Nigerian Research Journal of Engineering and Environmental Sciences 5(1) 2020 pp. 56-65

**Nigerian Research Journal of Engineering and Environmental Sciences**
**Journal homepage: www.rjees.com**

**Original Research Article**

# Cellulase Production using Fungi Grown on Sawdust

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**ARTICLE INFORMATION**

*Article history*:
Received 02 Apr, 2020
Revised 27 Apr, 2020
Accepted 02 May, 2020
Available online 30 June, 2020

*Keywords*:
Cellulase
Sawdust
Lignocellulose
*Penicillium chrysogenum*
Fermentation

**ABSTRACT**

*Agricultural wastes composed mainly of lignocellulosic biomass are mostly abundant materials in the environment and can be utilized to produce valuable products such as enzymes. The influence of pretreatment on sawdust media for cellulase production by fungi and the effect of varied pH were investigated in this study. The pretreatment methods used before fermentation of the media were thermal (using heat) and alkaline (using sodium hydroxide (NaOH)). The parameters analyzed were pH, fungal biomass and cellulase activity. The identified fungal isolates from sawdust with the highest zone of hydrolysis were Penicillium chrysogenum and Trichoderma harzianum. From the results, the highest fungal biomass and cellulase activity of 0.26±0.01 mg/g and 2.35±0.04 U/g respectively after 8 days, were obtained from the heat pretreated sawdust medium inoculated with Penicillium chrysogenum (HSPc). The least biomass and cellulase activity of 0.07±0.01 mg/g and 0.36±0.07 U/g were from the without sawdust (control) medium inoculated with Trichoderma harzianum (WSTh). Fungal growth and cellulase activity for HSPc were statistically significant when compared to other media ($p < 0.05$) after 8 days of fermentation. Thus, the study revealed that sawdust as a waste could be utilized as a promising substrate for the production of cellulase and yield can be enhanced using heat pretreatment.*



## 1. INTRODUCTION

Lignocelluloses, which are the major component of agricultural residues are the most abundant natural substance found on earth, but are highly underutilized (Lucia 2008; Reddy *et al.,* 2015). This material has been found to be a rich source of organic components comprising cellulose, hemicellulose and lignin with a significant proportion of 45-55%, 24-40% and 18-25 % respectively (Trevorah and Othman 2015). These agricultural wastes are discarded into the environment where they cause flooding, pollution and destruction of the aesthetic value of the environment, and a good way to overcome these problems is by utilizing them

as substrates for bioconversions to value added product such as enzymes (Oshoma *et al.*, 2017; Ahmed El-Imam *et al.,* 2019a,b).

One of such lignocellulosic waste materials is sawdust. Saw dust is a promising lignocellulosic waste composed of fine particles of wood that is generated during cutting and shredding of wood with saw and other wood processing machines (Eze *et al.*, 2011). It is the main by-product of wood processing in sawmills and can be reprocessed into particle board, burnt in a sawdust burner or used to generate heat for other milling operations. It is however generally regarded as a waste and is dumped in the environment. The utilization of saw dust by microbes for the production of an important industrial enzyme like cellulase is a welcome development that will help in reducing pollution and also valorize the waste (Behera *et al.*, 2017; Ahmed El-Imam *et al.,* 2019a,b).

Cellulases are a family of hydrolytic enzymes that hydrolyse β-1,4-glycosidic linkages of the cellulose polymer and other related cellooligosaccharide derivatives (Nadagouda *et al.*, 2016; Sari *et al.*, 2017). Cellulase is an important enzyme for the conversion of cellulosic materials into simple sugars that serve as feed-stock for the production of different chemicals and fuels via fermentation (Chinedu *et al.*, 2011). Cellulases are employed in many important processes such as in bioprocessing industries, preparation of medicines, food production, baking, waste treatment, perfumes, textile and paper industries (Nadagouda *et al.*, 2016; Behera *et al.*, 2017).

Microbial species that are capable of producing cellulase consists majorly of bacteria and fungi. These microbes are better isolated from the environment where the desired substrate is found, deposited or disposed (Lennox *et al.*, 2010). Some of these important microorganisms include *Aspergillus*, *Trichoderma*, *Phanerochaetes*, *Chytridiomycetes*, *Fibrobacteres*, *Clostridium* sp. and *Bacillus* (Watanabe and Tokuda, 2001; Motta *et al.*, 2013; Behera *et al.*, 2017). Although bacteria and fungi can produce cellulolytic enzymes, fungal enzymes are usually preferred because they are extracellular and are usually secreted in quantifiable amounts during growth in both submerged (Bhoosreddy, 2012) and solid state fermenters (Nwodo-Chinedu *et al.*, 2005; Bhoosreddy, 2012; Sari *et al.*, 2017). Fungal strains are the main producers of cellulase and secrete higher amounts of the enzyme than bacterial cells, with *Trichoderma* as the leading producer (Bischof *et al.*, 2016; Sari *et al.*, 2017).

Pretreatment is a necessary step in the utilization of lignocellulosic biomass for cellulase production (Trevorah and Othman 2015). Pretreatment changes the compositional and structural properties of the plant material by ensuring the complete disruption of the plant cell wall, reduction in its crystalline nature and increase in its pore size resulting in improved enzymatic contact with the polysaccharide (Gray *et al.*, 2006). Physical or mechanical, acid, alkali and enzymatic pretreatments are used in ensuring that there is an adequate amount of residual sugar released from the lignocellulosic material, that will be used by organisms for the bioconversion process (Gabhane *et al.*, 2014).

Currently, cellulase is commercially produced by submerged fermentation (SMF) processes due to ease of handling and simple control of environmental parameters. The limitation of cellulase production could be contamination rate of fermentation media from SMF processes (Mrudula and Murugammal, 2011). Solid state fermentation (SSF) has emerged as a potential technology for the production of microbial products, due to the ability of filamentous fungi to grow well on solid substrate and the process yield can be improved (Pandey *et al.*, 2000; Mrudula and Murugammal, 2011; Reddy *et al.*, 2015). The process is particularly applicable for developing nations because of its inherent advantages over conventional submerged fermentation which includes extremely lower operational costs, process simplicity and stabilities of the enzymes produced (Pandey *et al.*, 2000). Thus, in this work, the influence of pretreatment on sawdust biomass for cellulase production by fungi.

## 2. MATERIALS AND METHODS

### 2.1. Sample Collection and Preparation

Sawdust (*Alstonia boonei*) was aseptically collected from Ugbebo sawmill in Benin City, Nigeria. The sawdust was pretreated by weighing 100 g, sun-drying, oven drying at 80 $^{o}$C for 1h and then cooling. Another 100 g of saw dust was pretreated using 3 % (w/v) sodium hydroxide solution at a ratio of 1:10 (substrate: solution) for 3 h at room temperature. Afterward, the NaOH pretreated sample was rinsed using sterile distilled water until a neutral pH was attained, sun-dried and dried in oven at 80 $^{o}$C for 1 h until a constant weight is achieved. All the pretreated substrates were blended with a blender separately. All the blended samples were filtered to small particle sizes with 0.1 mm mesh sieve. The filtered samples were stored in an airtight polythene bags at room temperature until required.

### 2.2. Isolation and Characterization of Fungi

One gram of soil sample collected from the sawmill was serially diluted and plated on Potato Dextrose Agar (PDA) amended with 10 mg/l of chloramphenicol and incubated at 28 ± 2 °C for 5 days. Colonies from the culture were purified and sub-cultured. Stock cultures were maintained on PDA slant and stored at 4 $^{o}$C. The isolates were identified by phenotypic characteristics which included colony colour observation and growth pattern studies, as well as microscopic characteristics (Naveenkumar and Thippeswamy, 2013).

### 2.3. Screening for Cellulase Producing Fungi

Plates were spot inoculated with spore suspension of pure cultures on carboxymethyl cellulose (CMC) agar containing (g/L) NaCl (0.5), $KH_2PO_4$ (1.0), $MgSO_4.7H_2O$ (0.5), $MnSO_4.H_2O$ (0.01), $NH_4NO_3$ (0.3), $FeSO_4.7H_2O$ (0.01), CMC (10.0) and agar (12.0) for the secretion of cellulase and incubated at 28 ± 2 °C for 3 days. After incubation, the plates were flooded with 1% Congo red solution for 15 minutes then de-stained with 1M NaCl solution for 10 min. The diameter of zone of clearance around each colony was measured. Fungal isolates with highest zone of clearance were selected for cellulase production.

### 2.4. Determination of Inoculum Size

The fungal inocula were prepared from sub-cultured fungal isolates each on potato dextrose agar (PDA) plates and incubated for 5 days. The incubated cultured plates were flooded with 20 ml of sterile 1% v/v Tween 80 solution, to harvest the spores. The spore suspensions were filtered with sterile muslin cloths. The number of spores were counted using a haemocytometer and inoculum size of $10^6$ spore/mL of each fungal spore was used to inoculate all the media respectively (Dahot and Noomrio, 1992).

### 2.5. Fermentation Process for Cellulase Production

Heat and NaOH pretreated sawdust samples were used for the investigation. From the pretreated sample, 5 g was weighed into 100 ml Erlenmeyer flask containing 20 ml of standard Toyama's basal medium, (% w/v) $CaCl_22H_2O$, (0.05), $(NH_4)_2SO_4$, (1.0); $KH_2PO_4$ (0.3), $Mg(SO_4)_2.7H_2O$ (0.05) (Toyama and Ogawa, 1977) at a pH of 6. A control (without sawdust) experiment was set up containing the Toyama's basal medium solidified with agar in place of sawdust. The flasks were autoclaved at 121 $^{o}$C for 15 min and cooled. The flasks containing pretreated sawdust and control (without sawdust) were inoculated using the resultant spore suspension with inoculum size of $10^6$ spore/mL. The media were incubated at temperature of 28 ± 2 $^{o}$C and the fungal biomass, cellulase activity, pH of medium and reducing sugars were determined at every 2 days interval for 10 days.

### 2.6. Analytical Methods

Cellulase activity was determined by mixing 1.0 ml of 1% (w/v) CMC (prepared in 50 mM Na-acetate buffer pH 5.3) with 1.0 ml of crude extracellular enzyme source and incubating at 50 °C for 15 min. The reaction was stopped by the addition of 3.0 ml of 3, 5-dinitrosalicylic acid (DNS) and the contents boiled for 15 min. The colour developed was read at 540 nm using a spectrophotometer. The amount of reducing sugar liberated was quantified using glucose as standard. One unit of cellulase is defined as the amount of enzyme that liberates 1 µmol of glucose equivalents per minute under the assay conditions (Mandels *et al*., 1981). For every enzyme activity assay, a negative control experiment was set up with the same protocol except that the enzyme was replaced with 1 ml, 50 mM Na-acetate buffer (pH 5.3). A blank reaction mixture was set up in which both the enzyme and substrate was replaced with 2 ml 50 mM Na-acetate buffer. The result was recorded as the mean of the triplicate readings and activities were read on a glucose standard curve using net absorbance values.

Evaluation of pH was through the use of pH meter (3305 Jenway, England).

Reducing sugar was determined according to the method of Miller (1959). Three (3) ml of DNS reagent was added to 3 ml of crude enzyme in a lightly capped test tube to avoid loss of liquid due to evaporation, then heat mixture at 90 °C for 5-15 min to develop the red-brown colour and then 1 ml of a 40% potassium sodium tartrate (Rochelle salt) solution to stabilize the colour. After cooling to room temperature absorbance was read at 575 nm.

Fungal biomass was determined by direct estimation. Duplicate samples of 1 g fermented substrate from each flask were weighed into 15 ml centrifuge tubes and 5 ml of sodium sulphate (150 g/l) was added to each tube. The tubes were centrifuged at 12000 rpm for 15 min and were repeated thrice to ensure complete separation of fungal biomass from substrate. The fungal mass with lower density than the substrate floated while the substrate settles to the bottom, the biomass alone was transferred to a pre-weighed filter paper and dried in hot air oven at 85 °C to obtain a constant weight then the dried filter paper was weighed and the biomass was the difference in the filter paper (Desgranges *et al*., 1991)

### 2.7. Statistical Analysis

Assays were carried out in triplicates, means and standard deviations (SD) were determined using SPSS version 23.

## 3. RESULTS AND DISCUSSION

The fungal isolates identified and characterized from the saw dust contaminated soil were *Aspergillus niger, Aspergillus flavus, Fusarium oxysporum, Penicillium chrysogenum and Trichoderma harzianum* (Table 1). From the screened fungal isolates result (Table 1), all isolates had varied zone of clearance for cellulase production profile with *Trichoderma harzianum* having the highest (6.00±0.58 mm) followed by *Penicillium chrysogenum* (5.67±0.33 mm). Statistically, there was no significant difference among these two isolates ($p > 0.05$). The least zone of clearance was recorded for *A. flavus* (3.67±0.67 mm) and *Fusarium oxysporum* (2.67±0.33 mm). The various zones of clearing were significantly different ($p<0.05$) from *Penicillium chrysogenum and Trichoderma harzianum*.

The fungal isolates showed varied cellulolytic activity when screened on CMC Agar. This observation is similar to that of Fossi *et al*. (2015) that microbial species for enzyme production are best assayed for in the environments where the substrates are available. The result of cellulolytic activity screening showed that

*Penicillium chrysogenum* and *Trichoderma harzianum* had the highest zone of clearance and will be an efficient and high yielding cellulolytic fungi species.

Table 1: Screening of fungi isolates for cellulase production on CMC Agar

| Fungi isolates | Zone of clearance (mm) |
|---|---|
| *Aspergillus flavus* | $3.67^{ab} \pm 0.67$ |
| *Aspergillus niger* | $4.33^{b} \pm 0.33$ |
| *Fusarium oxysporum* | $2.67^{a} \pm 0.33$ |
| *Penicillium chrysogenum* | $5.67^{c} \pm 0.33$ |
| *Trichoderma hazianum* | $6.00^{c} \pm 0.58$ |

Key: Values are mean +- standard error of three replicates; a-c: different characters in the column indicate values with significant difference ($p<0.05$)

Various authors have reported these fungi as versatile cellulase producers in the bioprocessing industry (Chinedu *et al*., 2011; Okeke *et al*., 2015). Andersen *et al*. (2016) reported that among the *Penicillium* species, *P. chrysogenum* is a good candidate for cellulase high productivity yield while Nwodo-Chinedu *et al*. (2005) reported its ability to synthesize efficient cellulase system capable of solubilizing cotton fibers.

The isolates with the highest zone of clearance, *T. harzianum* and *P. chrysogenum* were employed in cellulase production via fermentation of pretreated sawdust. The pretreated media used were heat, NaOH and control (without sawdust). The media were designated as heat treated sawdust medium inoculated with *T. harzianum* (HSTh), NaOH treated sawdust medium inoculated with *T. harzianum* (NSTh), without sawdust (control) medium inoculated with *T. harzianum* (WSTh), heat treated sawdust medium inoculated with *P. chrysogenum* (HSPc), NaOH treated sawdust media inoculated with *P. chrysogenum* (NSPc) and without sawdust (control) mediium inoculated with *P. chrysogenum* (WSPc).

As shown in Table 2, there was an increase in the pH of all treatments except in the control. Also, all NaOH treated sawdust media showed higher pH with the highest recorded on day 10 by *T. harzianum* ($7.25 \pm 0.10$). For the heat-treated sawdust media, *P. chrysogenum* showed the highest pH ($6.05 \pm 0.06$) on day 8.

Table 2: Changes in pH of fungi isolates during production of cellulase using pretreated sawdust and without sawdust (control) media

| Treatment | Fermentation period (Days) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 6 | 8 | 10 |
| HSTh | $5.50 \pm 0.06$ | $5.36 \pm 0.08$ | $5.50 \pm 0.10$ | $6.05 \pm 0.08$ | $6.10 \pm 0.06$ | $6.10 \pm 0.05$ |
| NSTh | $5.50 \pm 0.06$ | $5.60 \pm 0.05$ | $6.5 \pm 0.04$ | $6.82 \pm 0.06$ | $6.97 \pm 0.08$ | $7.25 \pm 0.10$ |
| WSTh | $5.50 \pm 0.05$ | $5.53 \pm 0.05$ | $5.55 \pm 0.05$ | $5.54 \pm 0.05$ | $5.50 \pm 0.05$ | $5.30 \pm 0.05$ |
| HSPc | $5.50 \pm 0.06$ | $5.34 \pm 0.05$ | $5.42 \pm 0.08$ | $5.80 \pm 0.07$ | $6.05 \pm 0.06$ | $5.95 \pm 0.08$ |
| NSPc | $5.50 \pm 0.06$ | $5.40 \pm 0.05$ | $5.45 \pm 0.06$ | $5.66 \pm 0.09$ | $6.20 \pm 0.07$ | $6.40 \pm 0.09$ |
| WSPc | $5.50 \pm 0.05$ | $5.53 \pm 0.05$ | $5.55 \pm 0.05$ | $5.54 \pm 0.05$ | $5.50 \pm 0.05$ | $5.30 \pm 0.05$ |

Key: Heat-treated sawdust medium inoculated with *T. harzianum* (HSTh)
NaOH-treated Sawdust medium inoculated with *T. harzianum* (NSTh)
Without sawdust (Control) medium inoculated with *T. harzianum* (WSTh)
Heat-treated Sawdust medium inoculated with *P. chrysogenum* (HSPc)
NaOH-treated Sawdust media inoculated with *P. chrysogenum* (NSPc)
Without sawdust (Control) medium inoculated with *P. chrysogenum* (WSPc)

There was an initial drop in the pH of heat-treated sawdust media from day 2 to day 4 of fermentation before it rose again and this is likely due to the production of acidic metabolites (Deng *et al.,* 2012; Bischof *et al.,* 2016). The pH of the medium plays an important role in the growth and secretion of cellulase by the

microorganisms. Changes in pH observed during fermentation affects enzyme stability in the medium (Sethi and Gupta 2014). This investigation showed that maximum enzyme activity was observed in the medium at an approximate pH of 6 which is similar to reports by Deng *et al.* (2012) that observed highest total cellulase production at pH 5 and 6. Changes in pH values for NaOH treated sawdust media increased accordingly, this was due to the ionization state of $Na^+$ ion which neutralized the released acid formed thereby increased the pH value of the NaOH treated sawdust media (Sartori *et al*., 2015). This results contrasts that of Sridevi *et al*. (2015), who reported that NaOH treatment at room temperature resulted in a considerable removal of different components, resulting in an increase in cellulose content from 47.7 to 63.1 % in pretreated sawdust. Higher pH was reported by Sajith *et al*. (2016) to reduce biomass hence resulting to the inactivation of cellulase enzyme.

The various pretreated media were found to support growth of the fungal isolates as shown in Figure 1 The highest and lowest fungal biomass observed after 8 days were for HSPc and WSTh with values 0.26 ± 0.01 mg/g and 0.07±0.01 mg/g respectively. The fungal biomass from HSPc medium was statistically significant ($p< 0.05$) to that of other media at 8 days of fermentation.

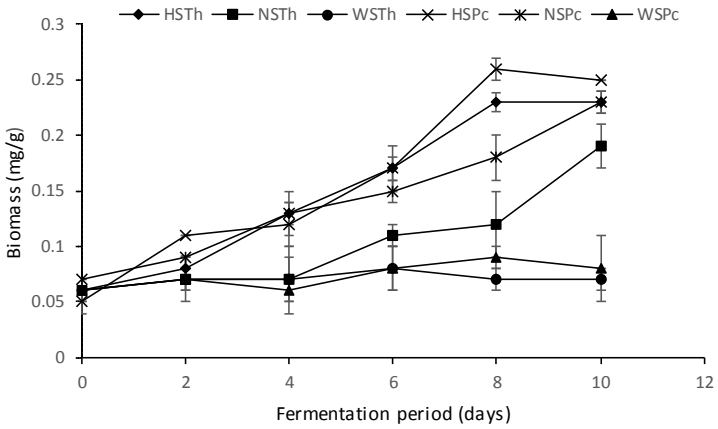


Figure 1: Fungal biomass produced during fermentation of pretreated sawdust medium by *T. harzanium* and *P. chrysogenum* and without sawdust (control) media

It was observed that the fungal biomass in the various pretreated media had a normal growth curve as the fungal biomass reading continued to rise, peaking at day 8 with heat having the highest fungal biomass afterwards dropping at day 10. The variation in fungal growth in the various media could be as a result of the varying concentration of cellulose released during various pretreatment (Suhas *et al*., 2013). The metabolism of cellulose makes carbon and energy available which induced fungal growth through enzyme synthesis hence, high yield of cellulase (Behera *et al*., 2017).

There was reduction in the reducing sugar activities of all treatment throughout the period of fermentation (Table 3). In heat treated sawdust media, the least concentration on day 10 was observed in *P. chrysogenum* (0.24 ± 0.01 g/mL) fermentation medium while *T. harzianum* fermentation medium was 0.2 ± 0.01 g/mL.

Table 3: Reducing sugar (g/L) of isolated fungi on pretreated sawdust media during fermentation and without sawdust (control) media

| Treatment | Fermentation period (Days) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 6 | 8 | 10 |
| HSTh | 0.42 ± 0.01 | 0.40 ± 0.01 | 0.36 ± 0.01 | 0.33 ± 0.01 | 0.32 ± 0.01 | 0.28 ± 0.01 |
| NSTh | 0.33 ± 0.01 | 0.30 ± 0.01 | 0.29 ± 0.00 | 0.29 ± 0.01 | 0.27 ± 0.01 | 0.27 ± 0.00 |
| WSTh | 0.11 ± 0.01 | 0.10 ± 0.01 | 0.09 ± 0.01 | 0.08 ± 0.01 | 0.07 ± 0.01 | 0.07 ± 0.01 |
| HSPc | 0.42 ± 0.01 | 0.40 ± 0.01 | 0.36 ± 0.01 | 0.32 ± 0.01 | 0.29 ± 0.01 | 0.24 ± 0.01 |
| NSPc | 0.33 ± 0.01 | 0.30 ± 0.01 | 0.28 ± 0.00 | 0.27 ± 0.01 | 0.26 ± 0.00 | 0.26 ± 0.01 |
| WSPc | 0.11 ± 0.01 | 0.10 ± 0.01 | 0.09 ± 0.01 | 0.08 ± 0.01 | 0.07 ± 0.01 | 0.06 ± 0.01 |

Key: Heat-treated sawdust medium inoculated with *T. harzianum* (HSTh)
NaOH-treated sawdust medium inoculated with *T. harzianum* (NSTh)
Without sawdust (control) medium inoculated with *T. harzianum* (WSTh)
Heat-treated sawdust medium inoculated with *P. chrysogenum* (HSPc)
NaOH-treated sawdust media inoculated with *P. chrysogenum* (NSPc)
Without sawdust (control) medium inoculated with *P. chrysogenum* (WSPc)

The levels of cellulase production were observed to be dependent on the type of pretreatment applied. Cellulase activity was found to be highest at 8 d of fermentation for the pretreated media inoculated with the isolates (Figure 2). The highest cellulase of 2.35 ± 0.06 U/g was produced from heat treated sawdust media inoculated with *P. chrysogenum* (HSPc) while the least (0.36±0.07 U/g) was from WSTh medium. The cellulase activity from HSPc medium was statistically significant ($p < 0.05$) to that of other media at 8 days of fermentation.

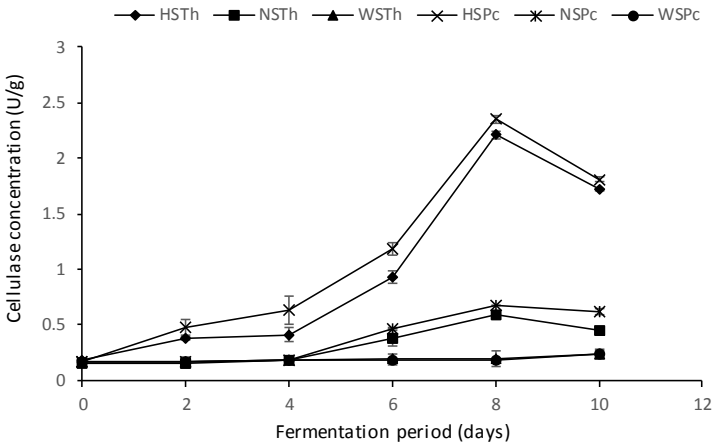


Figure 2: Cellulase concentration of *T. harzanium* and *P. chrysogenum* during the fermentation of pretreated sawdust and without sawdust (control)media

When compared with alkali treated samples, heat treated samples were observed to have lower pH, higher yield of fungal biomass, lower residual sugar and higher cellulase activity. Trevorah and Othman, (2015), reported that the use of moderate temperature in pretreatment can prevent the formation of furfural, hydroxymethylfurfural and organic acids, thereby reducing the loss of usable sugars. Preventing the formation of these products assists the successful operation of fermentation processes as they are inhibitory to fungal growth (Zhao *et al*., 2013). This study has shown that the method of pretreatment of lignocellulosic materials affects the efficiency of the saccharification process and that heat treatment resulted in a higher yield of reducing sugars during hydrolysis than alkali pretreatment. According to Acharya *et al.* (2008),

contact time and proper washing till neutral pH, are important for the improvement of hydrolysis of sawdust pretreated with NaOH as residual NaOH and released organic acids could hinder growth of fungi and therefore decrease cellulase production, as was observed in this study. Sari *et al.* (2017 reported that Penicillium is known to be a good candidate for cellulase production from pretreated lignocellulosic biomass. Chinedu *et al.* (2011) also reported that *Penicillium chrysogenum* had a higher cellulase activity of 0,67 U/mg than *Trichoderma harianum* (0.39 U/mg) from waste cellulosic materials. Heat treated sawdust media inoculated with *P. chrysogenum* had a more stable pH for cellulase production than *T. harzianum*. The high fungal biomass of *P. chrysogenum* as observed in this study could be the reason for the efficient utilization of fermentable sugars and consequential high cellulase activity. The result showed an increasing yield of cellulase activity up to day 8 of incubation, there after declined. The possible reason may be due to nutrient depletion and accumulation of toxic substances (Mai *et al*., 2018).

## 4. CONCLUSION

The investigation showed that sawdust supported the growth of fungal isolates and cellulase production via solid state fermentation. From the pretreatment methods used, heat pretreated sawdust media inoculated with *P. chrysogenum* recorded the highest yield of cellulase. This is a promising strategy for the efficient utilization of sawdust for cellulase production and will in turn curb environmental pollution caused by its improper disposal of the waste.

## 5. ACKNOWLEDGMENT

The authors acknowledge the assistance of the laboratory Staff of Mycofarm and allied synergy limited, Isiohor, Benin City, Nigeria, for the success of this study

## 6. CONFLICT OF INTEREST

There is no conflict of interest associated with this work.

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