The initial moisture content of BPS is critical as it facilitates nutrient and oxygen uptake by the cultivated microorganism in SSF. These differences in initial moisture content of the BPS on the cellulase enzyme system secretion suggest that optimum moisture content conducive for high cellulase induction depends on the adaptive mechanism of a particular fungus and the properties of the substrate used.

4.3. Effect of the Pre-Treatment of the Banana Pseudostem on the Production of the Cellulase Enzyme System

The production of cellulases is initiated when the fungi utilize plant polysaccharides as both energy and a carbon source [89,90]. The pre-treatment of BPS prior to SSF did not improve the overall production of the cellulase enzyme system as determined by FPase activity, but it enhanced the specific type of enzyme(s) by a particular fungal strain(s). The different pre-treated BPS showed an improvement in β-glucosidases by all fungal species. This might be because pre-treatment might have resulted in the partial hydrolysis of the polysaccharides in the BPS, releasing some soluble oligosaccharides or other disaccharides other than cellobiose, which act as inducers, or through transglycosylation had induced β-glucosidases and repressed endoglucanase and cellobiohydrolases. Another study by Salomão et al. [67] found that pre-treatement of sugarcane bagasse resulted in poor production of endoglucanases by fungal strains (*Trichoderma koningii* and *Penicillium* sp.) when compared to natural (untreated) sugarcane bagasse. This effect is attributed to the removal of amorphous regions within the cellulose by the pre-treatment; these regions are believed to play a role in inducing cellulase [64]. Shida et al. [91] explains that the presence of trace amounts of soluble sugars derived from cellulose or through constitutively expressed cellulase activity stimulates the transcription of genes encoding cellulases. The current results show an equal amount of β-glucosidase in SSF of hot water-pre-treated BPS and untreated BPS by *T. longibrachiatum* LMLSAUL 14-1. On the other hand, *T. harzianum* LMLBP07 produced a 9.4-fold increase in β-glucosidase in SSF of BPS pre-treated with 3% NaOH, while SSF of BPS pre-treated with 5% H_2SO_4 by *A. fumigatus* LMLPS 13-4 resulted in a 1.85-fold increase in β-glucosidases. These results confirmed that each fungus responds differently to this carbon source. Generally, SSF of untreated BPS maintained a high production of cellulase (FPase) in all the fungi evaluated. This observation was supported by the findings of [61], who reported that untreated agro-cellulosic wastes were effective in inducing cellulase production. This was due to the high nutrients found in

untreated BPS, such as proteins, potassium, phosphorus, and other micronutrients [92], and possibly trace amounts of glucose and starch supported fungal growth.

Brijwani and Vadlani [93] specifically reported that alkali-pre-treated soybean hulls had a significant reduction in enzyme production by both monoculture and mixed cultures of *T. reesei* and *A. oryzae* compared to untreated and acid- and steam-pre-treated soybean hulls. The possible explanation for the alkaline effect was that the inhibitory compounds that are generated during substrate pre-treatment have a deleterious effect on microbial growth and enzyme production. Sarkar and Aikat [94] reported that pre-treatment of rice straw with 2% NaOH resulted in an enhanced production of cellulase and endoglucanase and higher concentrations of sodium hydroxide, which was detrimental, supporting the above.

In general, pre-treatment of different agricultural waste products including sugarcane bagasse, cassava bagasse, wheat bran, and rice straw improved enzyme production more than the untreated substrates. Singhania et al. [95] found that the dilute alkaline pre-treatment of substrates mentioned led to the highest cellulase production. Pre-treated sugarcane bagasse was the best inducer of cellulase (FPase) at 154.58 FPU/g d.s from *T. reesei* NRRL 11640. Although pre-treated agricultural wastes enhanced the production of cellulases, currently there is no sufficient literature available on the use of pre-treated BPS for the production of cellulases by either bacteria or fungi in monoculture. However, the ability of untreated BPS to induce and promote the production of cellulase was reported [96,97]. Dabhi et al. [98] have also used untreated BPS as a substrate to produce cellulolytic enzymes under solid-state fermentation by a bacterial consortium. In SSF, by co-culturing fungi on alkaline-pre-treated BPS, an improved/increased production of cellulase was observed [99]. Thus, an in-depth understanding of the role of the physicochemical characteristics of the substrate on cellulase production in SSF would provide a comprehensive framework to facilitate cellulase production with enhanced hydrolytic activities.

4.4. Effect of Co-Cultivation of Trichoderma and Aspergillus Species on the production of the Cellulase Enzyme System

Another strategy to enhance the production of the cellulase enzyme system with efficient hydrolytic properties was through the use of two fungi, *Trichoderma* and *Aspergillus* species. *Trichoderma* species are known to be good cellulase producers but often lack sufficient β-glucosidase activity, while *Aspergillus* species produce high levels of β-glucosidase activity [100,101]. Co-cultivation of *T. longibrachiatum* LMLSAUL 14-1 and *A. fumigatus* LMLPS 13-4 negatively affected the overall levels of the cellulase enzyme system when compared to the monocultures, particularly with respect to cellulase (FPase) and β-glucosidase. The antagonistic behavior of *Trichoderma* towards the *A. fumigatus* and the competition for space and nutrients by both fungi might have contributed to non-improvement in the cellulase enzyme system. An *A. fumigatus* LMLPS 13-4 and *T. harzianum* LMLBP07 13-5 co-cultivation enhanced the production of cellulase (FPase) and endoglucanase activities, but β-glucosidase activity was lower than that observed in the monoculture of the respective fungi. In general, the co-cultivation of *T. longibrachiatum* LMLSAUL 14-1 with *A. fumigatus* LMLPS 13-4 and *T. harzianum* LMLPS 13-5 with *A. fumigatus* LMLPS 13-4 produced a balanced cellulase enzyme system, in terms of individual enzyme activities, which demonstrate the synergistic action required for the complete hydrolysis of holocellulose material. Nonetheless, co-cultivation of *T. longibrachiatum* with *A. fumigtus* produced a sufficient level of β-glucosidase, an important enzyme to complete hydrolysis. An increased level of β-glucosidase in the co-culture of *A. oryzae* and *A. niger* was observed in a study by Hu et al. [102]. Results from the current study suggest that co-culturing does not stimulate an increase in the overall cellulase enzyme system secretion, but it may activate (or induce) specific enzymes. This observed effect could be as a result of lack of compatibility. *Trichoderma longibrachiatum* and *T.* harzianum are known to produce other metabolites that inhibit the growth of some *Aspergillus* and *penicillium* species, including *Cladosporium spherospermum*, *Cryptococcus neoformans,* and *Fusarium oxysporum* [103–107],

making them dominant in co-cultivation conditions. Fungal strain compatibility is essential in order to promote better colonization and substrate penetration, and the synthesis of some metabolites by one organism may have a positive effect on the other organisms in terms of the increased production of cellulolytic enzymes [108,109].

Other co-culturing studies have also shown an improvement in enzyme production. It was noted that the co-culturing of the *T. reesei* QM 9414 mutant (*T. reesei* M) and *A. niger* improved β-glucosidase activity by 4-fold and 2-fold, respectively, compared to the monoculture on water hyacinth [110]. Ingale et al. [99] reported a cellulase activity of 4.05 FPU/g d.s and an endoglucanase activity of 13.15 U/g d.s by *A. ellipticus* and *A. fumigatus* cultivated on pre-treated BPS.

Dueñas et al. [111] reported a cellulase activity of 18.7 FPU/g d.s and β-glucosidase activity of 38.6 U/g d.s by co-culturing *T. reesei* LM-UC4 and *Aspergillus phoenicis* QM 329, which was higher than the activity found in the monocultures during SSF using sugarcane bagasse. There was also an improved production of the cellulase enzyme system in SSF of sugarcane bagasse supplemented with soymeal by co-culturing *T. reesei* LM-UC4E1 and *A. niger* [112]. *Trichoderma* and *Aspergillus* species have similar enzyme–gene regulation mechanisms and secrete large quantities of active cellulases; therefore, the co-culturing of multiple strains to produce diverse substances of biotechnological importance using plant polysaccharides requires further investigation [113].

In summary, the production of plant cell wall-degrading enzymes by fungi is a complex process that is dependent on obtaining the correct signal that triggers a cascade of transcriptional factors. The regulation of transcriptional factors is affected by physiological parameters, such as starvation, pH, temperature, etc. [114].

5. Conclusions

The *Trichoderma* and *Aspergillus* species investigated are mesophilic fungi with different temperature optima for the production of the cellulase enzyme system. The moisture content of the BPS substrate had an impact on the production of the specific enzymes. Pre-treatment of the BPS substrate did not improve the overall production of the cellulase enzymes for *T. longibrachiatum* LMLSAUL 14-1. SSF on pre-treated BPS by *T. harzianum* LMLBP07 13-5 and *A. fumigatus* LMLPS 13-4 enhanced the production of β-glucosidase compared with untreated BPS. The highest β-glucosidase activities by *T. harzianum* LMLBP07 13-5 and *A. fumigatus* LMLPS 13-4 were attained from SSF on alkaline- and acid-pre-treated BPS, respectively. There was an improved cellulase enzyme system with respect to FPase and endoglucanse activities by co-cultivation of *A. fumigatus* LMLPS 13-4 and *T. harzianum* LMLBP07 13-5. The results suggested that banana pseudostem has the potential as a cheap and abundant substrate for the production of cellulases.

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