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**Optimization of Exo-Β-Glucanase Production in *Aspergillus flavus* Using Rice Bran and Oil Palm Fibre as Substrates**

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**Abstract**

In this study, β-glucanases were generated from *Aspergillus flavus* isolated from rice and oil mill waste sites, utilizing rice bran as well as oil palm fiber as substrates through submerged fermentation. Optimal activity of the crude *Aspergillus flavus* exo-β-glucanases was observed at 2.36 and 2.34 µmol/ml/min after 96 hours (rice bran) and 120 hours (oil palm fiber) of incubation, respectively. Maximum activity occurred at pH 6.5 (rice bran) and 5.0 (oil palm fiber), with temperatures of 35°C (rice bran) and 40°C (oil palm fiber). The addition of yeast extract notably enhanced enzyme activity to 2.31 µmol/ml/min. Purification via 80% ammonium sulfate precipitation yielded purification folds of 1.18 (rice bran) and 1.15 (oil palm fiber), while dialysis tubing achieved folds of 1.50 (rice bran) and 2.50 (oil palm fiber). Enzyme activities were modulated by chloride salts and inhibitors, resulting in variable effects on activity levels. This study underscores the efficacy of rice bran and oil palm fiber in submerged fermentation for producing Aspergillus flavus exo-β-glucanase.

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*Exo-β-glucanases;*

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*saccharification;*

*submerge fermentation*

**Introduction**

Enzymes are biological substances, usually proteins that greatly increase the speed of nearly all chemical reactions (Stryer *et al*., 2002). These can be extracted from various sources such as plants, animal tissues, and microorganisms. Microbial enzymes are economically useful as they are easier to culture, grow quickly, and can be easily genetically modified compared to plants and animals. Some of the microbial enzymes produced commercially include amylases, lipases, proteases, β-glucanases, and cellulases. β-Glucanase specifically targets β-glucan, a component found in the cell walls of cereals and certain fungi, breaking it down into glucose monomers or saccharide oligomers (Pitson *et al*., 1993). This enzyme is effective against fungal pathogens by hydrolyzing β-glucan, thereby generating D-glucose as a carbon source (Tang-Yao, 2002). β-Glucan is a polysaccharide composed of D-glucose units linked by glycosidic bonds, predominantly seen in the endosperm and aleurone cells of plants in the Poaceae family (BeMiller *et al*., 2008). The highest concentrations of β-glucan are found in oats and barley, with smaller amounts present in cereals such as corn, rice, rye, wheat, and sorghum (Ragaee *et al*., 2008; Tiwari and Cummins, 2009). β-Glucan varies in how much you can get from different grains (Wood, 2010). Many β-glucans are useful in medicine and are targeted by antifungal drugs called echinocandins. Because β-glucan is a natural compound that affects biology, people are interested in using it as a food supplement, immune booster, and possibly as a drug (Vaclave *et al*., 2019). β-Glucanases are enzymes that break down β-glucans, which are major parts of plant cell walls like cellulose, callose, and cereal β-glucans. Fungal cell walls also have β-1,3;1,6-glucans (Bacic *et al*., 2009). Different types of β-glucanases are found in microbes, plants, snails, and sea urchins, such as beta-1,3-glucanase, beta-1,6-glucanase, cellulase, and xyloglucan-specific endo-beta-1,4-glucanase (Hrmova and Fincher, 2001; Leubner-Metzger, 2005). β-Glucanase is used in many industries, like making beer, adding to laundry soap, and breaking down waste from farming and industry to make syrup for animal feed (Bajomo and Young, 1992; Taniguchi and Honnda, 2009; Woodward and Wiseman, 1982; Arnau *et al*., 2020). *Aspergillus* species, including *A. niger*, *A. oryzae*, *A. awamori*, and *A. terreus*, are major producers of β-glucanase (Frisvad *et al*., 2018; Wang *et al*., 2012; Raza *et al*., 2011). However, the production process must be efficient and consider factors such as purity and safety, as well as the presence of mycotoxins. Exo-β-glucanase can be produced by solid state fermentation and extracted through centrifugation and filtration (Coughlan, 1985; Doughari, 2011).

**Materials and Methods**

**Collection of Samples**

Rice bran and oil palm fiber were gathered from a rice mill and an oil palm mill using clean plastic containers. Soil samples from these mills were placed in MacCartney bottles. These samples were then taken by private car to the Microbiology laboratory at Modibbo Adama University of Technology in Yola, Nigeria, for analysis.

**Isolation of Fungi**

To isolate fungi, a 1-gram soil sample was placed into a glass test tube with 9 ml of sterilized distilled water, resulting in a 10 ml suspension. This mixture was shaken for 10 minutes at room temperature. Subsequently, the suspension underwent serial dilution up to 10-7 to decrease microbial concentrations to a countable level. For fungal growth, 0.1 ml of the 10-6 dilution was plated onto Sabouraud Dextrose Agar (SDA) with the addition of 0.5 ml of chloramphenicol to inhibit bacterial growth. The culture was incubated at 30 °C for 3-5 days and then checked for fungal growth, which was then purified through sub-culturing three times onto SDA plates to obtain pure fungal isolates, which were stored on SDA slants at 4 ˚C (Adeleke *et al*., 2012).

**Identification of Fungi**

A drop of lactophenol cotton blue stain (LPCBS) was placed on a clean, grease-free microscope slide. Using a sterile needle, fungal growth from a 3-day-old culture was carefully picked and gently immersed in the LPCBS. A cover slip was then placed over the sample. The slide was viewed under a x40 objective lens of the microscope, allowing for detailed observation of fungal structures such as hyphae, spores, and other microscopic features. The arrangement of the hyphae and the nature of their fruiting bodies were examined and morphologically characterized, and these observations were compared to those in the Atlas of Mycology (Barnett and Hunter, 1972; James and Natalie, 2001).

**Screening of Fungal isolates for Exo-β-glucanase Production**

The isolates were grown on Petri dishes using a modified Czapek-Dox Agar known as MCDA, where beta-glucan oat powder replaced sucrose as the carbon source. Following incubation, 50mM iodine was applied to the plates to observe the clear zones around the colonies (Yogesh *et al*., 2009). The fungal strains that displayed noticeable clear zones in the beta glucan oat agar were selected as potential exo-β-glucanase producers (Ahmed and Mustafa, 2013).

**Characterizing the Molecular Properties of the Isolates Selected for Screening**

Following the DNA extraction procedure described elsewhere (Romos-Ibarra *et al*., 2021), molecular identification was conducted. The extracted DNA from *Aspergillus* was amplified using the universal primer set ITS 1 (5’ TCC GTA GGT GAA CCT GCG G 3’) and ITS 4 (5’ TCC TCC GCT TAT TGA TAT GC 3’). The amplified fragments underwent purification to eliminate PCR reagents, followed by verification of their integrity using a 1% Agarose gel. Subsequently, the PCR product was forwarded to Inqaba, a third-party company, for sequencing. The obtained results were edited using MEGA 6 and compared against Type strain sequences from the GenBank database, confirming the isolate as a eukaryote possessing 80S ribosomes. The large 60S subunit consisted of 5S rRNA, 5.8S rRNA, and 28S rRNA, while the small subunit was 40S RNA containing 18S rRNA. The Blast results revealed the presence of two internal transcribed spacer regions, ITS1 and ITS2, between the 18S, 5.8S, and 28S ribosomal subunits. This confirmed that the isolate was 99.66% identical to *Aspergillus flavus* (Table 1).

**Table 1. Confirmation of Aspergillius flavus isolate based on BLAST Analysis**



**Quantification of Fungi spores for Fermentation**

 The process of preparing a spore suspension involved transferring 1 ml of seed culture into 100 ml Erlen Meyer flasks containing 50 ml of fresh sterile Sabouraud Dextrose Broth. The mixture was then shaken for 10 minutes on a rotary shaker at 250 rpm. To count the spores, the hemacytometer and coverslip were thoroughly cleaned and dried with clean cotton wool. The coverslip was placed at the center of the hemacytometer, and approximately 9 microliters of the cell suspension were added to one of the two counting chambers using a clean micropipette and tip. Care was taken to avoid bubbles and to ensure the chambers were neither under-filled nor over-filled. Fragments in contact with the borders of the 0.1 mm³ corner squares were not counted. The spore count was determined by averaging the number of cells per square in the four corner squares and applying the formula: Spores/ml = (n) x 104, where 'n' is the average number of cells per square from the four corners counted (Sserumaga, 2012).

**Preparation of Substrates**

 The rice bran underwent washing, drying, milling using an electric blender, and was then sifted through a sieve with a mesh size ranging from 20 mm to 40 mm. Particles ranging from 0.42 mm to 0.85 mm were gathered to use as a solid base for submerge fermentation. Oil palm fiber was squeezed with a sugarcane juice machine to get rid of the juice. Once squeezed, the fiber was left in the sun for five days, cut into smaller bits, and sifted to ensure they were all less than 2 mm in size. The dried fiber was packed into plastic bags and stored at room temperature. It would later be used in the fermentation process to produce enzymes.

**Production of Crude Exo-β-glucanase**

The following steps were performed to create a medium for *Aspergillus flavus* growth: a 6 g sample of rice bran was mixed with 200 ml of 0.5% (w/v) (NH4)2SO4 solution using a magnetic stirrer in a 250 ml beaker. To the beaker, 0.1 g MgSO4 .7H2O, 0.2 g KH2PO4, and 0.6 g yeast extract were added. The pH was set to 5.5 using a 1N HCl solution. Subsequently, the solution was transferred to a sterilized 250 ml Erlenmeyer flask and autoclaved at 121 ºC and 15 psi for 15 minutes. Following cooling, 10 ml of *Aspergillus flavus* inoculum was introduced, and the mixture was left to ferment for 5 days at room temperature (30-32 ºC) on an electric rotary shaker set at 250 rpm. The identical process was then repeated using oil palm fibre in place of rice bran (Doughari, 2011).

**Extraction of Exo-β-glucanase**

After fermenting rice bran and oil palm fibre with the inoculum for 5 days, the culture fluids were filtered individually using Whatman filter paper (WFP 1). The filtrates were subsequently pH-adjusted to 5.5 and centrifuged at 5000 rpm for 30 minutes at 4 ºC to obtain crude enzyme extracts. These extracts were filtered once more with WFP 1, buffered to pH 5.5, and the resulting clear supernatant was employed as the source of crude exo-β-glucanase (Doughari, 2011; Ahmad and Mustafa, 2013).

**Crude Enzyme Activity Assay**

The activity of beta-glucanase in the culture filtrate was assessed by measuring the amount of reducing sugars produced in the reaction mixture using a modified Miller method (Miller, 1959) described by Doughari, 2011. The reaction mixture contained 500 μl of a 3% (wt/vol) beta-glucan substrate dissolved in 50 mM phosphate buffer at pH 7, supplemented with 500 μl of the crude enzyme solution. Prior to measurement, the spectrophotometer was calibrated using both an enzyme blank (positive control) and a reagent blank (negative control). The enzyme blank was prepared by combining 500 μl of substrate with 500 μl of crude enzyme solution, while the reagent blank consisted of 500 μl of phosphate buffer and 500 μl of substrate. After incubating all tubes, including experimental and control samples, at 37°C for 30 minutes, the reaction was stopped by adding 1 ml of 3,5-dinitrosalicylic acid and boiling the mixture in water for 15 minutes. After cooling, the absorbance of the reaction mixture was measured at 540 nm. The quantity of reducing sugar produced was determined using glucose as a standard. Beta-glucanase activity was quantified as the amount of enzyme needed to produce 1 μmol of glucose per minute under specified assay conditions, including pH and temperature (Shao-jun *et al*., 2001).

**Proteins Determination**

The Bradford method (1976) was employed to determine the concentration of proteins, which was calculated by extrapolating the standard curve using bovine serum albumin (BSA) as the standard.

**Optimization of Exo-β-glucanase Production**

The isolate underwent testing across different conditions to evaluate how the growth medium influenced exo-β-glucanase production. The study analyzed the effects of carbon and nitrogen availability, incubation period, temperature variations, and pH levels following the methods described by Thakur *et al*. (2010).

**Influence of Carbon Sources on Exo-β-glucanase Production**

The influence of carbon sources on enzyme production was investigated using 3% w/v concentrations of agricultural residues such as banana peels, orange peel, and yam peels, alongside pure chemicals including glucose, sucrose, maltose, and beta-glucan oat. The resulting culture supernatants were subsequently analyzed for exo-β-glucanase activity.

**Impact of Nitrogen Sources on Exo-β-glucanase Production**

The basal medium was supplemented with individual nitrogen sources such as peptone, urea, yeast extract, and (NH4)2SO4 to examine their effect on exo-β-glucanase production. Urea and peptone were replaced by alternative nitrogen sources. The production medium was prepared using methods similar to those used previously, but yeast extract and ammonium sulfate were substituted with different nitrogen sources. The culture supernatants were then assessed for exo-β-glucanase activity.

**The Optimal Incubation Time for Exo-β-glucanase Production**

The production media containing the fungal isolate were incubated for 24, 48, 72, 96, 120, 144, and 168 hours at 37°C and pH 5.5 to determine the most suitable time for production.

**The Optimal Temperature for Exo-β-glucanase Production**

The media containing the fungal isolate were incubated at various temperatures (30 ± 2°C, 35°C, 40°C, 45°C, and 50°C) to identify the optimal temperature for enzyme production.

**The Optimal pH for Exo-β-glucanase Production**

The production media containing the fungal isolate were incubated using 50mM buffers of varying buffering capacities, namely sodium acetate buffer (3.0-5.0), sodium phosphate buffer (5.5-7.5), and sodium carbonate (8.0-9), to identify the optimal pH for exo-β-glucanase production.

**Partial Purification of Exo-β-glucanase**

The salting out method was employed to precipitate proteins using ammonium sulfate fractionation, accompanied by gradual and continuous stirring. Two precipitation steps using ammonium sulphate were carried out in the creation of an enzyme concentrate. The crude enzyme was first brought to saturation with a 30% concentration of ammonium sulphate, followed by centrifugation at 4°C for 30 minutes at 5000 rpm. The supernatant was separated from the pellet and re-dissolved in 20 ml of sample buffer. An 80% concentration of ammonium sulphate was gradually added to the solution and stirred for an hour under cooling conditions. The mixture was transferred to a centrifuge tube and centrifuged, after which the supernatant was discarded and the pellet or precipitate was collected. The pellet was then suspended in the minimal volume of 50 mM phosphate buffer (pH 7), and the enzyme was subsequently dialyzed against 50 mM phosphate buffer (pH 7) (Bradford, 1976).

**Characterization of the Partially Purified Exo-β-glucanase**

**Impact of temperature on the partially purified *Aspergillus flavus* exo-β-glucanase**

Temperature effects on the partially purified *Aspergillus flavus* exo-β-glucanase were assessed following the protocol by Tari *et al*. (2008). The enzyme was placed in a 50 mM phosphate buffer and incubated at temperatures between 30 and 65 °C for 30 minutes. Samples were collected to measure residual β-glucanase activity using β-glucan oat, as detailed earlier (Doughari, 2011).

**Effect of pH on the partially purified *Aspergillus flavus* exo-β-glucanase**

The influence of pH on the partially purified *Aspergillus flavus* exo-β-glucanase was investigated using 50 mM buffers spanning pH 3 to 9, with β-glucan from oats utilized as the substrate, following established methods [18]. The buffer systems used in the study included sodium acetate buffer (pH 3.0-5.5), sodium phosphate buffer (pH 6.0-7.5), and sodium carbonate buffer (pH 8.0-9.0) (Thakur *et al*., 2010).

**The impact of surfactants and EDTA on the partially purified exo-β-glucanase from *Aspergillus flavus***

The impact of surfactants and EDTA on the partially purified exo-β-glucanase from *Aspergillus flavus* was assessed. The experiment involved using various surfactants and EDTA at 1%, 3%, and 5% concentrations, including Triton X-100, Tween 80, Cetrimide, and EDTA. The enzyme solution (500 μl) and the inhibitors (500 μl) were pre-incubated for 10 minutes at 40°C prior to starting the reaction with 3% oat glucan as the substrate. The remaining β-glucanase activity was then determined using previously described methods (Raza *et al*., 2011). A sample of the enzyme without surfactants or EDTA served as the control and was found to have 100% residual activity (Ramachandran, 2005).

**The influence of chloride salts on the partially purified β-glucanase from *Aspergillus flavus***

The influence of chloride salts on the partially purified β-glucanase from *Aspergillus flavus* was also evaluated. The assay was conducted in the presence of various chloride salts with concentrations of 1 mM, 2 mM, and 5 mM, including NaCl, CoCl2, CuCl2, MgCl2, NH4Cl, CaCl2, and Kcl2. The enzyme solution (500 μl) and salt concentrations (1 mM, 2 mM, and 5 mM) were pre-incubated for 30 minutes at 40°C before beginning the reaction with 3% oat glucan as the substrate. The residual β-glucanase activity was then assessed using established methods [20]. The enzyme sample without the salt served as the control and had 100% activity (Ramachandran, 2005; Doughari, 2011).

**Preparation of the Calibration Curve**

The glucose standard curve was constructed by varying the glucose concentration from 0 to 1 mg/ml. To do this, 0.1 g of glucose was dissolved in 100 ml of distilled water and aliquots of 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1 ml were taken and added to test tubes with an equal volume of distilled water, resulting in glucose concentrations of 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 μg/ml respectively. 1 ml of 3,5-dinitrosalicyclic acid reagent was then added to each sugar solution and the absorbance was measured at 540 nm. Finally, a graph of the optical density against glucose concentration was plotted (Bellasio *et al*., 2014).

**Statistical Analysis**

The data acquired from characterizing and studying the impact of diverse physiochemical factors—including pH, temperature, carbon sources, incubation duration, nitrogen sources, salt, and inhibitors—on exo-β-glucanase production and activity were statistically analyzed. Significant variations in enzyme activity or production due to different substrates (rice and oil palm fiber) were determined using One-Way ANOVA, which compares means across multiple independent groups. The results were presented as the standard error of the mean (SEM) and were calculated using GraphPad Prism 5 software.

**Results and Discussion**

In this research, twelve fungal strains were collected from natural sources, among which *Aspergillus flavus* displayed heightened exo-β-glucanase activity when cultivated on a specialized medium enriched with β-glucan derived from oats. Confirmation of the isolate's identity as *Aspergillus flavus* was achieved through microscopic examination and molecular analysis, revealing a genetic profile spanning 550 bases that matched the NCBI standard strain. Rice bran emerged as the most effective carbon source for exo-β-glucanase production (2.36 µmol/ml/min), outperforming oil palm fiber, yam peel, and banana peel. Conversely, maltose yielded the lowest enzyme production levels (Figureure 1). The findings indicated that organic nitrogen supported exo-β-glucanase production better than inorganic nitrogen did (Figureure 2). The duration of incubation influenced exo-β-glucanase production differently for rice bran and oil palm fiber, with peak levels observed on the 4th and 6th days of fermentation, respectively (Figureure 3). Optimal pH conditions for exo-β-glucanase production were found to be pH 6.5 for rice bran and pH 5.0 for oil palm fiber (Figureure 4), while maximum temperatures were 35 ºC and 40 ºC, respectively (Figureure 5). Following purification, the specific activity of the crude enzyme increased notably after both ammonium sulfate precipitation and dialysis, resulting in purification folds of 1.18 and 1.50, respectively (Table 2). Further characterization revealed that the enzyme exhibited peak activity at 40 ºC and 45 ºC, with optimal pH values of 5.0 and 5.5 for rice bran and oil palm fiber, respectively (Figureures 6 and 7). However, enzyme activity was significantly reduced in the presence of metal chlorides at concentrations of 1 mM, 2 mM, and 5 mM (Table 3). Cetrimide supported enzyme activity, while EDTA had an inhibitory effect (Table 4).

Figure 1. Effect of carbon source on *Aspergillus flavus* crude exo-β-glucanase production

Figure 2. Effect of nitrogenous source on *Aspergillus flavus* crude exo-β-glucanase production

Figure 3. Effect of length of incubation period on *Aspergillus flavus* crude exo-β-glucanase production

Figure 4. Effect of pH on *Aspergillus flavus* crude exo-β-glucanase production

Figure 5. Effect of temperature on *Aspergillus flavus* crude exo-β-glucanase production

**Table 2. Partial purification of Aspergillius flavus exo- β-glucanase**

|  |  |
| --- | --- |
| Parameter | Purification Steps |
| Rice bran substrate | Oil palm fibre substrate |
| Crude | (NH4)2SO4 Precipitation | Dialysis | Crude | (NH4)2SO4 Precipitation | Dialysis |
| Volume (ml) | 200 | 80 | 40 | 200 | 80 | 40 |
| Concentration of Protein (mg/ml) | 1.17 | 0.63 | 0.28 | 1.05 | 0.51 | 0.13 |
| Total Protein (mg) | 234.00 | 50.40 | 11.20 | 210.00 | 40.80 | 5.20 |
| Activity (U/ml) | 2.48 | 1.58 | 0.89 | 2.23 | 1.25 | 0.69 |
| Total Activity (U) | 496.00 | 126.40 | 35.60 | 446.00 | 100.00 | 27.60 |
| Specific Activity (U/mg) | 2.12 | 2.51 | 3.18 | 2.12 | 2.45 | 5.31 |
| Yield (%) | 100 | 63 | 36 | 100 | 56 | 30 |
| Purification fold | 1.00 | 1.18 | 1.50 | 1.00 | 1.15 | 2.50 |

Figure 6. Effect of temperature on partially purified Aspergillius flavus exo-β-glucanase

Figure 7. Effect of pH on partially purified Aspergillius flavus exo-β-glucanase

**Table 3. Effect of salts on partially purified Aspergillius flavus exo- β-glucanase**

|  |  |
| --- | --- |
| Salt | Exo- β-glucanase activity (U/mg) by substrate per salt concentration (mM) |
| Rice bran | Oil palm fibre |
| 1mM | 2mM | 5mM | 1mM | 2mM | 5mM |
| Control | 1.78±00 | 1.78±00 | 1.78±00 | 1.85±06 | 1.85±06 | 1.85±06 |
| NaCl | 1.73±03 | 1.59±14 | 0.61±00 | 0.94±11 | 0.57±01 | 0.46±00 |
| CoCl2 | 0.44±00 | 1.63±00 | 1.77±00 | 0.83±12 | 0.92±20 | 1.03±03 |
| CuCl2 | 1.61±00 | 1.60±02 | 0.15±12 | 0.75±00 | 0.71±04 | 0.18±11 |
| MgCl2 | 1.62±12 | 1.58±03 | 0.21±21 | 0.86±04 | 0.83±00 | 0.18±00 |
| CaCl2 | 1.62±12 | 1.57±014 | 0.44±03 | 0.85±03 | 0.84±00 | 0.66±02 |
| NH4Cl | 1.67±13 | 1.44±00 | 0.37±00 | 0.83±00 | 0.79±12 | 0.20±13 |
| KCl2 | 1.69±00 | 1.03±21 | 0.10±00 | 0.78±10 | 0.76±14 | 0.57±00 |

**Table 4. Effect of various concentrations (%) of different surfactants and EDTA on partially purified Aspergillius flavus exo- β-glucanase**

|  |  |
| --- | --- |
| Surfactant/EDTA | Exo- β-glucanase activity (U/mg) |
| Rice bran | Oil palm fibre |
| 1(%) | 3(%) | 5(%) | 1(%) | 3(%) | 5(%) |
| Control | 1.76±03 | 1.76±03 | 1.76±13 | 1.92±12 | 1.92±02 | 1.92±22 |
| Triton X | 1.69±01 | 0.51±16 | 0.45±13 | 1.83±05 | 0.81±09 | 0.66±07 |
| Tween 80 | 1.68±03 | 1.70±15 | 1.84±11 | 1.62±11 | 1.69±10 | 1.88±31 |
| Cetrimide | 1.82±06 | 2.28±06 | 1.34±13 | 2.00±05 | 1.97±06 | 1.46±08 |
| EDTA | 0.55±06 | 0.39±07 | 0.16±09 | 1.38±09 | 0.71±06 | 0.59±05 |

Figure 8. Effect of varying concentration of glucose on the initial reaction velocity

**Discussion**

*Aspergillus flavus* is a fungus known for its ability to degrade glucan, which is a type of polysaccharide that makes up the main component of starch. This degradation process is facilitated by the production of exo-β-glucanase, an enzyme that breaks down glucan molecules into smaller, more easily digestible sugars. The yield of exo-β-glucanase produced by *Aspergillus flavus* can vary depending on the substrate used. In this study, the organic waste and commercial carbon sources utilized as substrates for producing exo-β-glucanase may have different proportions of amylose to amylopectin, which can affect the yield of the enzyme.

Amylose is a type of starch that has a straight, linear structure, while amylopectin is a branched starch molecule. The ratio of these two types of starch can vary between different substrate sources, and this variation may impact the production of exo-β-glucanase. This study highlights the importance of understanding the relationship between substrate composition and exo-β-glucanase production in order to optimize the production of this enzyme. By using the right substrate, researchers may be able to increase the yield of exo-β-glucanase and improve the efficiency of glucan degradation by *Aspergillus flavus* (Doughari and Onyebarachi, 2019). Organic waste products like yam and banana peels have been found to increase the production of exo-β-glucanase in fungi. These products contain polysaccharides derived from glucose monomers, resulting in higher carbon content (Varki *et al*., 2009). Reports indicate that rice bran and oil palm fiber have a high concentration of glucan, which acts as an inducer for the production of glucanase (Doughari, 2011).

The use of these agricultural waste products in this study is an effective and cost-efficient way to eliminate the waste while providing an inexpensive and readily available source of carbon. The role of carbon sources in microorganism biosynthesis is significant, as they regulate enzyme synthesis through induction and repression (Kutateladze *et al*., 2009; Singh and Mandal, 2012). The versatility of *Aspergillus flavus* in utilizing different carbon sources is a testament to their ability to utilize these sources for enzyme production. This is supported by studies by Hussein *et al*., (2011), Rondanelli *et al.,* (2011) and Doughari (2011) who found oil palm fiber, rice bran, and rice bran respectively, to be good carbon sources for fungi. The ability to use agricultural waste in enzyme production could lead to a reduction in costs for exo-β-glucanase production. The study found that the addition of yeast extract to rice bran resulted in the highest production of β-glucanase by *Aspergillius flavus*, which is similar to the results reported by Xing-Jun (2004) who used barley flour and yeast extract to produce high amounts of β-glucanase by *Bacillus subtilis*. The high amounts of vitamins, minerals, and amino acids found in yeast extract play a crucial role in promoting cell growth and enzyme synthesis, leading to the induction of β-glucanase production (Zheng *et al*., 2011). The highest production of exo-β-glucanase by *Aspergillus flavus* was achieved when oil palm fiber was used as the substrate and peptone was added to the fermentation medium. The presence of peptone supported the growth of *Aspergillus flavus* by providing precursors for amino acid biosynthesis and cellular protein production. Exo-β-glucanase production by *Aspergillus flavus* from rice bran and oil palm fiber peaked at 96 hours and 120 hours of fermentation, respectively, using both organic and inorganic nitrogen sources.

The sufficient nutrients in the medium led to maximum enzyme production, while a decrease in production may have been caused by nutrient depletion. Similar results were observed in submerged fermentation for pectinase production (Yogesh *et al*., 2009). However, a different study found that the maximum production of β-glucanase occurred on the 7th day (168 hours) of solid-state fermentation (Yang *et al*., 2014). The moderate acidity of the media favored the growth of *Aspergillus flavus*, but further increases in pH reduced the production of exo-β-glucanase due to the sensitivity of enzymes to pH changes. Changes in pH can alter the shape of the active site of an enzyme and affect its charges, which can result in changes to the folding of the enzyme molecule and its shape (Doughari and Onyebarachi, 2019). Yang *et al.* (2014) however reported highest glucanase production at pH 5.0.

Previous studies conducted by Doughari (2011) on the production of fungal β-glucanase from rice bran have shown that *Penicillium citrinum* displayed maximum activity at pH 5.5, while *Penicillium oxalicum* displayed maximum activity at pH 6.0. The results suggest that moderate temperatures (mesophile) in the fermentation system promote the growth of *Aspergillus flavus* for exo-β-glucanase production. Higher temperatures can affect the active site of the enzyme, reducing its activity or causing denaturation. Temperature significantly influences both microbial growth and product formation. For instance, Jirku (1996) noted peak activity at 28°C, with decreased activity observed at temperatures exceeding 40°C. Similarly, Yang *et al*. (2014) demonstrated optimal β-glucanase production at 50°C using *Rhizomucor miehei* in solid state fermentation, contrasting with Klecius *et al.* (2006), who found *Bacillus halodurans* C-125 achieved maximum β-glucanase production at 60°C.

The purification process in this study demonstrated that the exo-β-glucanase produced by *Aspergillus flavus* could be purified to homogeneity using techniques such as ammonium sulfate precipitation and dialysis membrane tubing. Initially, the crude enzyme concentration in rice bran was 1.17 mg/ml, which decreased post-ammonium sulfate precipitation, indicating significant protein precipitation. Subsequent dialysis further reduced impurities, evidenced by the decrease in enzyme activity, highlighting effective removal of contaminants. The specific activity of the crude enzyme started at 2.12 U/mg and increased post-precipitation and dialysis. A similar trend was observed with oil palm fiber, where the initial enzyme concentration was 1.05 mg/ml, decreasing post-precipitation and dialysis. The specific activity also increased during these purification steps. These findings align with Doughari and Onyebarachi's (2019) assertion that successful purification should enhance specific enzyme activity, serving as a key indicator of purification efficacy.

The study analyzed how different pH levels affect partially purified exo-β-glucanase from *Aspergillus flavus*, revealing its acidic nature. Optimal enzyme activity was observed between pH 5 and 5.5, consistent with Doughari's findings (pH 5.5 to 6.0) (Doughari, 2011) and Sena *et al.'s* observations (pH 5.0 for *Moniliphthora perniciosa*) (Sena *et al*., 2011). Among β-glucanases from various microbes, the optimal pH typically ranges from 3.5 to 6.5, aligning with Yang et al.'s discovery (optimal pH 4.5 for *Rhizomucor miehei*) (Yang *et al*., 2014). Deviations from these pH ranges decrease enzyme activity, as pH changes affect the active site's conformation at the substrate binding site (Mahapatra *et al*., 2005).

The optimal temperature range for achieving the highest activity of purified exo-β-glucanase, using rice bran and oil palm fiber as substrates, was found to be between 40°C and 45°C. As the temperature increased (40-45°C), so did the enzyme activity. However, when the temperature exceeded 50°C, the activity of the enzyme decreased and continued to decrease with further increases in temperature. This indicates that *Aspergillus flavus* exo-β-glucanase is a thermostable enzyme, which agrees with Yang et al.'s (2014) observation that the optimal temperature for β-glucanase activity with *Rhizomucor miehei* was 55°C. On the other hand, Erfle et al. (1988) reported the optimal temperature for glucanase activity with *Bacillus halodurans* was 60°C. The rate of enzyme activity increases with temperature, but the increase is short-lived as the enzyme eventually denatures and stops functioning at high temperatures, leading to a sharp decrease in activity. The active site of the enzyme changes shape as the temperature increases, rendering it denatured (Anosike, 2001).

In this study, the activity of *Aspergillus flavus* exo-β-glucanase declines as NaCl concentration (1, 2, and 5 mM) increases. The elevated NaCl levels impact the protein structure, thereby influencing its activity. This phenomenon has been previously noted by Sena *et al.* (2011), who observed that glucanase activity is highest at lower NaCl concentrations and decreases as salt concentration rises. Conversely, enzyme activity shows an increase with higher concentrations of CoCl2, consistent with findings by Fukuda *et al*. (2008), who observed enhanced glucanase activity from *Flammulina velutipes* fruiting bodies in the presence of CoCl2. In contrast, enzyme activity decreases with increasing concentrations of CuCl2 and CaCl2. Doughari (2011) reported that CuCl2 slightly reduces β-glucanase activity in *Penicillium oxalicum* and *Penicillium citrinum*. MgCl2, NH4Cl, and KCl2 were found to weakly inhibit β-glucanase activity, as documented by Yasir *et al*. (2013) and Manczinger *et al*. (2001).

The impact of surfactant (cetrimide) and EDTA on purified *Aspergillus flavus* exo-β-glucanase grown on rice bran and oil palm fiber as substrate was investigated. For rice bran, activity was observed to increase from 1 to 3% with cetrimide, but then decrease at 5%. High concentrations of cetrimide were found to reduce the activity of β-glucanase produced from rice bran. Similarly, high levels of EDTA were recorded to decrease enzyme activity, with a decrease in activity seen at 0.25% EDTA concentration, as reported by Regmi *et al*. (2020). In the case of oil palm fiber as substrate, cetrimide increased activity at 1% concentration, but then decreased as the concentration increased. High levels of cetrimide also reduced the activity of β-glucanase produced from oil palm fiber. The lowest enzyme activity was recorded at 5% EDTA concentration, with an increase in EDTA concentration leading to a decrease in enzyme activity, which was in line with the findings reported by Regmi *et al*. (2020).

**Conclusion**

The results of this study indicate that both rice bran and oil palm fiber are viable for producing exo-β-glucanase through submerged fermentation with *Aspergillus flavus*. The enzyme produced by *Aspergillus flavus* has an optimal temperature of 35°C and pH of 6.5 for rice bran, and 40°C and pH of 5.0 for oil palm fiber. The purified enzyme has the potential to be used in various industrial and biotechnological applications, such as laundry detergents and beer production. The use of rice bran and oil palm fiber as substrates not only offers a plentiful source of glucan, but also minimizes production costs and reduces environmental waste as they are readily available at low or no cost.

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