Evaluation and Production of Cellulases from *Aspergillus Niger* Using Diverse Agro-Waste Substrates

Imran Saeed[1], Farooq Latif [2], Sumrah Maqbool[3], Mahjabeen Saleem[4], Navila Shaheen[5] and Muhammad Subhan[6]

https://doi.org/10.62345/jads.2025.14.1.46

Abstract

The study focused on optimizing cellulase production from Aspergillus niger, evaluating its enzyme activity against various agro-waste substrates, including corn cobs (Zea mays), orange peel (Citrus sinensis), sugarcane bagasse (Saccharum officinarum), wheat bran (Triticum aestivum), and wheat straw (Triticum aestivum). The highest cellulase activity, measured using CMCase, was observed with corn cob (0.68 U/mL), followed by orange peel (0.41 U/mL), sugarcane bagasse (0.35 U/mL), and wheat bran (0.17 U/mL). No visible fungal growth was observed on wheat straw. In the filter paper assay, orange peel exhibited the highest enzyme activity among all substrates (0.39 U/mL), followed by corn cob (0.32 U/mL), sugarcane bagasse (0.28 U/mL), and wheat bran (0.17 U/mL). This study showed the potential of agro-wastes as valuable substrates for industrial enzyme production, with Aspergillus niger proving effective in utilizing these materials for cellulase production.

Keywords: Solid State Fermentation, Aspergillus Niger, Agro-wastes.

Introduction

Agricultural wastes play a crucial role in the production of enzymes because they are inexpensive, renewable, and provide a microbial growth medium. These wastes, including rice straw, wheat bran, corn husks, and sugarcane bagasse, contains organic compounds such as cellulose, hemicellulose, and lignin that are complex, yet microorganisms can simplify them. Microbial processes on agricultural residues frequently yield enzymes, like cellulases, amylases, and lignases. Cellulose is a complex sugar that makes up a major constituent of plant cell walls and it is consider as the most abundant biological material on Earth. It consists of long bands of glucose molecules that are linked together in a straight, branched form. Cellulose comes from plants and helps make cell walls strong and stiff. Humans can't break down cellulose because we don't have the right enzymes, but it acts as dietary fiber. This helps digestion by adding bulk to stools and promoting normal bowel movements (Heinze, 2016). There are several industrial applications for cellulose. Since plant fibers with high cellulose content are used to make textiles like cotton and linen, cellulose is essential to the textile industry. Furthermore, cellulose has drawn interest as a

[1]*Department of Biochemistry, Minhaj University, Lahore, Pakistan.*
Corresponding Author Email: imransaeed8898@gmail.com
[2]*Department of Biochemistry, Minhaj University, Lahore, Pakistan.*
[3]*Department of Biochemistry, Minhaj University, Lahore, Pakistan.*
[4]*Department of Biochemistry, Minhaj University, Lahore, Pakistan.*
[5]*Department of Biochemistry, Minhaj University, Lahore, Pakistan.*
[6]*Department of Biochemistry, Minhaj University, Lahore, Pakistan.*

Compliance with ethical standards: There are no conflicts of interest (financial or non-financial). This study did not receive any funding.

renewable resource for biofuel production. An environmentally friendly alternative to fossil fuels is cellulosic ethanol, which is produced when cellulose breaks down into fermentable carbohydrates and then ferments into ethanol. Enzymes known as cellulases help in a number of industrial processes by speeding up the hydrolysis of cellulose into smaller molecules (Riseh et al. 2024).

Types of Cellulases

Enzymes called cellulases release glucose units with the breakage of β-1, 4-links in polymeric cellulose. (Hamid et al., 2015). The enzyme endoglucanase breaks down cellulose into smaller sugar unit molecules ultimately destroying cellulose structure converting it into glucose molecules (Rahman et al., 2018). An enzyme called exoglucanase cleaves off cellobiose units to break down cellulose. Because it selectively targets the terminals of cellulose strands, exoglucanase is an important enzyme in the degradation of cellulose. By breaking β-1,4-glycosidic connections it breaks down into smaller cellobiose units one after the other. During the process, exoglucanases constantly bind to the cellulose chain, releasing several cellobiose units before separating. It breaks down cellulose into smaller sugar molecules in concert with endoglucanas (Li et al., 2019). Beta-glucosidases (BGLs) are essential because the glucose they generate serves as the primary fermentation substrate. In order to achieve sustainable use of biomass, BGLs are important because they increase yields and decrease processing times in cellulose conversion (Singh et al., 2015).

Materials and Methods

Chemicals Used

The chemicals that were used in this study were analytical reagent grade chemicals. The whole research was performed at microbiology and biochemistry lab of Minhaj University Lahore.

Collection of Agro Wastes

Different agro-wastes, such as corn cobs (*Zea mays*), wheat straw (*Triticum aestivum*), sugarcane bagasse (*Saccharum officinarum*), and orange peel (*Citrus sinensis*), were collected from the local market for use in solid-state fermentation (SSF) for enzyme production.

Isolation of Fungi

Fungal isolates that were already present in the microbiology lab of Minhaj University Lahore were used for the study. Four fungal isolates, known for their cellulose-producing capabilities, were selected. Further isolation of these fungi was carried out using the streak plate method to obtain a pure culture of each fungal isolate.

The Streak Plate Method

This method is a widely used technique in laboratories to isolate microorganisms, such as bacteria and fungi, from mixed cultures for purification. This technique has proven effective for isolating pure bacterial and fungal cultures. A streak was made on the already present fungal isolates to further purify them using an inoculation loop, and they were then transferred to fresh PDA plates. This method simplifies the process of isolating different fungal species.

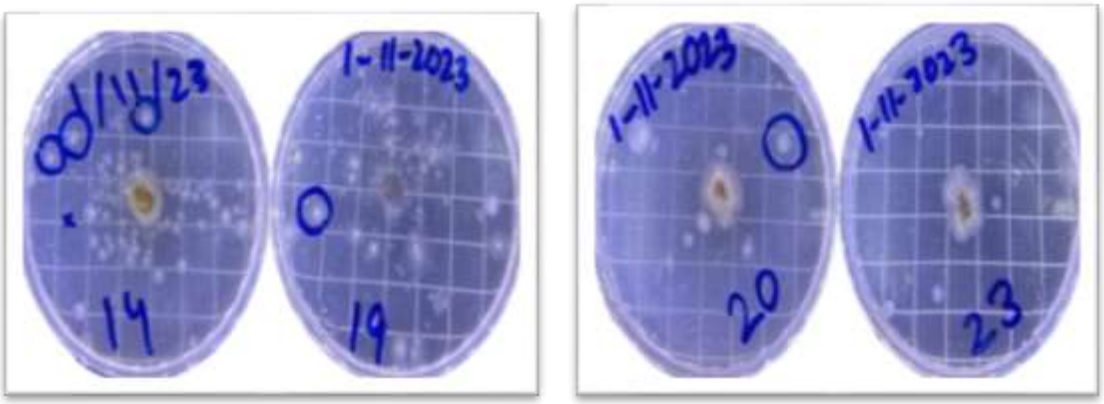
Culture Maintenance

PDA slants and glycerol stock solution of fungal isolates were made to keep the cultures fresh and were regularly transferred to fresh agar plates or slants every two or three months to keep the strain vital.

Primary Screening of Cellulose Degrading Fungi

Pure fungal cultures from various fungal colonies were added to 1% carboxymethylcellulose (CMC). Every fungal strain was positioned in the middle of the medium and injected about evenly. After that, the cultures were placed in a controlled setting at 37°C until they showed signs of growth (Teather and Word 1982). Throughout the incubation period, the fungal growth patterns were continuously observed. However, some strains did not develop in the CMC agar media, while others displaying significant growth and adaption to the CMC medium.

Figure 1: Different fungal growth on CMC Agar media



Congo Red Method

The Congo Red Method was applied. After being soaked with a 0.3% Congo red solution, the plates were left alone for fifteen minutes. The plates were then submerged in a 1M NaCl solution after being cleaned with water. After 15 minutes, there was a noticeable area around the colony that showed no signs of development (Sazci et al. 1986)

Solid State Fermentation (SSF)

In SSF method, different solid substrates five in total were used in Erlenmeyer flasks 5 g of each substrate was taken. A 1 cm hole was cut out of the center of the pure fungal culture. Preparation of media for the SSF in flasks was as follows, $(NH_4)_2\ SO_4$ 0.6 g, KH_2PO_4 0.4 g, K_2HPO_4 0.5 g, and $MgSO_4$ 0.2 g were added to a mineral salt medium to enable fungal growth (Sanghi et al., 2008). For the second experiment, the medium also included 5% peptone and 5% yeast extract to verify the superior nutrition supply. The solution was sterilized at a final pressure of 15 psi after being autoclaved for 15 minutes. After that, the material was transferred into containers and placed in an incubator set at 37 °C. The initial pH of the yeast medium and peptone media utilized was 6.0 and 5.7, respectively.

Figure 2: Different Substrates such as corn cob, wheat straw, wheat bran, sugar cane bagasse and orange peel for Solid State Fermentation

Assay for the Enzyme Endoglucanases (CMCase)

DNSA technique was used to quantify specific activity for endoglucanase. In this combination 1 mL of diluted enzyme, 1 mL of 1% CMC, 1 mL of acetate buffer (pH 5.0) all of the above mixed together and was put in a test tube for incubation the incubation temperature was set to 50 °C for 30 minutes. After incubation 3 mL of DNSA was added after that test tubes having enzyme and CMC substrate were put in boiling water for 5 to 6 minutes. Brick-red was the color of the reaction's indicators. Using 3,5-dinitrosalicylic acid and spectrophotometry at a wavelength of 550 nm, the enzymatic release of glucose was quantified (Deshavath et al. 2020). Enzyme activity was calculated using the given technique on a conventional glucose graph.

$$\text{Endoglucanase Activity (U/mL)} = \frac{\text{concentration of reducing sugar} \times \text{Dilution Factor}}{\text{Molar mass of glucose} \times \text{Volume of sample} \times \text{incubation time}}$$

Filter Paper Assay

The filter paper assay technique designed for the determination of carboxymethyl cellulase (CMCase) activity employed a strip of Whatman No. 1 filter paper as the substrate. A piece of filter paper was dipped in the enzyme solution and incubated under optimal conditions (at a specific temperature and pH) for a set period of time. After incubation, the quantity of reducing sugars liberated from the filter paper was quantified using the dinitrosalicylic acid (DNS) method. The enzyme activity was calculated based on the reducing sugar formed, which indicated the cellulase activity of the sample. The results were expressed in enzyme units (U/mL), with one unit defined as the amount of enzyme that liberated 1 µmol of reducing sugar per minute under the assay conditions (Xiao et al., 2004).

Standard Curve of Glucose Solution

Standard curve graphs was used to illustrate the relationship between a glucose solution's concentration and a quantifiable characteristic, such as absorbance or optical density

(spectrophotometry). In order to create a standard curve, concentration-based known glucose solutions were created, and spectrophotometer absorbance readings were taken at certain wavelengths. Plotting absorbance values versus glucose solution concentrations resulted in a linear plot, and the absorbance value could be used to calculate the concentration of an unknown glucose solution (Hu et al. 2008).

Statistical Analysis

Each assay was carried out three times and their results were expressed as the arithmetic mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA) to assess differences between groups. Post-hoc pairwise comparisons were conducted using the appropriate tests, and the results were visualized using box plots to highlight the distribution of data and the presence of significant differences between groups

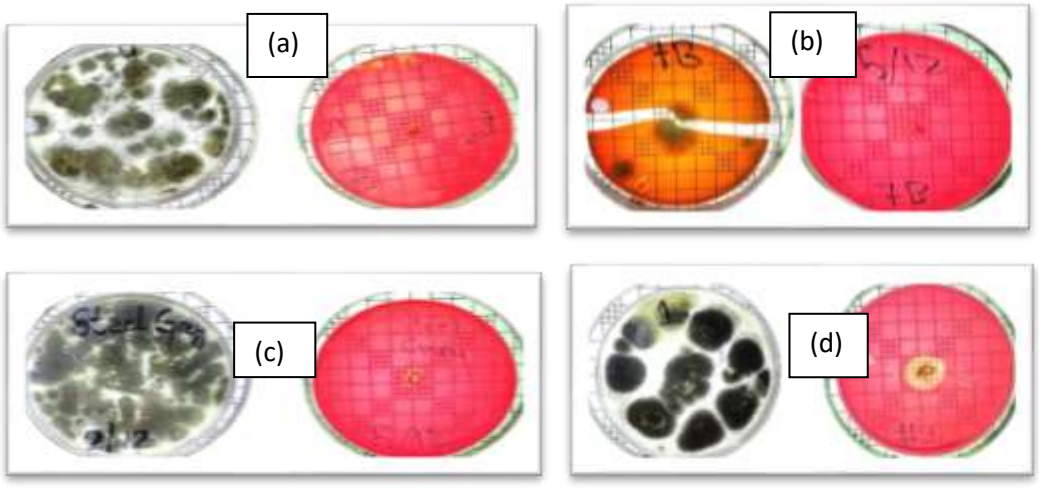
Results

Four different isolates were chosen to check their cellulolytic activity *A. niger* isolate was chosen from these isolates for further process like solid state fermentation. The isolate were subjected to a screening procedure on carboxymethyl methylcellulose (CMC) plates during the primary screening, which was conducted in accordance with the procedure previously reported by (Gohel et al. 2014). After that 0.3 % solution of Congo Red was put on the CMC agar plates only *A.niger* showed a zone of clearance.

Congo Red Method

In Fig. 3 the zone of hydrolysis on the CMC plate was observed for the fungal isolates negative results were yielded by other fungal isolates compared to *A. niger*. That showed *A. niger* is much more capable of producing cellulases than the other fungi obtained from this trial.

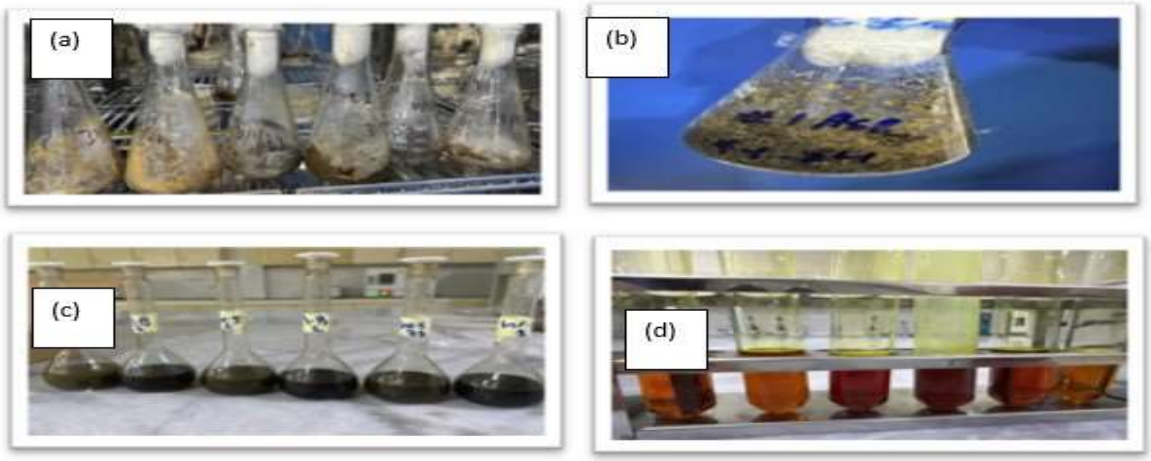
Figure 3: (a) Fungal isolate number #4 Congo red analysis (b) Fungal isolate number #7B Congo red analysis (c) Fungal isolate steel grey in colour Congo red analysis (d) Fungal isolate number #1 *A.niger* Congo red analysis



Solid State Fermentation(SSF)

SSF was performed on these substrates using *A. niger* fungal isolate that showed positive result in Congo Red Method. Substrates like orange peel and corn cob exhibited optimal fungal growth of *A. niger* but wheat straw did not showed any visible fungal growth. This result shows that the fungal isolates' capacities to grow on various substrates under SSF conditions varied. These results emphasize the significance of substrate selection and the indicate relationship between substrate composition and fungal metabolism in SSF operations.

Figure 4: (a) SSF in Flask showing fungal growth on different substrates (b) No visible fungal growth on substrate wheat straw (c) Crude enzyme after SSF (d) Enzyme after DNSA method



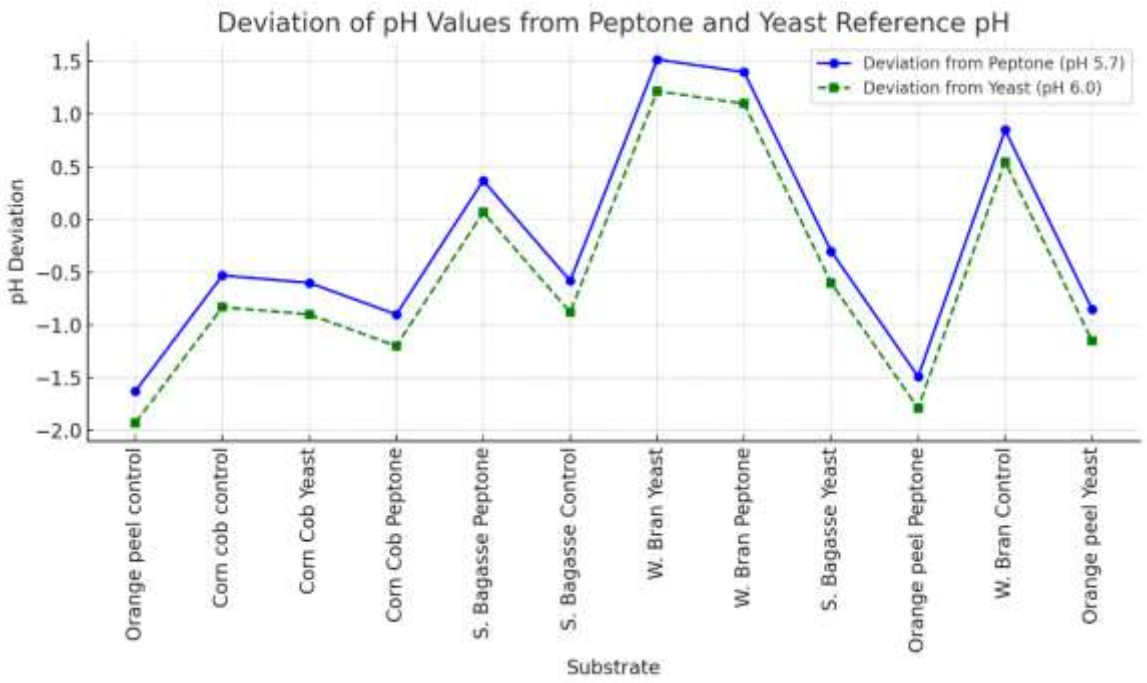
pH of Crude Enzyme

The initial pH of peptone medium used was 5.7 and yeast medium used was 6.0.

Table 1: pH of crude enzyme

Substrate	pH Value
Orange peel control	4.07
Corn cob control	5.17
Corn Yeast	5.10
Corn Cob peptone	4.8
S. Bagasse peptone	6.07
S.Bagasse control	5.12
W.Bran yeast	7.22
W.Bran peptone	7.1
S.Bagasse yeast	5.4
Orange peel peptone	4.21
W.Bran Control	6.55
Orange peel yeast	4.85

Figure 5: Deviation of pH values from Peptone and Yeast Extract Reference pH.



Two nutrient sources, peptone and yeast extract, were used in solid-state fermentation (SSF) to compare the effect of pH on cellulase activity. Above is the comparison of the two media. The initial pH was recorded and then compared with the pH after enzyme extraction using SSF.

Endoglucanase Activity

The result of endoglucanase activity is shown in table 2, which presents the enzyme activity for the three media types (Peptone, Yeast Extract, and Control) for each substrate. The table compares the mean enzyme activity values between the different media types for each substrate.

Table 2: Mean Values Endoglucanase Activity using CMC as Substrate

Substrate	Type of Medium	Mean (u/mL)	Standard Deviation (± SD)
Orange Peel – Peptone	Peptone	0.41	± 0.04
Orange Peel - Yeast Extract	Yeast Extract	0.41	± 0.04
Orange Peel – Control	Control	0.25	± 0.05
Wheat Straw – Peptone	Peptone	0.0	± 0
Wheat Straw - Yeast Extract	Yeast Extract	0.0	± 0
Wheat Straw – Control	Control	0.0	± 0
Wheat Bran – Peptone	Peptone	0.17	± 0.02
Wheat Bran - Yeast Extract	Yeast Extract	0.17	± 0.02
Wheat Bran – Control	Control	0.11	± 0.01
Corn Cob – Peptone	Peptone	0.68	± 0.03
Corn Cob - Yeast Extract	Yeast Extract	0.46	± 0.02

Corn Cob – Control	Control	0.35	± 0.02
S. Bagasse – Peptone	Peptone	0.35	± 0.02
S. Bagasse - Yeast Extract	Yeast Extract	0.25	± 0.03
S. Bagasse - Control	Control	0.12	± 0.03

Figure 6: Mean Values Endoglucanase Activity using CMC as Substrate

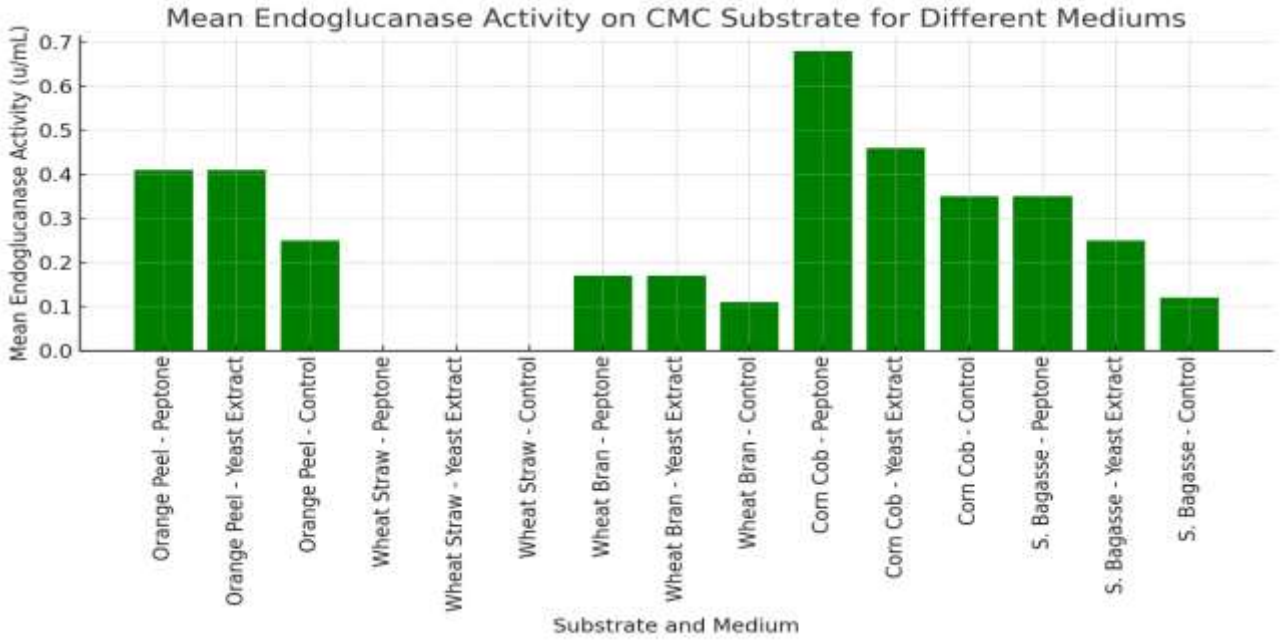
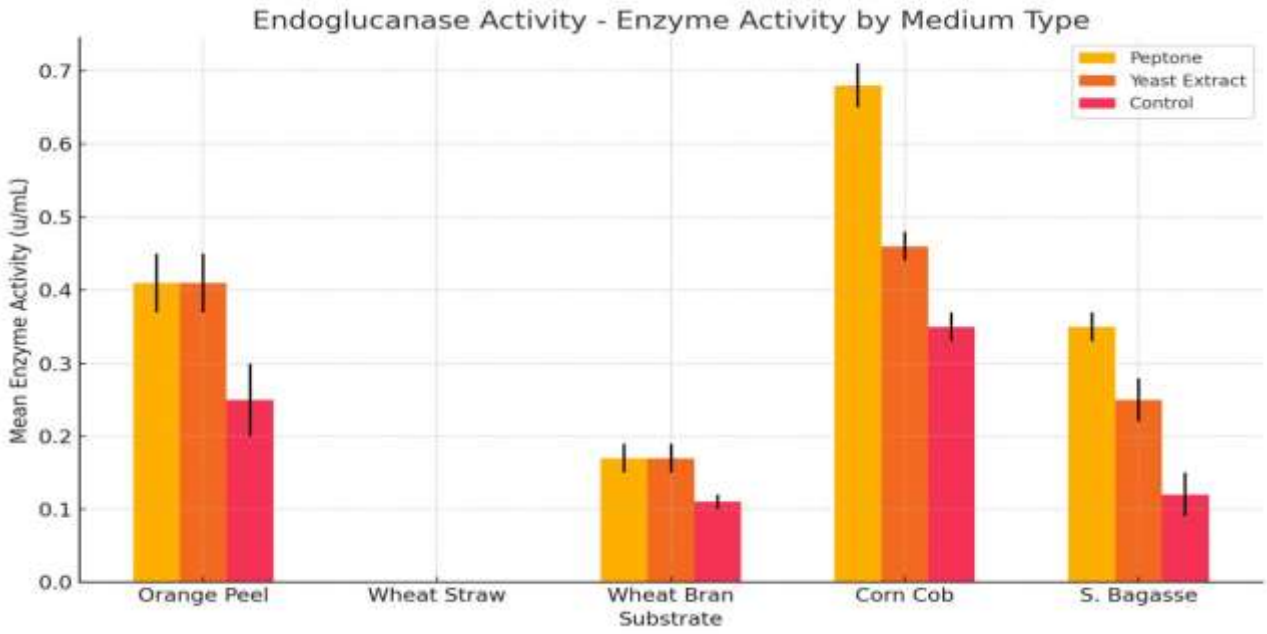


Figure 7: Figure: Enzyme Activity by Medium Type



Filter paper Assay

Table 3 contains measurements of mean enzyme activity (in µmL) for different substrates, such as Orange Peel, Wheat Straw, Wheat Bran, Corn Cob, and S. Bagasse, under three different media types (Peptone, Yeast Extract, and Control). The mean values and standard deviations were calculated for each group.

Table 3: Mean Values using Filter Paper Assay

Substrate	Type of Medium	Mean (u/mL)	Standard Deviation (± SD)
Orange Peel – Peptone	Peptone	0.39	± 0.03
Orange Peel - Yeast Extract	Yeast Extract	0.39	± 0.04
Orange Peel – Control	Control	0.29	± 0.03
Wheat Straw – Peptone	Peptone	0.0	± 0
Wheat Straw - Yeast Extract	Yeast Extract	0.0	± 0
Wheat Straw – Control	Control	0.0	± 0
Wheat Bran – Peptone	Peptone	0.15	± 0.02
Wheat Bran - Yeast Extract	Yeast Extract	0.17	± 0.02
Wheat Bran – Control	Control	0.07	± 0.02
Corn Cob – Peptone	Peptone	0.42	± 0.02
Corn Cob - Yeast Extract	Yeast Extract	0.43	± 0.03
Corn Cob – Control	Control	0.32	± 0.03
S. Bagasse – Peptone	Peptone	0.28	± 0.02
S. Bagasse - Yeast Extract	Yeast Extract	0.24	± 0.03
S. Bagasse – Control	Control	0.13	± 0.03

Figure 8: Mean values Across different substrate

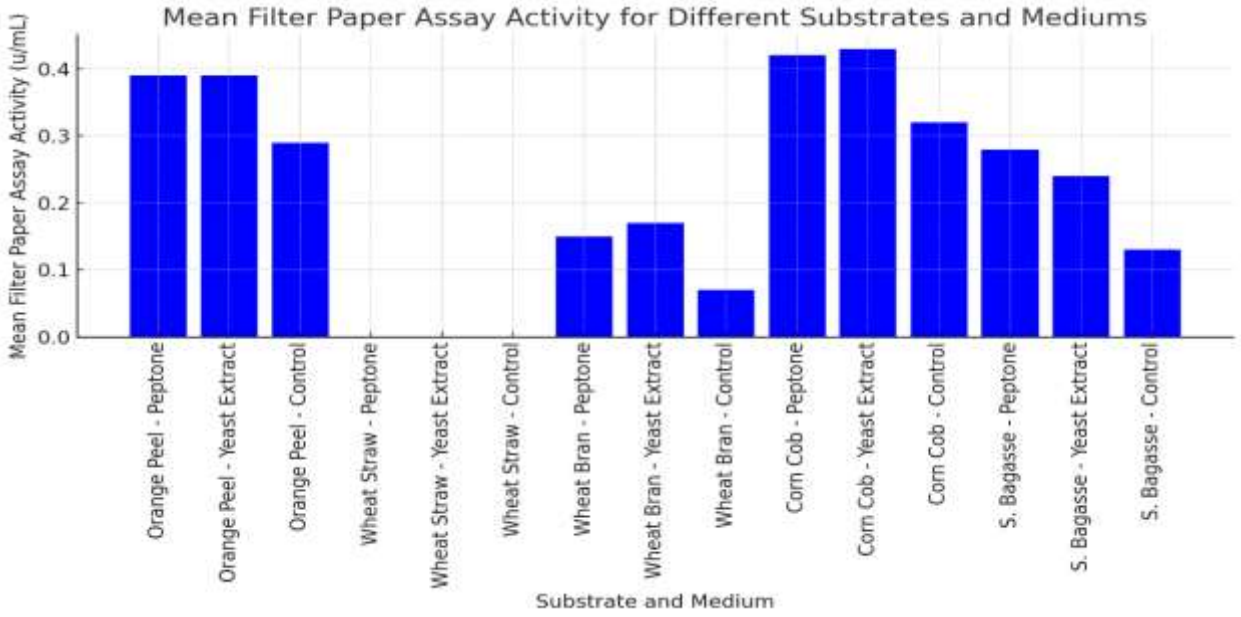
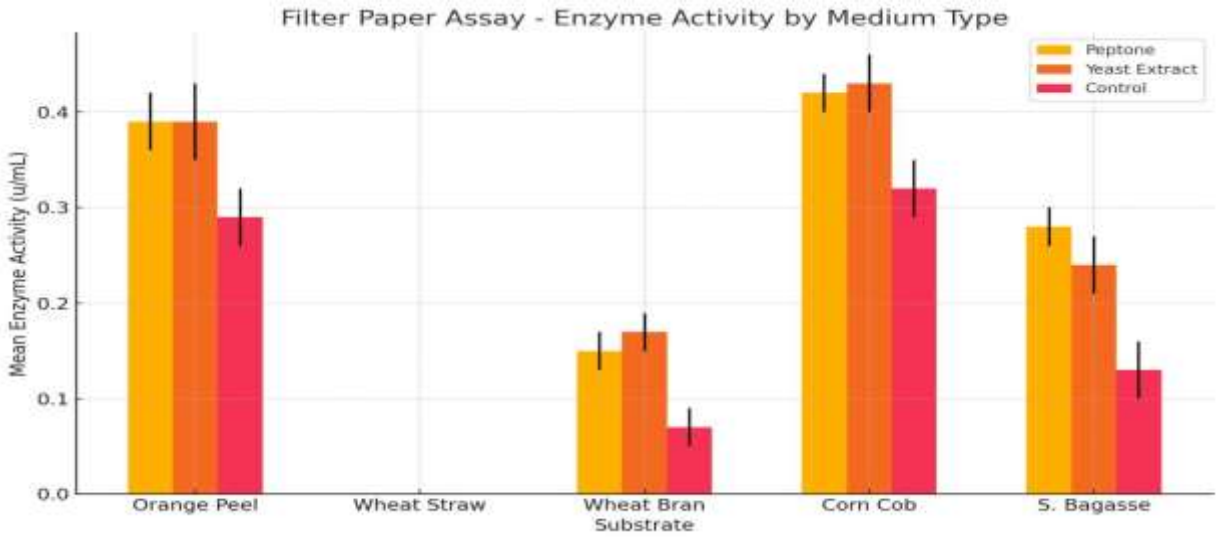
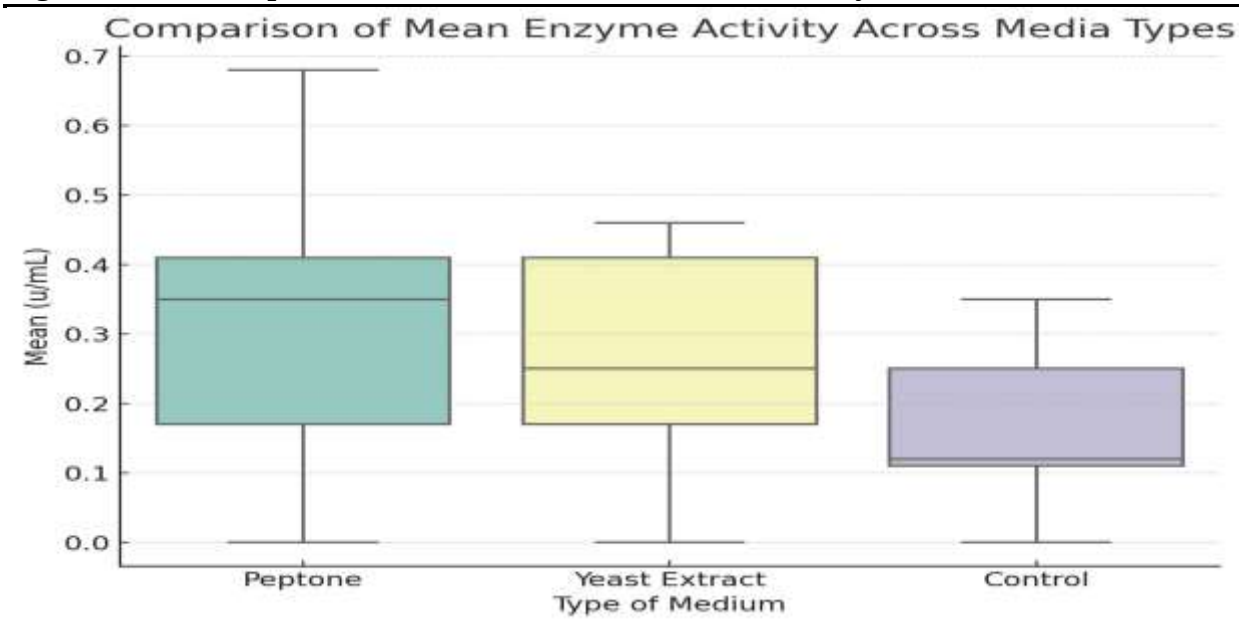


Figure 9: Filter paper Assay- Enzyme activity by medium type



Box-plot Comparison

Figure 10: The boxplot illustrates the distribution of mean enzyme



Discussion

Cellulases are important for biomass degradation and are enzymes of interest for many industrial applications including biofuel production and bioremediation. In this study we screened different fungal isolates for the production of cellulases by both a plate assay and SSF.

SSF is a biotechnological process in which microorganisms like bacterial and fungal isolates are grown on solid substrates with or without free water as discussed in the studies by (Barbosa et al. 1996). Its simplicity and cost effectiveness, coupled with the ability to use lignocellulosic waste as a substrate, makes this method of choice over other methods in terms of large scale enzyme production and other bioprocesses. Because fungi usually grow on solid organic sources, SSF is a

viable choice for cellulase production with *Aspergillus niger* as in the previous studies discussed by (Giese et al. 2005) role of different fungal species such as *Trichoderma harzianum* for enzyme production.

In this work, SSF was used to evaluate *A. niger* for its ability to produce cellulase under various conditions. On the basis of these results several factors were identified as important in the maximization of enzyme production such as pH, temperature, substrate composition and incubation period. Fungal growth, as well as enzyme secretion, is pH dependent; in our research A. niger showed optimal cellulase production at a slightly acidic pH, as it is known from earlier research (Kumar et al. 2018). Cellulase production was also substantially affected by temperature, as moderate temperatures produced the highest enzyme activity, including results from (Chandra et al. 2017) they too found that temperature also has a direct effect on fungal enzyme production.

Cellulase yields in SSF were dependent on the lignocellulosic material, agricultural residues chosen, thus highlighting the importance of substrate optimization in such SSF based bioprocesses (Pessoni et al. 2019), finally, claimed that the incubation period was important, and that long incubation periods would usually favor cellulase production.

Scaling up SSF to industrial production of enzyme requires optimization of these parameters. Enzyme production through SSF involves carefully controlling the environmental conditions so as to enhance efficiency of enzyme production thereby making SSF an industrially important method for cellulase production.

The filter paper assay stands as an essential method for endoglucanase activity measurements because it monitors β-1,4-glycosidic bond hydrolysis in cellulose. The substrate of choice in this assay consists of filter paper strips where enzyme cellulase degradation activity yields its results through quantification of released reducing sugar components. The standard procedure follows a protocol which allows detection of the released reducing sugars from cellulose through DNS reagent colorimetric analysis after incubation. (Decker et al., 2003).

Conclusion

A detailed approach to identify, cellulase producing fungal isolates with the help of qualitative analysis such as Congo Red Method and selecting the best among the fungal isolates for quantitatively analysis the such as SSF technique is presented in this study. Different agro wastes were used to grow the best fungal isolate that was *A.niger* to check its growth pattern . Favorable conditions for the growth of the fungi and the activity of the enzymes were created by the enhanced fermentation settings, resulting in the production of important enzymes and metabolites.

Endoglucanase activity was evaluated using the DNS approach and filter paper assay was also performed , allowing activity to be quantified and provide an estimate of the fungal isolate cellulase production efficiency. The making of standard curve with glucose solutions served to measure precisely to glucose levels, and often as a standard for many analytical and biochemical analyses. This paper presents a technique for the future biotechnological usage of fungus for bio detergents, biofuels production and different industrial sectors. Fungi offer the possibility of providing important resources for sustainable industrial processes and environmental management initiatives. Investigation into fungal capabilities and optimization of fermentation circumstances reveals opportunities for circumnavigating difficulties in disparate scientific disciplines. Further studies can be carried out for improving conditions for SSF that can maximize the cellulase production.

References

- Barbosa, A. M., Dekker, R. F. H., & Hardy, G. S. (1996). Veratryl alcohol as an inducer of laccase by an ascomycete, Botryosphaeria sp., when screened on the polymeric dye Poly R-478. *Letters in Applied Microbiology*, *23*(2), 93-96.
- Chandra, R., Shukla, A., & Sharma, R. (2017). Temperature and pH optimization for cellulase production by *Aspergillus niger*. *Biotechnology Letters*, *39*(5), 657–665.
- Decker, S. R., Adney, W. S., Jennings, E., Vinzant, T. B., & Himmel, M. E. (2003). Automated filter paper assay for determination of cellulase activity. In *Biotechnology for fuels and chemicals: The twenty-fourth symposium* (pp. 689–703). Humana Press.
- Deshavath, N. N., Mukherjee, G., Goud, V. V., Veeranki, V. D., & Sastri, C. V. (2020). Pitfalls in the 3,5-dinitrosalicylic acid (DNS) assay for the reducing sugars: Interference of furfural and 5-hydroxymethylfurfural. *International Journal of Biological Macromolecules*, *156*, 180–185.
- Giese, E. C., Covizzi, L. G., Borsato, D., Dekker, R. F. H., & Silva, M. L. C. (2005). Botryosphaeran, a new substrate for the production of β-1,3-glucanases by *Botryosphaeria rhodina* and *Trichoderma harzianum* Rifai. *Process Biochemistry*, *40*(5), 1711–1716.
- Gohel, H. R., Contractor, C. N., Ghosh, S. K., & Braganza, V. J. (2014). A comparative study of various staining techniques for determination of extracellular cellulase activity on Carboxy Methyl Cellulose (CMC) agar plates. *International Journal of Current Microbiology and Applied Sciences*, *3*(5), 261–266.
- Heinze, T. (2016). Cellulose: Structure and properties. In *Cellulose chemistry and properties: Fibers, nanocelluloses and advanced materials* (pp. 1–52). Springer.
- Hu, R., Lin, L., Liu, T., Ouyang, P., He, B., & Liu, S. (2008). Reducing sugar content in hemicellulose hydrolysate by DNS method: A revisit. *Journal of Biobased Materials and Bioenergy*, *2*(2), 156–161.
- Kumar, A., Singh, P., & Pandey, A. (2018). Influence of incubation period on cellulase production by *Aspergillus niger*. *Journal of Industrial Microbiology & Biotechnology*, *45*(2), 101–109.
- Ojumu, T. V., Solomon, B. O., Betiku, E., Layokun, S. K., & Amigun, B. (2003). Cellulase production by *Aspergillus flavus* Linn isolate NSPR 101 fermented in sawdust, bagasse, and corncob. *African Journal of Biotechnology*, *2*(6), 150–152.
- Rahman, M. S., Fernando, S., Ross, B. M., Wu, J., & Qin, W. (2018). Endoglucanase (EG) activity assays. In *Methods in molecular biology* (pp. 169–183). Springer.
- Riseh, R. S., Vazvani, M. G., Hassanisaadi, M., & Thakur, V. K. (2024). Agricultural wastes: A practical and potential source for the isolation and preparation of cellulose and application in agriculture and different industries. *Industrial Crops and Products*, *208*, 117904.
- Sanghi, A., Garg, N., Sharma, J., Kuhar, K., Kuhad, R. C., & Gupta, V. K. (2008). Optimization of xylanase production using inexpensive agro-residues by alkalophilic *Bacillus subtilis* ASH in solid-state fermentation. *World Journal of Microbiology and Biotechnology*, *24*, 633–640.
- Sazci, A., Erenler, K., & Radford, A. (1986). Detection of cellulolytic fungi by using Congo red as an indicator: A comparative study with the dinitrosalicyclic acid reagent method. *Journal of Applied Microbiology*, *61*(6), 559–562.
- Sharada, R., Venkateswarlu, G., Venkateswar, S., & Rao, M. A. (2014). Applications of cellulases—Review. *International Journal of Pharmaceutical, Chemical & Biological Sciences*, *4*(2), 1–10.
- Singh, G., Verma, A. K., & Kumar, V. (2015). Catalytic properties, functional attributes, and industrial applications of β-glucosidases. *3 Biotech*, *6*(1), 1–14.
- Teather, R. M., & Wood, P. J. (1982). Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Applied and Environmental Microbiology*, *43*(4), 777–780.