Optimum culture conditions of microbial isolates obtained from pap processing waste for cellulase production

ABSTRACT

The cost of cellulase is high due to cost of its substrate and producer microorganisms. The aim of this study was therefore, to investigate the cellulolytic potential of two indigenous isolates from pap processing wastes. The two indigenous isolates of bacteria and fungi strains were screened for cellulase production by growing on carboxymethyl cellulose (CMC) agar plate. Parameters such as substrate concentration, pH, temperature, incubation period were determined for cellulase production. The two organisms were isolated and identified as *Aspergillus niger* and *Bacillus* sp. The pap processing waste medium inoculated with *Aspergillus niger* had the highest cellulase activity of 7.2U/ml after 96 hours of incubation than the medium inoculated with *Bacillus* sp. that had highest cellulase activity of 4.31U/ml after 48 hours of incubation. As the concentration of pap processing waste increased up to 20%, cellulase activity increased with the maximum of 14.0 U/ml and 18.0 U/ml by *Bacillus* sp. and *Aspergillus niger* respectively. As the initial pH increased from 4 to 6 the cellulase activity increased with pH 6 as the optimum for maximum cellulase activity of 20.0 U/ml and 16.0 U/ml respectively for *Aspergillus niger* and *Bacillus* sp. The incubation temperature of 35° C was the optimum for the maximum cellulase activity of 22.0 U/ml and 18.0 U/ml respectively for *Aspergillus niger* and *Bacillus* sp. In conclusion, this research work has established the potential of pap processing waste that, if well harnessed can be used for the production of cellulase.

**Keywords:** culture conditions, Aspergillus niger, Bacillus sp, pap processing waste and cellulase

**Introduction**

“Cellulases are hydrolytic enzymes that are produced by microbes during the degradation process of cellulose or plant fibrous parts. Bacteria and fungi are good producer of Cellulase” (Henriksson *et al*., 1999; Naher *et al*., 2021).

“Cellulases are used in the textiles industry for cotton softening and denim finishing; in laundry detergent for color care, cleaning; in the food industry for mashing during beer and ethanol production; in the pulp and paper industry for drainage improvement and fibre modification, and they are even used for pharmaceutical applications” (Cherry and Fidants 2003; Naher *et al*., 2021; Sethi *et al*., 2013; Siva *et al*., 2022; Jayasekara and Ratnayake 2019; Gupta *et al*., 2015).

“Successful production of cellulase from cellulosic materials as renewable carbon sources is dependent on the development of economically feasible process technologies and optimization of culture condition parameters” (Sethi *et al*., 2013; Ellila *et al*., 2017; Islam and Roy 2018; Siva *et al*., 2022; Khadka *et al*., 2022). “Cellulase production is the most expensive step during ethanol and other metabolites production from cellulosic biomass, it account for approximately 40 % of the total cost” (Naher *et al*., 2021; Islam and Roy 2018; Jayasekara and Ratnayake 2019). “Significant cost reduction is required in order to enhance the commercial viability of cellulase production technology” (Naher *et al*., 2021; Sethi *et al*., 2013; Ellila *et al*., 2017).

“Cellulase with its immense importance and uses is being imported for use in Nigeria at very high cost” (Milala *et al*., 2005). “The local production of such enzymes using locally available agricultural wastes which can serve as substrate will reduce the cost of importation and encourage self-reliance” (Milala *et al*., 2005; Mrudula and Murugammal 2011; Ezea *et al*., 2022). “Pap processing wastes is one of the agricultural wastes generated during pap fermentation or production. It has enough cellulosic material which can be used as substrate for the production of cellulase. Aside cellulase production, pap processing waste was reported to be a good substrate for citric acid and other metabolites production because it has high starch and cellulose content” (Ezea, 2022). This research work was therefore, designed to Determine the optimum conditions of pap processing waste for cellulase production in submerged culture.

**Materials and Methods**

Collection of samples for Isolation

Compost of pap processing waste samples were collected from different pap sellers at Eke Agbani in Enugu State of Nigeria. The composts were dried under the sunlight and ground using clean grinding machine before transporting to the laboratory in a sample bottle.

 Isolation of Microorganisms from pap processing waste

One gram of ground pap processing waste compost was transferred in aliquots of 9 ml sterile distilled water in test tubes. It was shaken vigorously for 15 min. The pap compost waste suspension was then subjected to 10-fold serial dilutions in test tubes. For fungi isolation the serially diluted samples were inoculated on potato dextrose agar in Petri dishes and were incubated for 7 days at room temperature. For bacteria isolation, a ten-fold serial dilution was made for each pap processing waste, after which 1 ml of 10-5 dilutions was plated on Nutrient agar using pour plate method in petri dishes and incubated at 37 ° C for 48 hours. Single colonies were picked up and sub cultured on Nutrient agar and Potatoes dextrose agar slants respectively for bacteria and fungi. The agar slants were maintained at 4 ° C and subcultured at intervals.

Screening for cellulase producing microorganisms

Cellulase producing fungi were screened according to Devis and Kumar (2012) and Gautam *et al*. (2010) on carboxymethyl cellulose agar containing 2.0 g peptone, 1.0 g KH2PO4, 0.5 g MgSO4.H2O, 0.5 g KCl,10.0 g carboxymehtyl cellulose sodium salt, and 17.0 g agar in 1000 ml distilled water (pH 5.5). The carboxymethyl cellulose agar medium was sterilized at 121O C for 15 min. After cooling, the plates were spot inoculated with isolates and incubated at room temperature for 48 hours. After 48 hours the plates were flooded with 0.1% Congo red solution for 15 minutes and then de-stained with 1 M NaCl solution for 15 minutes. Those showing a zone of discoloration or clearance around the isolate (which indicates hydrolysis) were selected.

Inoculum preparations

To the 5 days old culture slant, 10 ml of 0.1 % Tween 80 solution was added and spores were dislodged using an inoculation needle under sterile condition. A 10 ml of 5 x 107 spores per ml of the spores was estimated using haemocytometer and used as inoculum as described in Ezea *et al*. (2022). Bacterial inoculum was prepared by inoculating two loops full of the bacteria isolate into 10 ml of Nutrient broth and incubated at 37° C for 48 hours.

Cellulase production

Cellulase enzyme was produced using nutrient medium as described in Ezea *et al* (2022) containing 2.0 g peptone, 1.0 g KH2PO4, 0.5 g MgSO4.7H2O, 0.5g KCl, in 1000 ml distilled water and 5 % of pap processing waste flour was suspended in each 100 ml of the basal medium in 250 ml Erlenmeyer flask and the medium was sterilized at 121° C for 15 minutes. After cooling, the flask was inoculated and incubated at 37° C and room temperature for bacteria and fungi respectively for 120 hours.

Optimization of culture conditionsfor cellulase production from pap processing waste

Different percentage of pap processing waste flour (5, 10, 15, 20 and 25 %) were investigated for cellulase production by suspending in 100 ml nutrient medium into 250 ml foam-plugged Erlenmeyer flask and incubated for 120hours.The effect of initial pH on cellulase production was carried out by adjusting the pH to 3, 4, 5, 6, 7 and 8 using 0.1M HCl and 0.1M NaOH before Pretreatment. The effect of incubation temperature on cellulase production was investigated under the following temperature; 20 ° C, 25 °C, 30 °C, 35 ° C and 40 ° C for 96 hours

Determination of Cellulase activity

Cellulase activity (CMCase) was assayed using the method of Ghose (1987). The activity was estimated using 1 % solution of carboxymethyl cellulose in 0.05 M citrate buffer (pH 4.5). The reaction mixture contained 1 ml citrate buffer, 0.5 ml of substrate solution (carboxymethyl cellulose) and 0.5 ml enzyme solution. The reaction was carried out at 50 °C for 30 minutes. The reaction was stopped by addition of 3 ml of 3, 5 dinitrosalicylic acid reagents and boiled for 5 minutes followed by addition of 3 ml of distilled water. Thereafter, the absorbance was taken at 540 nm (Miller, 1959). One unit of Cellulase activity was defined as the amount of enzyme releasing 1 µmol of reducing sugar from carboxymethyl cellulose per ml per min under the assay conditions. A calibration curve was established with glucose from which reducing sugars were calculated.

Identification of the isolates

The isolates obtained from pap processing waste were identified using microscopy, biochemical test and fermentation profile method of identification according to Bergey's manual of determinative bacteriology.

Statistical analysis

Data obtained were subjected to one- way analysis of variance (ANOVA) and the means were separated using the least significant difference.

**Results**

The two isolates from pap processing waste with code QQ4 and PP5 were identified based on their colony morphology, microscopic appearance, and Gram staining reaction, biochemical and sugar fermentation test as *Aspergillus* *niger* and *Bacillus* Sp respectively (table 1).

Fig. 1 shows the cellulase activity of both *Aspergillus* *niger* and *Bacillus* Sp isolates. The pap processing waste medium inoculated with *Aspergillus* *niger* had the highest cellulase activity of 7.2 U/ml after 96 hours of incubation than the medium inoculated with *Bacillus* Sp that had cellulase activity of 4.31 U/ml after 48 hours of incubation.

Different pap processing wastes had different cellulase activities. As the concentration of pap processing waste increased up to 20 %, cellulase activity increased with the maximum of 14.0 U/ml by *Bacillus* Sp after 48 hours of incubation before a declined at 25 % substrate concentration (fig. 2). *Aspergillus niger* had the highest cellulase activity of 18 U/ml from 20 % pap processing waste. As the substrate concentration increased, the cellulase activity increased (fig. 3).

Table 1 Identification of the isolates

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Code | Colony morphology | Microscopic appearance or Gram reaction | catalase | coagulase | oxidase | indole | citrate | SFT | Probable organisms |
| QQ4 | Velvety, black creamy and white with time | Septate hyphae, brush-like conidiophores chain conidia | NT | NT | NT | NT | NT | NT | *Aspergillus niger* |
| PP5 | Milky, sticky, flat, rough, opaque colonies | Positive rod | +Ve | -Ve | -Ve | -Ve | +Ve | -Ve | *Bacillus* Sp |

SFT = Sugar fermentation test, NT= Not tested, -Ve= Negative, +Ve= Positive

**Fig 1:** Cellulase activity of *Bacillus* Sp and *Aspergillus nigrer* isolated from pap processing waste

**Fig. 2:** Effect of different concentrations of pap processing waste on cellulase activity by *Bacillus* Sp

**Fig. 3:** Effect of different concentrations of pap processing waste on cellulase activity by *Aspergillus niger*

Fig. 4 shows that as the initial pH increased from 4 to 6, the cellulase activity increased with pH 6 as the optimum for the maximum cellulase activity of 20.0 U/ml and 16.0 U/ml for Aspergillus niger and Bacillus Sp respectively. Cellulase activity declined at pH 7 and 8 for both *Aspergillus niger* and *Bacillus* Sp respectively.

The effect of different incubation temperature ranging from 25 ° C to 45 ° C. The incubation temperature of 35 ° C was the optimum for both *Aspergillus niger* and *Bacillus* Sp with the maximum cellulase activities of 22.0 U/ml and 18.0 U/ml respectively. Cellulase activity of both *Aspergillus niger* and *Bacillus* Sp were inhibited at 40 ° C and 45 ° C (fig. 5).

Fig. 4; Effect of different pH on cellulase activity by *Aspergillus niger* and *Bacillus* Sp on pap processing waste

Fig. 5; Effect of different temperature on cellulase activity by *Aspergillus niger* and *Bacillus* Sp on pap processing waste

**Discussion**

In this study, pap processing waste was used as a source for obtaining desirable cellulase-producing microorganisms. Upon screening the isolates for cellulase activity, *Aspergillus niger* and *Bacillus* sp. were the isolates found to have good potential for cellulose degrading ability. This result is in agreement with the previous reports from Opere *et al.* (2021) and Sukumaran *et al.* (2015). Similar isolations have been reported by Gupta *et al.* (2016), Nwogwugwu *et al.* (2018) and Devi *et al.* (2018), who “isolated various cellulosic organisms from sugarcane bagasse, sawdust, corn cob, bagasse, wheat straw yam and cassava peels and rice straws dumping sites. Microorganisms that produce cellulase could be isolated from places such as soil around mills, cassava farms and processing factories as well as flour markets” (Islam and Narayan, 2019).

The pap processing waste medium inoculated with *Aspergillus niger* had the highest cellulase activity of 7.2 U/ml after 96 hours of incubation than the medium inoculated with *Bacillus* sp. that had cellulase activity of 4.31U/ml after 48 hours of incubation. This result establishes the fact that the isolates can decompose pap processing waste used as substrate from which they were isolated. This is in line with the study of Imran *et al.* (2016) and Gunathilake *et al.* (2012).The results suggested that these isolates might be potential agents in the biotransformation of cellulosic wastes to industrial products such as biofuel and biofertilizers, since glucose is the major monomer unit of cellulose (Nwogwugwu *et al.,* 2018). Fungi (*Aspergillus niger*) tend to have greater cellulolytic abilities than bacteria (*Bacillus* sp.) as reported by Li *et al.* (2019). The fungal isolates might have produced more cellulases because of their abilities to survive in harsh environmental conditions, which are major characteristics of lignocellulosic materials that are found in pap processing waste.

Different concentrations of pap processing wastes had different cellulase activities. As the concentration of pap processing waste increased up to 20%, cellulase activity increased with the maximum of 14.0 U/ml by *Bacillus* sp. after 48 hours of incubation while *Aspergillus niger* had the highest cellulase activity of 18 U/ml. As the substrate concentration increased, the cellulase activity increased. This result is in agreement with Rathnan *et al.* (2012) who reported an increase in the activity of cellulase when the carbon source concentration was increased during production of cellulase by some bacteria and fungi in submerged fermentation. Legodi *et al.* (2019) reported cellulolytic activities of fungi isolated from natural compost in a submerged fermentation (SmF). Gunathilake *et al.* (2013) reported isolation of cellulolytic fungi and bacteria from soils, composts and leaf litter and screened for simple sugar production. Carbon source and its concentration had a great influence on cellulase production depending on the nature and type of carbon used for fermentation.

**Conclusion**

Pap processing waste has the potential to be used as source of microorganisms and substrate in the production of cellulase. *Aspergillus niger* culture significantly had more cellulase activity than *Bacillus* sp. The concentrations of pap processing waste had a positive signifcant effect on the cellulase production. Initial pH of Pap processing waste and incubation temperature had signifcant positive effect on cellulase production. Utilization of Pap processing waste into cellulase production will guarantee market and prices and reduce the cost of cellulase production. This study has demonstrated that Pap processing waste, which is sufciently found in the Eastern region of Nigeria can be used in cellulase production.

**Conference details:**

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Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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