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Optimization of Fermentation Conditions for Cellulase Production by *Trichoderma harzianum* PK5 Obtained from Decaying Palm Kernel Cake

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Abstract

Cellulases are considered to be among the most important enzymes in the commercial market and in various industries. Their applications are widespread, leading to increased demand and high associated costs. This necessitates the search for more cost-effective cellulases from microorganisms. Therefore, the aim of this study was to optimize cellulase production by Trichoderma harzianum PK5 using the One Factor at a Time (OFAT) approach. The effects of carbon, nitrogen, and various environmental factors were studied in both submerged and solid-state fermentation setups by adjusting one factor at a time based on the optimal conditions established from the previous condition. Copra meal and KNO_3 were identified as the best complex carbon and nitrogen sources, respectively, for cellulase production by Trichoderma harzianum PK5. The optimal pH of 4.0, moisture concentration of 125% (v/w), inoculum size of 8%, temperature of 30°C, and an incubation time of 7 days were determined as the optimal conditions for cellulase production by this isolate, resulting in an enzyme titre of 252.54±7.73 U/gds in solid-state fermentation. It was found that cellulase enzyme production by the isolate was constitutive. In conclusion, cellulase production by T. harzianum PK5 was significantly optimized using the OFAT approach.

Keywords: Trichoderma harzianum; Optimization; Solid State Fermentation; Cellulase; OFAT

INTRODUCTION

The most prevalent polysaccharide in the world is cellulose (Wang *et al.*, 2020). This highly organized cellulobiose polymer, also known as 4-D-glucopyranose or D-glucopyranosyl-ß-1, makes up more than half of the bulk of wood. About 10,000 glycosyl units make up the cellulose chain in native wood cellulose, forming fibrils—long molecule bundles held together by several strong hydrogen interactions between neighboring hydroxyl groups. According to Malherbe and Cloate (2003), cellulosic materials have crystalline units divided by less organized amorphous regions that could be targets of chemical and biological attacks. The enzymes known as cellulases are responsible for breaking down cellulose.

Jahangeer *et al.* (2005) describe cellulase as a class of hydrolytic enzymes that degrade cellulose to its glucose substituents. Several enzymes that work in concert make up the multi-enzyme system known as cellulase (Grassin and Fauquembergue, 1996). In the process of depolymerizing cellulose, three primary enzymes are involved: endoglucanase, exoglucanase, and ß-glucosidase. These three enzymes, endoglucanase and exoglucanase, are referred to as "real cellulase" since they produce cellubiose and glucose through their actions. Endoglucanase primarily targets the amorphous portions of the ß-1,4-glucan chain of cellulose by acting randomly on it, leading to the formation of most oligosaccharides (Walsh, 2002). Exoglucanase, on the other hand, releases cellubiose by acting on the non-reducing end of cello-oligosaccharides. The depolymerization process is completed when cellubiase, also known as ß-glucosidase, breaks down the resulting cellubiose into two glucose molecules (Himmel *et al.*, 1994). The critical role of

cellulases in biomass breakdown cannot be overstated.

Cellulases are produced by a variety of species, with fungi and bacteria being the most prominent producers (Immanuel *et al.*, 2007; Antia *et al.*, 2018). Many industries, including detergent, textile, food, leather, paper, bioethanol, and pharmaceuticals, have found numerous uses for cellulases.

From their natural habitat, Jahangeer *et al.* (2005) isolated 115 fungal isolates, of which 78 (67.83%) were determined to be cellulolytic. The majority of the organisms belonged to the genera *Aspergillus, Trichoderma, Fusarium, Alternaria, Penicillium,* and *Rhizopus*. These isolates, originating from the environment, exhibited maximum cellulase activity at 37°C and within the pH range of 4.0 to 4.8. In fermentation trials, it was demonstrated that *Aspergillus niger* could utilize paper cellulose for cellulase production, with optimal production conditions observed at day 7, pH 5.0, and 45°C (Devi and Kumar, 2012). Ahmed *et al.* (2009) investigated the cellulase enzymes (EXG, EG, and BGL) of *Trichoderma* harzianum, showing that the organism achieved optimal production at pH 5.5, temperature of 28°C, and incubation time of 5 days.

The focus on cellulase production by many researchers is driven by the high cost of commercial cellulases. It is essential to study cellulase production using diverse, inexpensive, and readily available biomasses as the carbon source. Therefore, this study aims to optimize cellulase production by *T.* harzianum PK5 using the One Factor at a Time (OFAT) approach.

MATERIALS AND METHODS

Collection of Samples

Source of the isolates, substrates, and other chemicals

A fungus, *Trichoderma harzianum* PK5 identified to molecular level by PCR (with accession number KR871217), which was previously screened and selected for cellulase production was obtained from the culture collection unit of the Department of Microbiology, Akwa Ibom State University for this research. Palm Kernel Cake, Potato Peels and Copra Meal were obtained from retailers around the Akwa Ibom State. Carboxymethyl cellulose was purchased from Sigma Chemicals (St. Louis, Mo, USA. All other chemicals used were also of analytical grade.

Preparation of Inoculum

The spores of *Trichoderma harzianum* PK5 were obtained from a five (5) day old slant by shaking them off the surface of matured mycelia using 10 mL of sterile distilled water. The total spore count was standardized using a neubauer counting chamber at $1x10^6$ spores/mL (Sae-lee, 2007).

Enzymes Assay

The cellulase activity assay was conducted following the protocol outlined by Mabrouk and El Ahwany (2008), and the quantification of reducing sugars was carried out using the Dinitrosalicylic Acid (DNS) method as described by Miller (1959). The assay mixture for cellulase activity consisted of 0.5 mL of 0.5% (w/v) carboxymethyl cellulose (CMC) in 50 mM sodium citrate buffer at pH 5.0, combined with 0.5 mL of the culture broth. The reaction mixture was then incubated at 50 degrees Celsius for 30 minutes.

One milliliter of DNS reagent was added after the incubation. The reaction mixture was boiled for 10 minutes. The development of the red-brown color was measured at 540 nm using a spectrophotometer. One unit of enzyme activity (U) is defined as the amount of enzyme that releases one µmol of glucose per minute under the test conditions.

Optimization and production characteristics of cellulase by the *Trichoderma harzianum* PK5

The different conditions that impact the production of cellulase by *Trichoderma harzianum* PK5 were systematically varied to identify the optimal conditions for its production. These conditions encompassed the carbon source, nitrogen source, fermentation time, inoculum size, initial moisture content of the substrates, etc.

Production of cellulase enzyme using different carbon sources

The influence of various carbon sources on cellulase production by *Trichoderma harzianum* PK5 was examined. Birchwood xylan, locust bean gum, carboxymethyl cellulose, glucose, xylose, and arabinose (refined carbons) were studied in submerged fermentation, while copra meal,

palm kernel cake, and soybean meal (agro-waste) were investigated in solid-state fermentation (Mabrouk and El Ahwany 2008).

Cellulase Production by the Trichoderma harzianum PK5 using Solid State Fermentation

Ten (10) grams of each of the agro-waste complex carbon sources, dried to a constant weight, were wetted with 5 mL of ML1 medium (pH 5.0), representing a 50% (v/w) moisture concentration (Santiago *et al.*, 2007). The selective basal medium ML1 was composed of the following (g/l): yeast extract, 0.5; casein peptone, 1.0; KH_2PO_4, 1.0; $(NH_4)_2HPO_4$, 1.0; $MgSO_4.H_2O$, 0.7. The flask contents were autoclaved for 15 minutes at 121°C, then cooled to 30°C. Once the substrates had cooled, fungal spores were added at a 4% (v/w) inoculum concentration (1 mL of inoculum broth contained $1x10^6$ spores), and the inoculum was incubated at 30°C for 72 hours in a stationary mode.

The extraction of the produced enzyme was carried out by adding 50 mL of 50 mM Sodium citrate buffer (pH 5.0) to the fermented material. The contents of each flask were then centrifuged at 10000 rpm for 10 minutes. The resulting supernatant was considered the crude enzyme preparation, and the cellulase activities were assayed as previously described.

Submerged Fermentation of Refined Carbon Substrates for Cellulase Production by the Trichoderma harzianum PK5

Each of the refined carbon sources was added to 50 mL of the basal ML1 medium (pH 5.0) at 1% (w/v). The flasks' entire contents were autoclaved at 121 degrees Celsius for fifteen minutes and then cooled to 30°C. Each isolate was added to the setup at 4% (v/w) of the fungal spores (1 mL of the inoculum broth contained $1x10^6$ spores). They were then incubated at 30 °C for 7 days under either a static or stationary condition. After the fermentation process, the entire contents of the broth were centrifuged for ten minutes at 10,000 rpm. The supernatant was assayed for cellulase activity as previously outlined.

Effect of nitrogen sources on the production of Cellulase by the Trichoderma harzianum PK5

In order to study the effect of nitrogen sources on cellulase production by the fungal isolate, eight nitrogenous nutrient components - six of which were organic (urea, peptone, yeast extract, casein, tryptone, and soybean meal) and two of which were inorganic (KNO3 and NH4NO3) - were added to 10 grams of the solid substrate at a 4% (w/w) ratio, while maintaining 30% (v/w) moisture content, pH 5.0, and 7 days of incubation at 30°C (Darah and Omar 2010).

Effect of initial pH on the production of Cellulase by the Trichoderma harzianum PK5

Regulating the substrate's pH was achieved by using buffered basal media at various pH values (4.0, 5.0, 6.0, and 7.0). We employed 50 mM sodium citrate buffer (pH 3.5–6.0) and 50 mM sodium phosphate buffer (pH 6.0–7.0) for this purpose. The system was sterilized for 15 minutes at 121°C before inoculation with 4% (v/w) fungal spores (1 mL of the spore solution contained $1x10^6$ spores) and subsequent incubation at 30°C for 7 days.

Effect of Moisture Content on the Production of Cellulase by the Trichoderma harzianum PK5

The effect of the substrate's moisture content on enzyme production was evaluated at five different moisture levels (50, 75, 100, 125, and 150%; v/w) by adding basal medium to the substrates before sterilization. Subsequently, the setup was incubated at 30°C for 7 days post-inoculation with 4% (v/w) inoculum. The pH of the basal media was maintained at 4.0 using sodium citrate buffer (50 mM).

Effect of Inoculum Size on the Production of Cellulase by the Trichoderma harzianum PK5

By inoculating 10 grams of sterilized substrates with 1%, 2%, 4%, 8%, and 10% (v/w) spore solutions, we investigated the effects of various inoculum sizes on cellulase formation. Prior to this, we moistened the substrates to the specified moisture content of 125% (v/w) using ML1 broth made with 50 mM sodium citrate buffer (pH 5.0). The setups were then sterilized at 121°C for 15 minutes, cooled to 30°C, and inoculated with different concentrations of fungal spores. Each milliliter (1 mL) of the

inoculum broth contained 1x10^6 spores. The setups were then incubated at 30˚C for 7 days.

Effect of Temperature on the production of Cellulase by the Trichoderma harzianum PK5

Four different incubation temperatures - 27˚C, 30˚C, 35˚C, and 40˚C - were used to cultivate the isolate for cellulase production. Ten (10) grams of the substrates were moistened with ML1 broth (prepared with 50 mM sodium citrate buffer; pH 5.0) at moisture levels of 125% (v/w). The setups were then sterilized at 121˚C for 15 minutes, after which they were cooled to 30˚C before being inoculated with 8% (v/w) fungal spores of the respective enzyme-producing isolates. One milliliter (1 mL) of the inoculum broth contained 1x10^6 spores/mL. The setup was incubated at 30˚C for 7 days.

Effect of Incubation Time on the Production of Cellulase by the Trichoderma harzianum PK5

The time course of fermentation for cellulase production was studied by assaying the amount of each enzyme produced in the crude enzyme filtrates obtained at specific time intervals. Ten (10) grams of the substrates were moistened as previously determined with ML1 medium (prepared with 50 mM sodium citrate buffer; pH 4.0), sterilized at 121˚C for 15 minutes, and then cooled to 30˚C before being inoculated with 8% (v/w) fungal spores of the respective enzyme-producing isolates. Each milliliter (1mL) of the inoculum broth contained 1x10^6 spores. The setups were then incubated at 30˚C for 14 days, with samples taken for enzyme assay on days 3, 5, 7, 10, and 14.

Regulation of Cellulase production in the Isolate

The impact of easily utilizable carbohydrates on cellulase production was investigated using glucose, galactose, arabinose, and xylose. Ten (10) grams of each complex substrate were supplemented with 0.2 g/g of the mentioned simple sugars at various stages. The substrates were appropriately moistened with ML1 medium (prepared with 50 mM sodium citrate buffer; pH 5.0) and sterilized at 121˚C for 10 minutes. Following sterilization, the fermentation flasks containing the substrates were cooled to 30˚C and then inoculated with 4% (v/w) fungal spores of the respective cellulase-producing isolates. Incubation occurred at 30˚C for 7 days, after which the enzymes produced were collected, assayed, and compared to the enzyme quantities generated in the absence of these simple sugars (Mabrouk and El Ahwany, 2008).

RESULTS

Effect of different carbon sources on the production of cellulase by the *Trichoderma harzianum* PK5

The isolate was capable of growing on both refined and complex substrates, producing cellulase enzymes in varying quantities. The highest cellulase production by *Trichoderma harzianum* PK5 was observed in the presence of carboxymethyl cellulose, with an activity of 106.06±6.47 U/gds. Copra meal, a cost-effective substrate, also exhibited a significant inductive effect on cellulase production by this isolate, with an activity of 95.09±1.65 U/gds. Cellulase production in the presence of soybean meal,

Table 1: Cellulase Produced from Different Carbon Sources by *Trichoderma harzianum* PK5 via Solid State Fermentation at 50% Moisture Level.

	Enzyme/ Enzyme Activity (U/gds)
Substrates	Cellulase
Soy-Bean meal	*36.14±2.60$^{e\neq}$
Copra meal	95.09±1.65^{b}
PKC	30.60±2.84^{e}
Xylan	36.34±0.46^{e}
CMC	+**106.06±6.47^{a}**
LBG	28.39±2.16^{e}
Xylose	77.87±2.53^{c}
Arabinose	50.07±3.20^{d}
Glucose	28.72±5.99^{e}

**Results are shown in triplicate as Mean±SE of the data.*
≠Using Duncan's Multiple Range Test Plus, values with the same superscript do not differ significantly (P<0.05).
+In the same column, values in bold type are higher than other values.

palm kernel cake, xylan, locust bean gum, and glucose did not show significant differences (Table 1).

Effect of different nitrogen sources on the production of cellulase by the *Trichoderma harzianum* PK5

The nitrogen sources tested included peptone, yeast extract, urea, casein, soybean meal, and tryptone as organic nitrogen sources, while KNO_3 and $(NH_4)_2SO_4$ were used as inorganic nitrogen sources (Table 2). Control fermentations without additional nitrogen were also conducted. The presence of KNO3 significantly enhanced cellulase production, resulting in an enzyme activity of 127.71±1.96 U/gds, which was 34.00 units higher than the control experiment. Cellulase production with other nitrogen supplements was lower than that of the control, with ammonium sulphate providing the lowest support for cellulase production by *Trichoderma harzianum* PK5, yielding an enzyme activity of 16.50±0.16 U/gds.

Effect of pH on the production of cellulase by *Trichoderma harzianum* PK5

The impact of the initial pH of the culture medium on cellulase production was examined across the pH range of 3.0 to 7.0 (Table 3). Optimal cellulase production was achieved at pH 4.0, resulting in an enzyme activity of 137.46±0.71 U/gds. A notable decline in enzyme activity was observed at pH 5.0 (80.35±2.34 U/gds). Enzyme activity at pH 6 and 7 was recorded at 66.14±0.57 and 66.49±0.44 U/gds, respectively, with no significant differences noted among these values.

Effect of different moisture levels on the production of mannan-degrading enzymes (MDEs)

Table 4 shows the effect of moisture content on the production of cellulase by the fungus. There was a steady increase in cellulase production from 80.94±3.24 U/gds at 50% (v/w) moisture level to 140.08±7.57 U/gds at 100% (v/w) moisture level. Cellulase production peaked at 125% (v/w) moisture with a recorded activity of 225.04±6.31 U/gds. Eighty (80.00) units of this activity was lost at 150% (v/w) moisture level. Moisture concentration of 125% (v/w) favoured the production of cellulase by *T. harzianum* PK5.

Effect of different inoculum sizes on the production of cellulase by the *Trichoderma harzianum* PK5

Inoculum sizes based on a standardized number of spores ($1x10^6$ spores/mL) were examined using spore concentrations of 1, 2, 4, 8, and 10% (v/w) of dry substrates. Cellulase production increased sharply with an increase in inoculum size; the recorded activity was 11.86+0.33 U/gds at 1% (v/w) inoculum concentration (Table 5). This increased significantly to 237.85+0.65 U/gds at 8% (v/w). However, at 10% (v/w), enzyme production reduced by 64.00 units. The optimal production of cellulase was observed at an inoculum concentration of 8% (v/w) (with the inoculum broth containing $1x10^6$ spores/mL).

Effect of different incubation temperature on the production of cellulase by the *Trichoderma harzianum* PK5

The effect of incubation temperature on cellulase production by the fungal isolate was examined. Cellulase production was highest at 30°C, and the productions at 27°C and 35°C were not significantly different. The highest activity recorded for these enzymes was 247.51±18.23 U/gds. There was a gradual reduction in the quantities of enzymes produced from 35°C to 40°C (Table 6).

Effect of incubation time on the production of cellulase by the *Trichoderma harzianum* PK5

Cellulase production reached a peak enzyme activity of 252.54±7.73 U/gds after 7 days of incubation. The activity at day 4 was low (64.59±5.32 U/gds). After the 7th day of production, there was a gradual reduction in the quantity of enzymes produced. Enzyme activity of 223.14±10.50 U/gds was recorded on day 18; however, this value was not significantly different from the 206.81±1.34 U/gds at day 10 and 232.55±0.40 U/gds at day 14 (Table 7). Production of cellulase peaked on the seventh (7th) day of production.

Repressive and Inductive effects of simple sugars on the production of cellulase by the *Trichoderma harzianum* PK5

Cellulase production was not repressed in the presence of the simple sugars used. Without any

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supplemented simple sugar, cellulase activity of 230.73±0.37 U/gds was recorded, and this value remained constant throughout the experiment. However, the highest value of 239.01±0.16 U/gds was achieved when galactose was used as the simple sugar (Table 8).

Table 2: Cellulase Produced in the Presence of Different Nitrogen Sources on by *Trichoderma harzianum* PK5 using the best supporting complex carbon source in a solid state fermentation at 50% moisture level.

	Enzyme/Enzyme Activity (U/gds)
Nitrogen Sources	Cellulase
Peptone	*54.69±0.92$^{d\neq}$
Yeast Extract	37.62±5.77^{f}
Urea	24.79±0.90^{g}
KNO_3	+127.71±1.96^{a}
Casein	72.81±0.06^{c}
Soybean Meal	74.21±0.64^{c}
Tryptone	48.08±0.54^{e}
Ammonium Sulphate	16.50±0.16^{h}
Control	93.60±0.86^{b}

**Results are shown in triplicate as Mean±SE of the data.*
≠Using Duncan's Multiple Range Test Plus, values with the same superscript do not differ significantly (P<0.05).
+In the same column, values in bold type are higher than other values.

Table 3: Cellulase Production at different pH by *Trichoderma harzianum* PK5 using the best carbon and nitrogen sources in a solid state fermentation at 50% moisture level.

	Enzyme/Enzyme Activity (U/gds)
pH	Cellulase
3	*38.76±0.71$^{d\neq}$
4	**+137.46±0.71^{a}**
5	80.35±2.34^{b}
6	66.14±0.57^{c}
7	66.49±0.44^{c}

**Results are shown in triplicate as Mean±SE of the data.*
≠Using Duncan's Multiple Range Test Plus, values with the same superscript do not differ significantly (P<0.05).
+In the same column, values in bold type are higher than other values. Carbon sources: Copra Meal; Nitrogen sources: KNO3

Table 4: Cellulase Production at different moisture content by *Trichoderma harzianum* PK5 in solid state fermentation incubated at 30 ºC for 7days

	Enzyme/Enzyme Activity (U/gds)
Moisture (%, v/w)	Cellulase
50	*80.94±3.24$^{d\neq}$
75	111.90±5.23^{c}
100	140.08±7.57^{b}
125	**+227.04±6.31^{a}**
150	143.15±0.08^{b}

**Results are shown in triplicate as Mean±SE of the data.*
≠Using Duncan's Multiple Range Test Plus, values with the same superscript do not differ significantly (P<0.05).
+In the same column, values in bold type are higher than other values. Carbon sources: Copra Meal; Nitrogen sources: KNO_3,

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Table 5: Effect of inoculum size (% v/w) on production of cellulase by *Trichoderma harzianum* PK5 in solid state fermentation incubated at 30°C for 7days using the best moisture levels (125% (v/w).

Inoculum size (%, v/w)	Enzyme/Enzyme Activity (U/gds) Cellulase
1	*11.86±0.33^{e}≠
2	40.34±2.75^{d}
4	141.99±0.52^{c}
8	+**237.85±0.65^{a}**
10	173.09±6.63^{b}

Carbon sources: Copra Meal; Nitrogen sources: KNO_3; 1 ml of the inoculums contained about 1x10^6 spores
**Results are shown in triplicate as Mean±SE of the data.*
≠Using Duncan's Multiple Range Test Plus, values with the same superscript do not differ significantly (P<0.05).
+In the same column, values in bold type are higher than other values.

Table 6: Cellulase Production at different incubation temperatures on by *Trichoderma harzianum* PK5 in solid state fermentation incubated for 7days using the best moisture levels and inoculum sizes.

Temperature (°C)	Enzyme/Enzyme Activity (U/gds) Cellulase
27	*212.89±0.27^{b}≠
30	+247.51±18.23^{a}
35	207.29±2.12^{b}
40	108.14±0.19^{c}

Carbon sources: Copra Meal; Nitrogen sources: KNO3, (Cellulase)
**Results are shown in triplicate as Mean±SE of the data.*
≠Using Duncan's Multiple Range Test Plus, values with the same superscript do not differ significantly (P<0.05).
+In the same column, values in bold type are higher than other values.

Table 7: Cellulase Production at different incubation time (days) by *Trichoderma harzianum* PK5 in solid state fermentation incubated at 30°C for 7days using the best inoculums, moisture and initial pH levels.

Incubation Time (days)	Enzyme/Enzyme Activity (U/gds) Cellulase
4	*64.59±5.32^{d}≠
7	+ **252.54±7.73^{a}**
10	206.81±1.34^{c}
14	232.55±0.40^{b}
18	223.14±10.50^{bc}

Carbon sources: Copra Meal; Nitrogen sources: KNO3 *(Cellulase)*
**Results are* presented in triplicate as *Mean±SE of the* data.
Using Duncan's Multiple Range Test Plus, values with the same superscript do not differ significantly (P<0.05).
+In the same column, values in bold type are higher than other values.

Table 8: Repressive and Inductive effects of simple sugars (0.2 g/g) on the production of cellulase by *Trichoderma harzianum* PK5 in a solid state fermentation.

Sugars (0.2 g/g)	Enzyme/Enzyme Activity (U/gds) Cellulase
Control	*230.73±0.37^{c}≠
Glucose	230.91±0.16^{c}
Galactose	+**239.01±0.16^{a}**
Arabinose	236.09±0.57^{b}
Xylose	229.24±0.20^{d}

Carbon sources used in the experiment were Copra Meal, and the nitrogen source was KNO3.
**Results are* presented in triplicate as *Mean±SE of the* data.
Using Duncan's Multiple Range Test Plus, values with the same superscript do not differ significantly (P<0.05).
+values in bold type within the same column indicate higher values compared to other values.

DISCUSSION

Copra meal supported the production of cellulose more in a solid-state fermentation experiment. The use of simple sugars such as glucose and mannose as sole carbon sources resulted in a low yield of this enzyme. This observation is consistent with the findings of Youssef *et al*. (2006), who studied the effect of carbon sources on mannan-degrading enzyme production by fungal isolates. They found that copra meal was the most preferred carbon source, leading to the highest enzyme and protein yields in all isolates. Conversely, cultures containing simple sugars as sole carbon sources yielded the lowest enzyme levels (Youssef *et al*., 2006; Onilude *et al*., 2012a).

Trichoderma harzianum PK5 required urea for enhanced cellulase production compared to other nitrogen sources. Kheng and Omar (2005) observed that urea increased xylanase production in A. niger USM A1I more effectively than peptone, yeast extract, ammonium sulfate, and ammonium nitrate. Various studies have highlighted the use of diverse nitrogen sources as supplements in enzyme production from lignocellulosic materials, each showing different levels of support for enzyme production (Onilude *et al*., 2012b).

The initial pH of the production medium significantly influenced cellulase production by *T. harzianum* PK5, with the optimum pH being 4.0. Previous studies on fungal enzyme production have also identified the pH range of 4.0 to 6.0 as optimal for enzyme production, aligning with the optimal growth pH of these fungi (McTigue *et al*., 1994; Ahmed *et al*., 2009; Devi and Kumar, 2012). Extracellular microbial enzymes are produced at high titers when the growth medium pH matches the optimal growth pH of the microorganisms involved (Antia *et al*., 2019).

In solid-state fermentation for the production of microbial metabolites, moisture plays a vital role and significantly influences the fermentation process (Pandey, 2003). Depending on the substrate and organism used, the moisture requirement can range from as low as 50% (v/w) to as high as 150% (v/w). This variability may be due to differences in the rate of water absorption by different substrates (Pandey, 2003). Moisture levels below the optimum can decrease the solubility of nutrients in solid substrates, reduce swelling, and create higher water tension (Ikasari and Mitchell, 1994). These factors can lead to insufficient nutrient availability, resulting in decreased microbial growth and enzyme production (Venkateswarlu *et al*., 2000).

From the results obtained as well as those of other researchers, the optimum inoculum density for enzyme production in solid-state fermentation varies widely depending on the growth rate of the isolate used and the substrate they are meant to colonize and utilize. An inoculum size below the optimum will affect the time needed for cells to proliferate, colonize, and utilize the substrate to produce the desired products (Ramachandran *et al*., 2004). Conversely, a higher inoculum density above the optimum has been generally observed to adversely affect enzyme production (Ibrahim *et al*., 2012).

The cellulase in this study was best produced at a temperature of 30°C. This is in line with the observations of other authors (De Loannes *et al*., 2000; Koseki *et al*., 2006; and Fritz *et al*., 2008) that most fungal extracellular enzymes are optimally produced at 30°C. A temperature of 30°C has been widely reported as the best temperature for enzyme synthesis by fungal isolates (Rashid *et al*., 2012; Ufot *et al*., 2022). Enzyme production at 30°C is applauded because it is still within room temperature, thus energy costs can be saved.

In a similar study on cellulase production by *T. harzianum*, Ahmed *et al*. (2009) reported an optimum production time of 5 days for this enzyme. Generally speaking, the time course required to reach maximum levels of enzyme production may be affected by various factors, including the presence of different ratios of amorphous to crystalline segments in the production substrates. Wanderley *et al*. (2004) asserted that the incubation time for enzyme production by microorganisms depends on the nutrients present in their growth medium as well as other cultural conditions.

Cellulase production by *T. harzianum* PK5 was not affected in the presence of these simple sugars. One reason why its production of cellulase remained unaffected in the presence of simple and easily utilizable sugars could be that the gene responsible for cellulase production in *T. harzianum* PK5 is not subject to catabolite repression (Biswas *et al*., 1990). Furthermore, the work of Großwindhager *et al*. (1999) may explain this deviation from the norm as observed in *T. harzianum* PK5. Großwindher

et al. (1999) worked on the production of MDEs by S. rolfii under derepressed conditions and they reported that continuously feeding low non-repressing amount of glucose in a culture of S. rolfii led to increase in production of mannanase and mannosidase.

CONCLUSIONS

Using the OFAT approach, the production of cellulase by *T. harzianum* PK5 was greatly optimized. The cellulase enzyme concentration in the production medium increased from 106.06 ± 6.47 to 252.54 ± 7.73 U/gds when the fermentation conditions were maintained at pH 4.0, temperature of 30°C, inoculum size of 8% v/w, and an incubation time of 7 days, utilizing copra meal and KNO_3 as carbon and nitrogen sources. Although the production of cellulase in this organism was found to be constitutive, the variation of nutritional and environmental factors significantly induced higher levels of the enzyme to be synthesized.

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