Microbial Diversity, Enzyme Profile and Substrate Concentration for Bioconversion of Cassava Peels to Bioethanol

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Posted Date: 8 November 2023

doi: 10.20944/preprints202311.0494.v1

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Article

Microbial Diversity, Enzyme Profile and Substrate Concentration for Bioconversion of Cassava Peels to Bioethanol

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Abstract: In light of the hazardous effects of global warming brought on by climate change, bioethanol production is one of the key alternatives in which the world has expressed a great deal of interest. Two factors—indigenous production and price—are crucial for bioethanol to be appealing in Africa and, for that matter, Ghana. The objective of this research is to produce bioethanol from cheap lignocellulosic materials using onsite enzymes produced from cassava peels degrading fungi. Due to Ghana's prominent position as one of the leading cassava producers in the world, the study focused on utilizing cassava peels, which are underutilized in the country. The research was conducted in multiple stages. In the first stage, the peels were prepared by washing, drying, and manually crushing them using mortar and pestle. The second stage involved analyzing the chemical composition of the peels, including starch and insoluble fibre, which were determined as Acid Detergent Fiber (ADF), Neutral Detergent Fiber (NDF), and Acid Detergent Lignin (ADL). The results showed significant levels of cellulose (39.78%) and starch (31.21%), indicating that cassava peels are valuable raw materials for bioethanol production. The content of hemicellulose (21.11%) and lignin (3.84%) were also determined. In the third stage, using a Petri dish, three fungi (ICPF1, ICPF2, and ICPF3) were isolated from two different cassava peels. $ICPF_1$ was only identified from fresh cassava peels (FCP), while all three fungi were identified from decayed cassava peels (DCP). Morphologically, these fungi were identified as *Aspergillus niger*, *Aspergillus flavus*, and *Rhizopus stolonifer*. The fourth stage focused on optimizing the enzyme activity of the three isolates for potential applications, with *A. niger* demonstrating the highest enzyme activity with a diameter of zone of clearance of 16 mm. Stage five involved optimizing the production of the onsite enzyme in a 50ml flask using *A. niger,* basal salt medium (BSM), and cassava peels as a substrate. The DNSA method was used to measure the absorbance of maltose and glucose at 540 nm for various substrate concentrations (1%, 3%, 5%, 8%, and 10%) at specific intervals of 2, 4, 6, 8, and 10 days with a spore concentration of 2.1 x 10^5 cells/ml. The maltose and glucose concentrations were calculated as 7.138mg/ml and 6.398 mg/ml, respectively, and the corresponding enzyme activity was determined as 4.759U/ml and 4.265U/ml. The optimal conditions of Day 4 and a substrate concentration of 10%, along with a fixed temperature of 30°C and a pH of 5.5, were used to prepare the onsite enzymes in a 500 ml flask for the fermentation process. The onsite enzymes were used for saccharification and *Saccharomyces cerevisiae* for fermentation under simultaneous saccharification and fermentation (SSF) process. A mixture of 20 ml of onsite enzymes and 1.5 g of *S. cerevisiae* were added to substrate concentrations of 5%, 10%, and 20%, and the ethanol concentration was analyzed daily for 7 days using Gas Chromatography (GC). The highest ethanol concentration (1.316%) was observed on Day 5 with a substrate concentration of 20%, while the lowest concentration (0.123%) was recorded on Day 1 with a substrate concentration of 5%.

Keywords: Bioethanol; cassava peels; substrate; onsite enzyme; optimization; cassava-degrading fungi

1. Introduction

The focus of the United Nations' sustainable development goal number 13 is to address climate change, as its severe impact is affecting every country worldwide. Greenhouse gas emissions have

 doi:10.20944/preprints202311.0494.v1

increased by over 50% since 1990, and 2019 marked the second warmest year on record and the end of the warmest decade ever recorded. Carbon dioxide (CO_2) and other greenhouse gas levels in the atmosphere reached new records in 2019. Although there was a temporary reduction in emissions due to the pandemic, emissions are expected to rise as the global economy recovers. Urgent and coordinated action is necessary to combat both the pandemic and climate change for the sake of human survival. Additionally, Goal 7 of the United Nations Sustainable Development Goals emphasizes the importance of affordable and sustainable energy for all, ensuring access to reliable and inexpensive energy sources. The use of fossil fuels without measures to reduce greenhouse gas emissions contributes to global climate change. It is evident that enhancing energy efficiency and promoting renewable energy sources is crucial for mitigating climate change and reducing risks to ecosystems. The Intergovernmental Panel on Climate Change (IPCC) highlighted the need to halve fossil fuel emissions within 11 years to limit global warming to 1.5 degrees Celsius above pre-industrial levels (IPCC, 2018). The concentration of atmospheric carbon dioxide has increased by 49% from 1750 to 2020 (Global Carbon Project, 2021). Non-renewable energy sources alone are insufficient to meet the current and growing energy demand (Pinaki et al., 2015). Motor vehicles are a significant source of pollution, responsible for 70% of carbon monoxide and 19% of carbon dioxide emissions globally (Deenanath et al., 2012). The International Monetary Fund (IMF) estimates that there are currently over 1 billion cars worldwide, with projections indicating a rise to approximately 2.9 billion cars by 2050, mostly in developing countries (World Energy Outlook, 2022). These statistics underscore the need to take action to reduce the impact of greenhouse gas emissions from motor vehicles.

Global ethanol production has been increasing overall, but it experienced a decline in 2020 due to the COVID-19 pandemic. The United States is the largest producer of ethanol, followed by Brazil, the European Union, China, and Canada (Edeh, 2021). The rest of the world generated a significant amount of ethanol as well (Edeh, 2021). In Africa, where many countries are net oil importers, there is a growing need to develop alternative fuels for energy self-sufficiency and socio-economic benefits (Sekoai & Yoro, 2016). Biofuels are seen as a potential solution, and governments worldwide have implemented policies to promote their use and reduce CO_2. In Ghana, there have been attempts to establish biofuel policies, such as using biodiesel blends for government vehicles and increasing the production of jatropha and cassava/sugarcane for bioethanol (Sielhorst, 2008). However, the initiatives faced challenges and failed to reach their targets due to factors like the discovery of crude oil, changes in political power, and a lack of political commitment (Abubakari et al, 2016). Various crops, including cassava, sugarcane, maize, and jatropha oil seeds, have been identified as potential feedstocks for bioethanol and biodiesel production in Ghana (Sekoai & Yoro, 2016).

Bioethanol is a leading biofuel that has experienced significant market growth. It is produced by fermenting the starch and sugar components of plant and biomass materials (Edjekouane et al., 2020). Bioethanol serves as a high-octane fuel and a replacement for lead in gasoline, enhancing its octane rating (Kumar & Kataria, 2019). When bioethanol is blended with gasoline, it oxygenates the fuel mixture, resulting in cleaner combustion and reduced pollutant emissions (Adetunji, 2015). Compared to other generations of bioethanol production, the focus of this study is on second-generation bioethanol, which utilizes lignocellulosic biomass. Lignocellulose found abundantly in both woody and non-woody plants, is the most common renewable energy source globally and a by-product of various plant processing materials (Visser et al, 2013). Its primary components are cellulose, hemicellulose, and lignin. The use of second-generation bioethanol offers numerous advantages over other generations due to the widespread availability and sustainability of lignocellulosic biomass (Kumar & Kataria, 2019). Cassava, scientifically known as *Manihot esculenta*, is a starchy root crop commonly used as a food source in developing nations (Kehinde et al., 2021). It is extensively cultivated in tropical and subtropical regions of Asia, South America, and Africa. Nigeria, the Democratic Republic of Congo, Thailand, Indonesia, Brazil, and Ghana are among the top cassava producers, with global production reaching 291 million tonnes in 2019 according to United Nations FAOSTAT. In Ghana alone, approximately 3.8 million metric tons of cassava peels are generated annually (Aboagye et al., 2021).

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Cassava peels are an ideal feedstock for bioethanol production due to their low lignin content, facilitating the separation of fermentable sugars for bioethanol processing. Moreover, their abundant availability and affordability make cassava peels a viable option for bioethanol production (Aboagye et al., 2021).

Fungi are widely recognized as effective microorganisms for breaking down lignocellulose into its main components. Cellulolytic enzymes, such as cellulases and amylases, are crucial for efficient cellulose hydrolysis, and fungi, particularly species like Aspergillus, Rhizopus, and Penicillium, are known to produce these enzymes abundantly. *Aspergillus niger*, in particular, is highly regarded for its enzyme production capabilities and is considered safe for use in various biotechnological applications by the US FDA (Schuster, 2002). Enzymes play a significant role in the cost of bioethanol production and producing them on-site can help reduce expenses. Among the enzyme-catalyzed approaches, Simultaneous Saccharification and Fermentation (SSF) is preferred due to its ability to achieve higher yields and concentrations of ethanol. This method combines enzymatic cellulose hydrolysis with the simultaneous fermentation of glucose to ethanol, resulting in improved efficiency and cost-effectiveness. The SSF technique also avoids the need for expensive equipment and minimizes the risk of contamination by organisms sensitive to ethanol. The use of yeast in the cellulolytic enzyme complex helps enhance yields and saccharification rates by reducing the accumulation of inhibitory sugars in the reactor. The objective of this research is to analyze the composition of cassava peels, isolate and characterize degrading fungi from fresh and decaying peels, produce a crude enzyme blend using cassava peels, optimize enzyme production, and utilize the enzyme cocktail for the production of bioethanol through simultaneous saccharification and fermentation using cassava peels as the substrate.

2. MATERIALS AND METHODS

CHAPTER 1

2.1. Location of study

The present study was conducted at the laboratories of the Department of Biochemistry and Biotechnology and the Department of Chemical Engineering all in Kwame Nkrumah University of Science and Technology (KNUST), Kumasi in the Ashanti region of Ghana.

2.2. Sources of experimental materials

The feedstock of cassava peels that served as substrate was procured from the Gari processing facility at Asuboi in the Ayesuano district close to Suhum on the Accra-Kumasi Highway in the Eastern Region of Ghana. From the same cassava processing factory's disposal site, two more samples of Fresh Cassava Peels (FCP) and Decayed Cassava Peels (DCP) for fungal isolation were collected.

2.3. Sample preparation

The cassava peels used as feedstock were taken through a series of steps. Firstly, they were washed with water to remove any unwanted particles and dust. Then, the peels were dried in the sun for a period of 7 days to eliminate moisture. Once dried, the cassava peels were manually crushed using a mortar and pestle, resulting in fine particles. These fine particles were then placed in a sealed poly bag and stored under room conditions for future processing. Additionally, fresh and partially decayed cassava peels were washed, crushed separately, and stored for further processing as well.

2.4. Chemical analysis of cassava peels

This was carried out in the Nutrition Laboratory of the Animal Science Department, KNUST. The insoluble fibre in cassava peel which is, cellulose, hemicellulose, and lignin content was determined as Acid Detergent Fibre (ADF), Neutral Detergent Fibre (NDF), and Acid Detergent Lignin (ADL) according to the method of analysis used by Van Soest (van Soest, 1995). The analysis of starch from the cassava peels was also conducted using the wet method as described by Noorfarahzilah et al., (2014).

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2.5. Isolation of fungi

The isolation of the fungi was undertaken at the Soil Science Laboratory of Kwame Nkrumah University of Science and Technology The investigation employed the simple dilution plate method. Each sample, weighing 1 g, was diluted three-fold in 9 ml of sterile distilled water. The final dilution (10^{-3}) was then flooded onto three separate sterile Potato Dextrose Agar (PDA) medium plates that were supplemented with chloramphenicol antibiotic to inhibit bacterial growth. The plates were incubated at room temperature (25°C-28°C) for a duration of 7 days. The resulting fungal growth was counted using a colony counter and identified using the Illustrated Genera of Imperfect Fungi Manual by (Barnett and Hunter, 1972). The frequencies of occurrence for each identified fungus were determined. Subsequently, the cultures were sub-cultured until pure isolates were obtained.

2.6. Macromorphological and micromorphological characteristic identification

The fungal isolates were identified based on their macromorphological and micromorphological characteristics. For macromorphological identification, colony traits such as colour, texture, and spore structure were observed, following the guidelines provided in the handbook for the identification of fungi (Alexopoulos, 1996; Barnett and Hunter, 1972). Micromorphological characteristics were examined using the Conventional Lactophenol Cotton Blue Technique (LPCB). Slides for microscopic analysis were prepared using five-day-old pure cultures. A small number of mycelia was placed on a slide, and a drop of lactophenol blue was added. A cover slip was then applied, and the sample was examined under a light microscope at a magnification of x400. Identification was conducted by comparing the observed features with relevant micrographs.

2.7. Quantitative analysis of the three fungal isolates for enzyme activity

The three fungal isolates were cultivated on Potato Dextrose Agar (PDA) medium. Subsequently, the isolates were tested for enzyme activity by incorporating 3g of cassava peels into a PDA medium. Three separate media were prepared, each inoculated with one of the three fungi and then incubated at 30°C. An iodine solution containing iodine (0.2 ml), potassium iodide (0.4 ml), and distilled water (100 ml) was flooded onto each medium. The presence of a clear zone around the fungal growth indicated the production of amylase. To identify cellulase enzymes, the plates were flooded with a 1% Congo red solution and left at 28°C for 20 minutes. Subsequently, the plates were thoroughly rinsed with a 1M sodium chloride solution. The formation of a clear zone surrounding the cellulase-producing colonies against a dark red background indicated cellulase activity.

2.8. Substrate for the enzyme production

Starch powder and Carboxymethylcellulose (CMC) powder were used for the assay of amylase, and cellulase enzymes respectively. The purpose of the assay above was to check for the presence and quantity of these enzymes which were the enzymes of interest.

2.9. Preparation of Aspergillus niger spore suspension

After the mould had been incubated for five days, its spores were harvested by saturating the culture with 10 ml of sterilized distilled water and then scraping the spores out. The spore suspensions were then decanted and combined to the appropriate volume in a 100 ml Erlenmeyer flask.

2.10. Determination of spore concentration

Using sterile distilled water, 1 ml of spore suspension was transferred from the stock into a measuring cylinder with a capacity of 100 ml. After shaking the diluted sample, a portion was pipetted into the grooves of haemacytometer and placed under a light microscope to count the spores. The average of the spore counts from the haemacytometer five zones was calculated. The average

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value was then multiplied by a dilution factor of 10 and 10^4 spores per millilitre. 2.1 x 10^5 spores/ml were determined to be the spore concentration.

2.11. Preparation of Growth Medium

Basal Salt Medium (BSM) which is the main medium used in this experiment was prepared according to the combinations used by (Efeovbokhan et al., 2019) with slight modifications. The following concentrations of components; 1.4 g of $(NH_4)_2SO_4$, 0.3 g of $MgSO_4.7H_2O$, 2.0 g of KH_2PO_4, 0.5 g of $FeSO_4$, 0.3 g of $CaCl_2$, 0.3 g of Urea, 1 ml of Tween 20, 0.16g of $MnSO_4$, 0.14 g of $ZnSO_4$, 0.2 g of $CoCl_2$, and 10 g of Starch in 1 L of deionized water for the production of enzymes. No additional carbon or nitrogen source was added. The pH of the medium was adjusted to 5.5.

2.12. Enzyme production (α-amylase and cellulase) from Aspergillus niger

Temperature and pH were the fixed conditions maintained during the enzyme production. The optimum temperature and pH were used in this research. For the enzyme production by *Aspergillus niger*, an optimum temperature of 30°C and a pH of 5.5 respectively were used. These fixed conditions are within the range for optimum production of amylase and cellulase enzymes as reported in the literature for *Aspergillus niger*.

> *Substrate concentration and incubation time, two essential variables, were varied in this study. Varying amounts of 1, 3, 5, 8 and 10% (w/v) of the dry-weight biomass of the cassava peels were used as the substrate concentrations. On the other hand, the incubation time was varied as; 0, 2, 4, 6, 8, and 10 days. It was essential to do this to obtain the ideal substrate concentration and incubation time for the scale-up manufacture of Crude Enzyme Extract (CEE), the enzyme pool that was used to produce the bioethanol.*

The five distinct substrate concentrations, 1, 3, 5, 8, and 10% w/v of the cassava peels, were added in triplicate to five different 50 ml flasks that contained 25 ml of culture media. The flasks and their contents were autoclaved for 15 minutes at 121°C and were then cooled to room temperature. After that, the flasks were inoculated with 5 ml of spore suspension of A. niger containing 2.1 x 10^5 spores/ml, and they were incubated in an orbital shaker at 150 rpm and 30 °C for the indicated varied number of days: 0, 2, 4, 6, 8, and 10 days. Amylase and cellulase activity tests were performed after samples were collected at the specified time intervals.

2.13. Recovery of crude enzyme extract (onsite enzymes)

At various periodic intervals indicated, samples were taken from the flasks and filtered. The filtrate which contains the crude enzymes was recovered through centrifugation at 7,000 rpm for 30 mins at 30°C. The supernatant was then decanted into a test tube for further analysis.

2.14. Measurement of enzyme activity (U/ml)

The amylase and cellulase assays were performed using the Dinitrosalicylic Acid (DNSA) technique. For the amylase activity measurement, 0.5 ml of soluble starch solution and 1 ml of potassium phosphate buffer (pH 6.8) were mixed in five separate test tubes. Similarly, for cellulase activity measurement, 0.5 ml of CMC solution and 1 ml of sodium acetate buffer (pH 5.5) were mixed in five independent test tubes. To initiate the reaction, 0.5 ml of the raw enzyme extract was added to each mixture. The tubes were then incubated at 50°C for 30 minutes. All five samples, which were collected at regular intervals for examination over the course of ten days, followed this procedure. A control test tube was also prepared for both assays. After the incubation period, 1 ml of DNS reagent was added, and the reaction was stopped by placing all the tubes in a boiling water bath at 95°C for 5 minutes. The amount of reducing sugar released (glucose) was determined by measuring the absorbance at 540 nm using a spectrophotometer with the DNS technique. The concentration of reducing sugars was calculated by comparing the results with a standard glucose curve. In this

 doi:10.20944/preprints202311.0494.v1

context, one unit of amylase and cellulase activity is defined as the amount of enzyme that can release 1 mole of reducing sugars per minute under the specified assay conditions. (Monga et al., 2011).

2.15. Optimization of onsite enzymes

The optimum conditions obtained in the outcome of the initial enzyme extraction process as outlined earlier were used to scale up the enzyme extraction in a 500 ml flask. The initial experiment results proved that 10% substrate concentration and a 4-day period were the optimum variable conditions for enzyme extraction in this experiment as a temperature of 30°C and pH of 5.5 remains constant with the same already prepared Basal Salt Medium (BSM). The dinitrosalicylic acid (DNSA) method was used for the amylase and cellulase enzyme assays. The absorbance at 540nm was measured against a standard curve as was done in the initial process. The CEE was stored for the hydrolysis process.

2.16. Bioethanol production

Simultaneous Saccharification and Fermentation (SSF)

The crude enzymes produced (amylase and cellulase) were used to test for ethanol production efficiency by flask cultivation in other to get the optimum conditions for the scale-up of the final bioethanol production in a 10 L bioreactor. This initial experimental process was done in shake flasks (250 mL). An enzyme dose of 20 mL, a temperature of 50°C, and a pH of 5.0 were maintained as fixed conditions throughout the experimental period. Substrate concentrations of 5%, 10%, and 20% w/v were varied in the saccharification process. The best condition among the three that produced the highest amount of bioethanol against the fixed conditions was chosen for the scale-up in the bioreactor.

Three different conical flasks were utilized for the given substrate concentrations of 5%, 10%, and 20% w/v. In each of the three conical flasks, the already prepared cassava peel flour was dissolved in an initial volume of distilled water. To reach the target, more water was poured into the flask. The flasks were sealed with cotton wool, vigorously shaken, and then autoclaved at 121°C for 15 minutes. After that, 20 ml of the enzyme mixture which contains the cellulase and amylase was added to each flask as an inoculant, and a brewer's yeast (obtained from Anchor Yeast, South Africa) with a concentration of 1.5 g was added to the fermentation broth. Each conical flask's mixture was sealed with aluminium foil and kept under anaerobic conditions for 7 days. After 7 days, samples were taken daily, and filtered, and Gas Chromatography (GC) was used to determine the ethanol concentration. In triplicate, each experiment was carried out, and a control experiment was also prepared.

2.17. Statistical analysis

All analyses were done in triplicates and results were presented as mean standard values. One-Way Analysis of variance (ANOVA) was used for the comparison of means using Microsoft Excel 365 ProPlus. The significance of $p < 0.05$ was accepted.

3. Results And Discussions

CHAPTER 1

3.1. Lignocellulosic and starch characterization

Table 1 indicates the parameters of the percentage insoluble portion of the cassava peels and that of starch. At 0.5 g of the total cassava peels analyzed for the insoluble portion, the composition of cellulose was the highest (39.78%), and lignin content was found to be the lowest (3.84%). There was also a 31.21% starch determined according to the protocol as described by (Noorfarahzilah *et al.*, 2020).

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Table 1. Lignocellulosic and starch composition (%) of cassava peels.

Parameter	Percentage of dry cassava peel components (%) (on as-fed basis)
Cellulose	39.78
Hemicellulose	21.11
Lignin	3.84
Starch	31.21

Cassava peels are utilized as a raw material in the production of second-generation bioethanol, as they can be hydrolyzed to produce crucial sugars required for bioethanol production. Understanding the initial levels of these constituents in the materials used is important. In this experiment, the dried cassava peels sample was analyzed using the Neutral Detergent Fibre (NDF), Acid Detergent Fibre (ADF), and Acid Detergent Lignin (ADL) methods to determine its lignocellulosic composition. Table 1 presents the percentage composition of cassava peels, which includes starch (31.21%), cellulose (39.78%), hemicellulose (21.11%), and lignin (3.84%). Similar results were reported by (Widiarto et al., 2019) for cellulose (40.5%) and hemicellulose (21.4%), but there was a significant difference in lignin content (11.7%). Conversely, (Suleman et al., 2021) reported lower cellulose content (11.30%) and similar hemicellulose and lignin content (22.39% and 3.09% respectively). (Bayitse et al., 2015; and Kongkiattikajorn, 2012) reported a wider range of starch content in their experiments (47.16% and 15.82% respectively). The results of the current experiment, as shown in Table 1, highlight two important characteristics of cassava peels that make them suitable for bioethanol production. Firstly, they have a relatively high composition of cellulose and starch, which, when properly hydrolyzed, can yield significant amounts of glucose and maltose. Secondly, the biomass has a low lignin content, which reduces the recalcitrance of the lignocellulosic material during enzymatic pretreatment.

Microorganisms, including bacteria and fungi present in the environment, play a crucial role in the degradation of cassava peels. The presence of fungal organisms in the soil, particularly in gari processing sites, aligns with a report by (Omemu et al., 2007) indicating that soil serves as a reservoir for plant-degrading organisms. This research focused on the isolation of fungi for microbial analysis. Table 2 presents the results of fungal isolation from two different samples obtained from the same source. In fresh cassava peels (FCP), only one type of fungal isolate, identified as $ICPF_1$, was found. In decayed cassava peels (DCP), three different fungi were identified $ICPF_1$, $ICPF_2$, and $ICPF_3$. Previous studies conducted by (Ebah, 2021 and Akinruli et al., 2022) also reported the presence of *Aspergillus niger* and Rhizopus spp. in the fungi isolated from cassava peels. Table 3 demonstrates the clear degradative abilities of the isolated fungi, with all three isolates showing evidence of enzyme production. Among the three isolates, $ICPF_1$ identified as *Aspergillus niger* exhibited the largest zone of clearance around the colony, indicating its high enzyme production capability.

Table 2. Fungi isolated from cassava peels.

Sample	$ICPF_1$	$ICPF_2$	$ICPF_3$
FCP	1	0	0
	0	0	0
	1	0	0
DCP	0	6	1
	1	1	0
	0	2	0

ICPF = Isolated Cassava Peels Fungi; FCP = Fresh Cassava Peels; DCP = Decaying Cassava Peels. Figures represent colony counts for isolated pathogens.

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Table 3. Zone of clearance results.

Sample	Diameter of zone of clearance (mm)
$ICPF_1$	16
$ICPF_2$	9
$ICPF_3$	13

3.2. Qualitative analysis of the isolates for enzyme production

All three isolates were screened for amylase and cellulase production using the PDA plate method resulting in a clear zone of enzyme hydrolysis in the Petri dishes after treatment with iodine and Congo red solutions respectively. The zone of clearance analysis of all the isolates as indicated in Table 3, revealed that on average, *Aspergillus niger* got the highest zone of clearance whiles *Aspergillus flavus* obtained the least. *Aspergillus niger* was then chosen and preserved for further analysis.

A crude enzyme blend was generated using cassava peels as a substrate in a 250ml flask. The presence of an enzyme cocktail in the crude extract was confirmed by assessing the activity of amylase and cellulase enzymes. These enzymes belong to a group that breaks down complex sugars into simpler forms. Amylase and cellulase play a vital role in the saccharification process of various natural substrates for biofuel production, as highlighted by (Mohy et al., 2014). The cost of enzymes constitutes a significant portion (10%–20%) of the overall expenses involved in producing ethanol from biomass. This financial aspect has prompted numerous studies to explore ways to reduce enzyme costs in bioethanol production. One suggested approach is the on-site production of economical lignocellulosic raw materials, which can help alleviate the financial burden associated with enzyme procurement (Barta et al., 2010). In line with this, the present study aims to address this cost-efficiency concern by utilizing cassava peels for the on-site production of a crude enzyme blend. This approach offers a viable and economical means of obtaining enzymes for bioethanol production.

Enzymatic activity can be measured using two approaches: assessing the remaining amount of substrate or quantifying the produced product resulting from the enzyme-catalyzed reaction. In the case of amylase and cellulase, the enzymatic reactions yield maltose and glucose, respectively. To measure the product concentrations, a colorimetric assay was employed. This involved determining the absorbance values, as presented in Tables 4 and 5 and utilizing these values to calculate the corresponding product concentrations, as shown in Figures 2 and 3. Finally, the enzyme activity was determined and reported in Figures 4 and 5. To determine the concentrations of amylase and cellulase present in the assayed samples, a standard curve for glucose was constructed, as depicted in Figure 1. A standard curve allows for the determination of the concentration of an unknown sample by comparing its absorbance to known standards. A well-constructed standard curve should exhibit a high R-squared value, preferably close to 1, indicating a good fit. In the case of this research, the glucose standard curve displayed an R-squared value of 0.9821, indicating a well-fitting curve (Figure 1).

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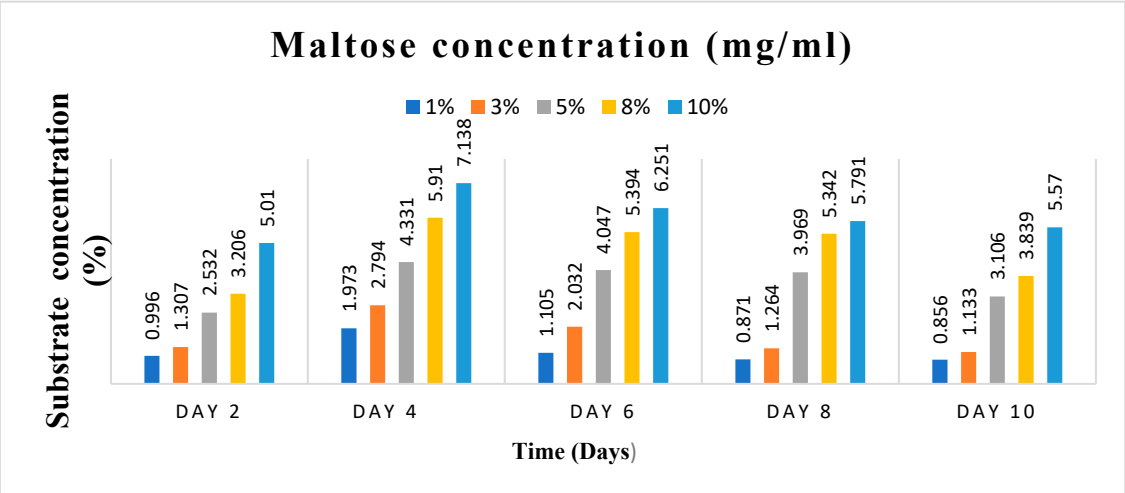

Figure 1. Maltose concentration (mg/ml) from the absorbance.

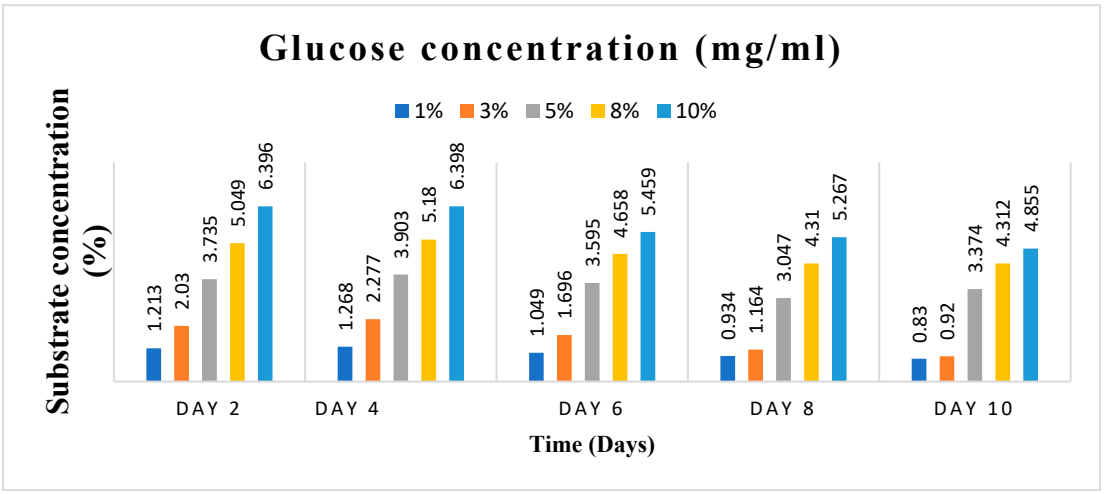

Figure 2. Glucose concentration (mg/ml) from the absorbance.

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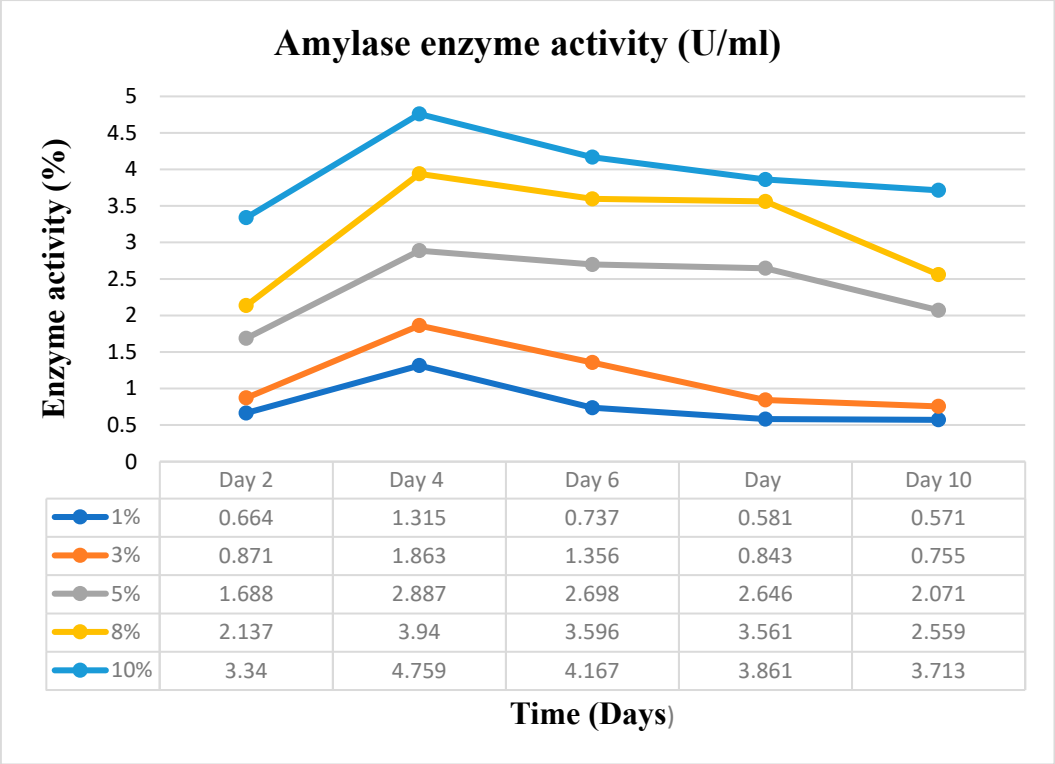
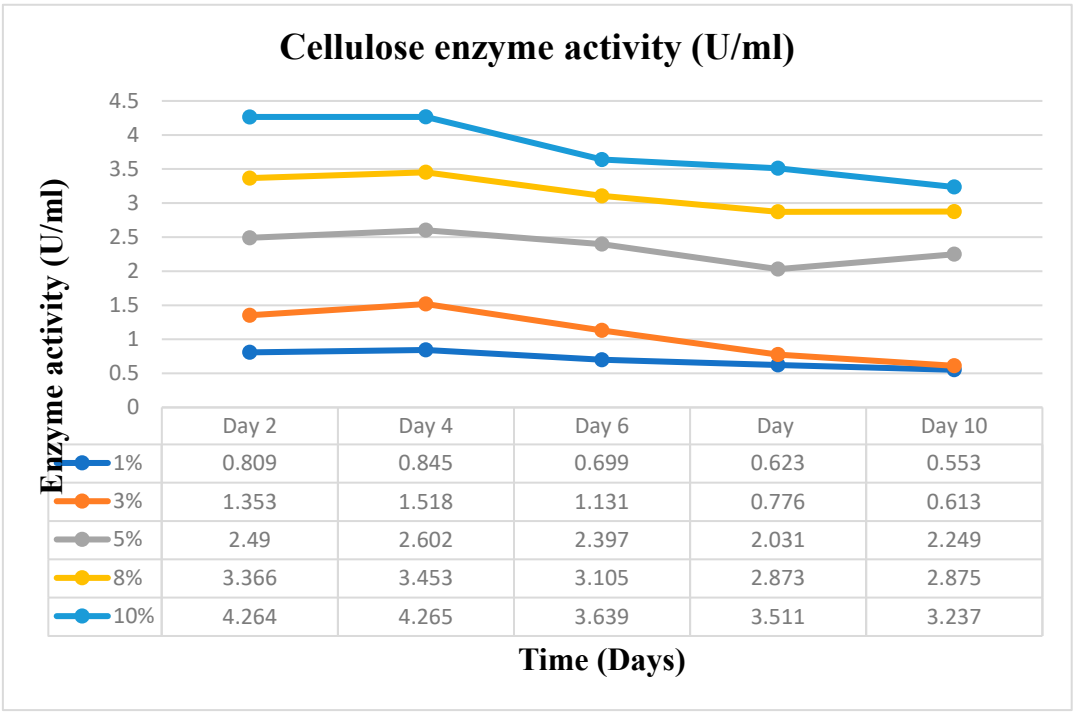


	Day 2	Day 4	Day 6	Day	Day 10
1%	0.664	1.315	0.737	0.581	0.571
3%	0.871	1.863	1.356	0.843	0.755
5%	1.688	2.887	2.698	2.646	2.071
8%	2.137	3.94	3.596	3.561	2.559
10%	3.34	4.759	4.167	3.861	3.713

Figure 3. Amylase enzyme activity obtained from the maltose concentration.



	Day 2	Day 4	Day 6	Day	Day 10
1%	0.809	0.845	0.699	0.623	0.553
3%	1.353	1.518	1.131	0.776	0.613
5%	2.49	2.602	2.397	2.031	2.249
8%	3.366	3.453	3.105	2.873	2.875
10%	4.264	4.265	3.639	3.511	3.237

Figure 4. Cellulase enzyme activity was obtained from the glucose concentration.

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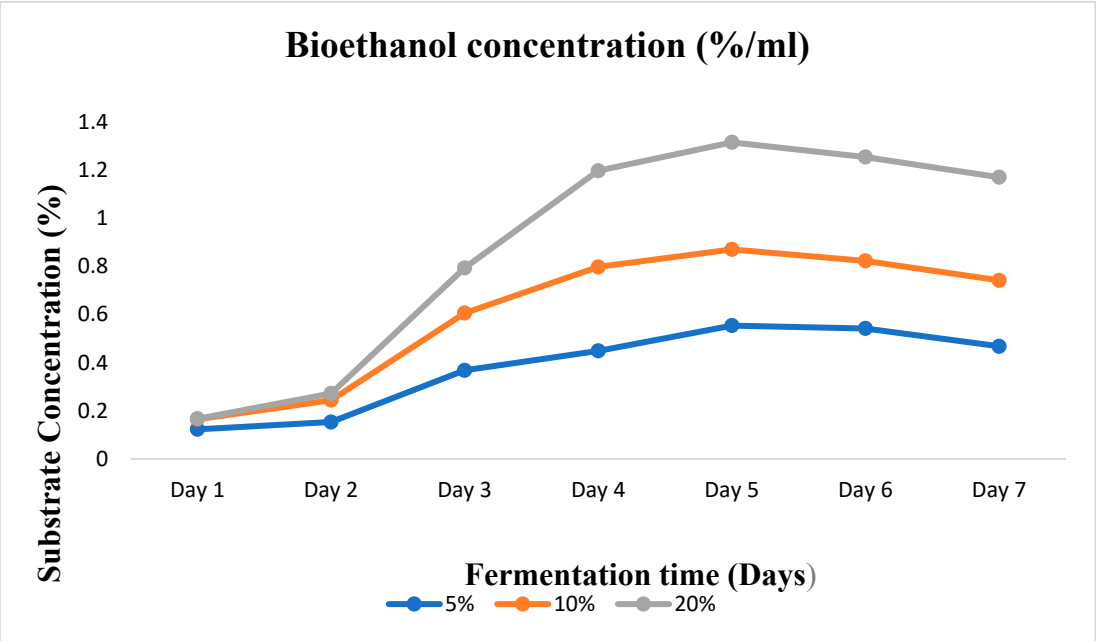

Figure 5. Bioethanol concentration obtained from flask fermentation using Gas Chromatography.

Tables 4 and 5 present the absorbance values (OD) of maltose and glucose, respectively, as determined using a spectrophotometer at a wavelength of 540 nm. Both tables demonstrate that as the substrate concentration increases, the absorbance values also increase. Notably, there is a pronounced rise in values from Day 2 to Day 4 across all samples in both tables. Subsequently, the values start to decrease from Day 6 to Day 10 in both maltose and glucose measurements. The highest absorbance values were observed on day 4 with a substrate concentration of 10%, yielding values of 5.435 for maltose and 4.876 for glucose. Referring to the OD values in Tables 4 and 5, the corresponding concentrations of maltose and glucose were calculated and recorded in Figures 2 and 3, respectively. It is worth noting that the highest concentrations, namely 7.138 for maltose and 6.398 for glucose, were obtained on day 4 with a substrate concentration of 10%, aligning with the OD values presented in Tables 4 and 5.

Enzyme activity refers to the overall catalytic capability of an enzyme, representing the amount of substrate converted by the enzyme in moles per unit of time. Figures 2 and 3 provide the concentrations of maltose and glucose, respectively, which were used to determine the enzyme activity. Figures 4 and 5 reveal the results of the enzyme activity measurements, with values of 4.759 and 4.265 obtained for amylase and cellulase, respectively. These results demonstrate that both amylase and cellulase exhibited their highest enzyme activity on day 4 with a substrate concentration of 10%. This finding is consistent with the research conducted by (Bellaouchi et al., 2021), who also reported the highest enzyme activity on day 4. Given that the primary aim of these initial experimental procedures was to identify the optimal conditions in terms of the number of days and substrate concentration, the 10% substrate concentration and the 4-day duration were chosen as they produced the highest concentrations of maltose and glucose, along with their corresponding highest amylase and cellulase activities. These conditions were selected for the scale-up production of the crude enzyme extract (CEE) in a 500ml flask. In addition to substrate concentration and number of days, other factors such as temperature and pH were taken into account since they can influence the rate of enzyme activity. Therefore, a temperature of 30°C as reported by Veerapagu et al., (2016 and Mohy El-Din et al., (2014) and a pH of 5.5 as reported by Bellaouchi et al., (2021) were considered and maintained throughout the experiment as the optimal conditions.

The production of ethanol is closely associated with the growth of beneficial microbial cells. According to (Zely, 2014), the number of yeast cells and the substrate amount have an impact on ethanol production. In this study, the enzymatic production of bioethanol from cassava peels was examined using *Aspergillus niger*, a mould isolated from the same cassava peels, for substrate

 doi:10.20944/preprints202311.0494.v1

hydrolysis. The fermentation process involved the use of Brewer's yeast (Saccharomyces cerevisiae). The results presented in Figure 6 indicate that a substrate concentration of 20% and a fermentation period of 5 days resulted in the highest ethanol yield (1.316%/ml) from the cassava peels during flask fermentation. The percentage of ethanol production increased significantly for all samples during the initial five days of fermentation. Day 1 showed the lowest ethanol levels, while Day 5 demonstrated the highest levels. After Day 5, a gradual reduction in ethanol concentration was observed, continuing until Day 7. (Duangwang and Sangwichien, 2015) explained that the initial increase in ethanol levels during the first five days is attributed to the presence of sufficient fermentable sugars, allowing yeast to convert sugars into ethanol through digestion. The subsequent decline in ethanol levels indicates the depletion of available fermentable sugars necessary for yeast growth. The use of *Aspergillus niger* for cassava peel hydrolysis and Saccharomyces cerevisiae for bioethanol production has been reported as effective by (Adetunji, 2015) and (Efeovbokhan, 2019). Higher maximum ethanol yields of 1.91% (Osemwengie, 2020) and enzyme concentrations of 1.89% (Peláez, 2013) have been reported in other studies, slightly surpassing the 1.32% yield obtained in the current flask experiment.

4. Conclusion

Firstly, the onsite production of a crude enzyme cocktail from cassava peel using *Aspergillus niger* proves to be an innovative method for obtaining enzymes for the hydrolysis of lignocellulosic materials such as the cassava peels. Secondly, Cassava peel proves to be favourable substrate for bioethanol production. Furthermore, the flask fermentation experiments indicate that a substrate concentration of 20% cassava peel, along with 1.5g of *Saccharomyces cerevisiae* and 20 ml of Crude Enzyme Extract (CEE), is suitable for scaling up bioethanol production in a bioreactor. These optimized conditions demonstrate the potential for industrial bioethanol production in Ghana, suggesting that larger-scale production is feasible.

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