Optimization of bioconversion processes for ethanol production from agrowastes using immobilization technology: a comparative study of cassava peel and rice husk

[1]Udom, F. A., [1]Asamudo, N. U., [1]Udoh, D. I., [1,2*]Ofon, U. A. and [1]Ndubuisi-Nnaji, U. U.
[1]Department of Microbiology, University of Uyo, Akwa Ibom State, Nigeria
[2]Center for Energy and Environmental Sustainability Research, Akwa Ibom State, Nigeria
Corresponding author: utibeofon@uniuyo.edu.ng

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Abstract: The production of ethanol from agricultural waste was investigated using simultaneous saccharification and fermentation (SSF). Cassava peel (CP) and rice husk (RH) were selected as substrates and analyzed for their proximate composition before pretreatment by steam explosion. *Aspergillus niger*, which exhibited the highest cellulose conversion potential among isolates from a waste dump, was employed for saccharification, while *Saccharomyces cerevisiae* was used to convert sugars into ethanol. S. cerevisiae cells were immobilized in sodium alginate for fermentation. The fermentation process was carried out at ambient temperature for 120 h, using a fungal inoculum size of $2.6 \pm 0.04 \times 10^6$ cellsmL^{-1} and 5% yeast concentration. Reducing sugar levels were monitored every 24 hours using the dinitrosalicylic acid (DNS) method, and ethanol yield was determined via specific gravity measurements. Proximate analysis revealed that CP contained higher moisture (6.81%), protein (4.77%), lipid (4.01%), and carbohydrate (70.64%) levels compared to RH. Initially, CP produced higher reducing sugar (0.899 mg/ml) and ethanol yield (4.20%) than RH (0.764 mgmL^{-1} and 1.75%). Fermentation conditions were optimized, with CP achieving a maximum ethanol yield of 23.02% at 30°C, pH 5.0, 25% substrate concentration, and 20% yeast concentration, while RH reached 15.92% at 32°C, pH 5.0, 30% substrate concentration, and 20% yeast concentration. Free yeast cells resulted in lower ethanol yields for both CP (19.92%) and RH (12.96%). Overall, CP proved to be a more effective substrate, and both substrates showed potential for bioethanol production.

Keywords: Ethanol, optimization, yeast immobilization, saccharification, fermentation, cassava peel, rice husk

Introduction

One of the most crucial determining variables affecting global prosperity is energy. The world's current economy is heavily reliant on diverse yet finite fossil energy sources like oil, coal, natural gas, and so on, which are used to produce fuel, power, among other goods (Nogueira et al., 2020). The production of biofuel for use in automobiles is one defining attribute of the 20th century (Alabdallal et al., 2023). Due to the enduring rise in energy prices and the over-reliance on fossil-based fuels coupled with its attendant environmental pollution problems, a great deal of attention has shifted to alternative yet sustainable energy sources. As a result, the conception of liquid biofuels, example ethanol has been promoted as a potential sustainable solution to the issues associated with traditional fuel sources. (Nwosu- Obieogu, 2016).

Due to their little or no cost, high availability, and renewable nature, lignocellulosic waste materials are highly preferred for bioconversion into biofuels (Baral et al., 2019; Ndubuisi-Nnaji et al., 2021; Adewuyi, 2022). Of the lignocellulosic biomass, agricultural wastes are the most significant and can be biologically converted or transformed into single-cell proteins, glucose, and ethanol — products with a marketable appeal. Microorganisms and their enzymes that can which consume the various sugar forms found in lignocellulosic hydrolysates ensure the catalytic conversion (breakdown) of these waste into biofuel and other value-added bioproducts In Nigeria, a significant number of agricultural biowastes are largely under-utilized, and often disposed of indiscriminately at open dumpsites. It has been estimated that Nigeria generates about 4.34 million tonnes of rice straw and 900 thousand tonnes of rice husk (Awogbemi et al., 2021), and it is evident that with the increasing population, the quantity of agrowastes will continue to increase exponentially in the coming decades. For this reason, the purposeful exploitation, utilization and transformation of these huge waste conversion potential for bioenergy presents a significant and viable economic opportunity for the country with no adverse effect. To date, immobilized cell technology has been proposed as a suitable and successful way to increase ethanol production (Moreno-García et al., 2018; Prado et al., 2024). It permits the simultaneous saccharification of biomass and conversion of sugar to ethanol, lowering the cost with increasing rate of production. Several studies have been conducted on ethanol production using immobilized yeast to ferment lignocellulosic wastes employing processes such as electro-fermentation, enzyme hydrolysis, and immobilization via coaxial electrospinning (Sowatad et al., 2020; Nisha and Vidya, 2024; Gherbi et al., 2023; Cadiz et al., 2023; Chen et al., 2024). In this study ethanol was produced from agrowastes by simultaneous saccharification and fermentation process using yeast cells immobilized in calcium alginate beads. The production process was optimized for greater yield by adjusting varying factors like pH, temperature, substrate concentration and yeast concentration thus contributing to the ever-expanding body of knowledge on alternative biofuel and bioenergy generation.

Materials and Methods

Figure 1 shows the flowchart of the experimental design of this study from sample collection to production and estimation of ethanol.

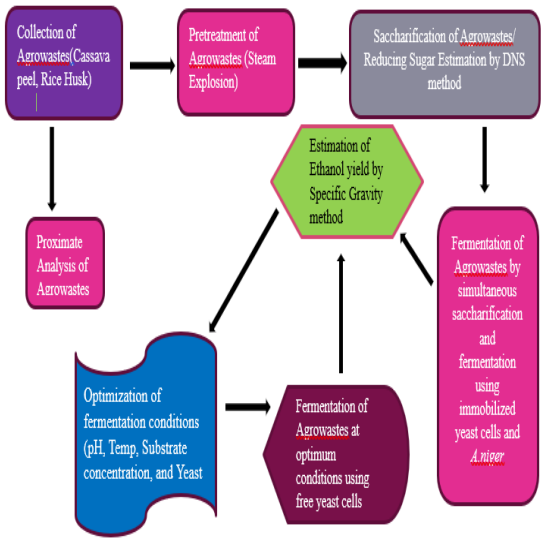


Figure 1: Flowchart of experimental workflow

Collection of Samples

About 500g of Rice husk (RH) was obtained from a local rice mill in Ini Local Government Area of Akwa Ibom State, and 800g of Cassava peel (CP) was obtained from farm sites in Uyo. The agrowastes were washed properly to remove dust particles then spread over a surface and allowed to sun-dry for 8- 10 h to standardize the moisture level. The dried agrowastes were ground into fine particles of sizes 1 - 2 mm and used as substrates in the experiment. Soil samples from municipal dumpsites collected at depth (15 cm) were used for the isolation of cellulose-degrading fungi for the bioethanol production assay.

Proximate Analysis of Agrowastes

Proximate composition of the agrowastes such as percentage moisture, fibre, ash, lipid, protein and carbohydrate were determined according to *Association of Official Analytical Chemists* AOAC (2010) standard.

Pretreatment of Agrowastes

The ground agrowastes were pretreated by steam explosion method. Steam pretreatment can be utilized for different kinds of biomass, it requires little energy and there is no need for use of organic solvents and chemicals. Five (5g) of each ground sample was weighed and poured into a 500ml conical flask. Sterile distilled water was added separately to make up to 200ml and the flasks were covered with sterile cotton wool wrapped in aluminum foil to avoid contamination. The mixture was hydrolyzed and sterilized by autoclaving at 121ºC for 15 minutes. The pretreated samples were then filtered using a No. 1 Whatman filter paper in a 500ml conical flask as described by Bassey (2023).

Isolation and Characterization of Cellulose Degrading Fungus

Six soil samples were collected from three different waste dumpsites in Uyo. Ten grams (10g) of soils was mixed with 90ml of sterile water, and placed on a vortex shaker to dislodge the soil particles. Exactly one (1) ml of each aliquot was transferred into 9ml of sterile water, and was serially diluted to factor 6 (10^{-6}). Total of 0.1ml from the 10^{-5} and 10^{-6} were spread on the surface of sterile Sabouraud dextrose agar (SDA) plates supplemented with 0.5ml of streptomycin to suppress bacterial growth. The plates were incubated at 28 ˚C for 5 – 7 days. Different fungal colonies observed were subcultured onto fresh sterile SDA plates and incubated at 28 ˚C for 5-7 days (Knudsen et al., 1995).

Cellulase Producing Potential of Fungal Isolates

This was done according to the method described by Ahmad et al. (2021) with slight modification. The fungal isolates were point inoculated on Carboxymethylcellulose (CMC) agar plates with 2 - 3 days incubation at room temperature. After incubation, the plates were flooded with Congo red stain for 15 – 20 minutes and then rinsed with 1M NaCl. A clear halozone around the isolate on the CMC agar indicated cellulose hydrolysis by cellulase enzyme. Based on zone of clearance, the most efficient cellulose hydrolyzing isolate was selected for further identification and study.

Identification of Cellulose Degrading Fungal Isolate

Macroscopic and Microscopic Examination of Fungal Isolate.

Morphological features were observed which included: colour, growth pattern and physical appearance on SDA (Ali et al., 2024). The method of Barnett and Hunter (1972) was employed for microscopic examination of properties such as nature of hyphae, conidial head, vesicle shape and special vegetative structure.

Molecular Identification of Fungal Isolate

According to the method of Ali et al. (2024), genomic DNA from the isolate was extracted using ZR fungal/bacterial DNA Miniprep (Manufactured by Zymo Research) and extraction was done according to the manufacturer's instructions. Agarose gel was supplemented with loading buffer and stained with EZ vision DNA stain. Electrophoresis was carried out at 100 V for approximately 1 h to ascertain the presence of extracted DNA. A UV transilluminator was used to visualize DNA fragments. The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA X were used for all genetic analysis.

Activation of Yeast Cell

Fermentation was carried out using Bakers' yeast rehydrated at 30 ˚C for 30 min then inoculated onto a plate of Sabouraud Dextrose Agar (SDA) followed by continuous subculture at 2 days interval. Stress tolerance tests which included thermotolerance, ethanol tolerance as well as sugar utilization test were carried out on the yeast cell (Gidado et al., 2016).

Thermotolerance Test

The yeast was inoculated on SDA plates and incubated at 30, 33, 36 and 39 ˚C and the inoculated plates were observed for growth after 48 h (Babiker *et al.*, 2010).

Ethanol Tolerance Test

A 24-h washed yeast cells was used, aliquot of 0.1ml of the washed cells was inoculated into yeast extract broth containing 3, 6, 9, 12 and 15% ethanol. The tubes were incubated at 30 ˚C for 48 h, the optical density of the cells was taken using a spectrophotometer to determine the number of cells/ml (Breisha, 2010).

Immobilization of Yeast Cells

For immobilization in beads, 3% (w/v) sodium alginate was prepared by dissolving 3g of sodium alginate in 100ml of water and added to a 100ml suspension of *S. cerevisiae* in a beaker. The mixture was added dropwise using a 10ml syringe into 150ml of 2% (w/v) $CaCl_2$ solution prepared in a separate beaker. The beads were hardened in this solution for 1 h then rinsed with sterile

water to be used thereafter in the fermentation experiments (Duarte *et al.*, 2013).

Estimation of Reducing Sugar (RS)

Reducing sugar estimation was carried out using dinitrosalicylic acid (DNS) method as described by Tambuwal *et al.* (2018). A standard glucose calibration curve was plotted and used to obtain the reducing sugar concentration every 24h.

Preparation of Fermentation Medium

The fermentation medium that was used for ethanol production was prepared using the following (per 100ml): yeast extract (1g), peptone (0.1g), ammonium Sulphate $(NH_4)_2SO_4$ (0.4g), $MgSO_4.7H_2O$ (0.75g), KH_2PO_4 (3.5g), $CaCl_2$. $2H_2O$ (1.0g) and ferrous sulphate $FeSO_4$. $4H_2O$ (0.01g). The media was autoclaved for sterilization at 121˚C for 15 min.

Simultaneous Saccharification and Fermentation (SSF) of Substrates

Simultaneous saccharification and fermentation of the substrates was carried out using 5% substrate and 5% immobilized yeast concentration and 10mL of fungal suspension of OD 0.26±0.04 (equivalent to 2.6 x 10^7 cells/ml). This was achieved based on the procedure adopted by Bello et al. (2022). Five percent (5%) substrate concentration (2.5g in 50mL of sterile water) was inoculated with 10mL of fungal suspension of OD 0.26±0.04 (equivalent to 2.6 x 10^7 spore cells/ml) along with 5% immobilized yeast concentration then incubated at ambient temperature for 120 h and sampled every 24 h to determine reducing sugar (RS) and ethanol yield (EY).

Estimation of Ethanol Yield (EY)

The original specific gravity and final specific gravity was obtained after fermentation occurred every 24 h as described by Suhail *et al*. (2013). The percentage alcohol by volume (%ABV) was determined using the Balling equation as written by Maskell *et al*. (2017) which is expressed thus:

$$\%ABV = \frac{76.08(OG-FG)}{1.775-OG}\ X\ (\frac{FG}{0.794})$$

OG = Original specific gravity before fermentation

FG = Final specific gravity after fermentation

Optimization of Fermentation Conditions for Bioethanol Production

Optimization of fermentation conditions involves varying the values of the process variables in order to find the optimal value to maximize fermentation and hence increase ethanol yield. The process variables optimized in this study were pH, temperature, substrate concentration and yeast concentration. Temperature and pH affect enzymatic activity of the yeast and ultimately affects fermentation time. The optimal values of these variables depend on the type of organism and substrates involved. Readily biodegradable substrates can be converted most efficiently under mesophilic conditions.

Influence of pH

To determine the effect of pH on ethanol production, 5% concentration of each substrate was fermented using 5% yeast concentration at varying pH values of 4.0, 4.5, 5.0, 5.5 and 6.0 at ambient temperature for 72 h. 1N HCl and 1N NaOH was used to adjust the pH.

Influence of Temperature

To determine the effect of temperature on ethanol yield, 10% concentration of each substrate was fermented at varying temperatures of 30, 33, 36, 39 and 42 ˚C at the optimum pH and standard inoculum size.

Influence of Substrate Concentration (SC)

Different concentrations of each substrate such as 10%, 15%, 20%, 25% and 30% were used to carry out fermentation at optimum temperature and pH and observed for a period of 72 h.

Influence of Yeast Concentration (YC)

Different concentrations of the immobilized yeast such as 10, 15, 20, 25 and 30% w/v were used in the fermentation of the substrates at optimum substrate concentration, temperature and pH for a period of 72 h.

Fermentation Using Free Yeast Cells

To compare the ethanol yield from each substrate obtained using immobilized yeast and free yeast cells, the different substrates at optimum concentration were fermented using free yeast cells at optimum pH and temperature for a period of 72 h.

Statistical analysis

All experiments were performed with replication and values reported as mean data of datasets with standard deviation (SD). A one-way analysis of variance (ANOVA), Tukey and Duncan's Post-hoc tests were carried out using Statistical Package for Social Sciences (SPSS). Significance was accepted at $p \leq 0.05$.

Results and Discussion

Proximate composition of the agrowastes

The result of the proximate analysis of the agrowastes which included percentage moisture, protein, lipid, ash, crude fibre, and carbohydrate are shown in Table 1. It was observed that CP had the higher percentage moisture content (6.81%), percentage protein (4.77%), percentage lipid (4.01%) and percentage carbohydrate (70.64%) while RH had the lower percentage moisture (2.79%), percentage protein (2.53%), percentage lipid (2.12%) and percentage carbohydrate content (47.03%). RH also had the higher crude fibre 28.23% compared to CP.

This implies the presence of more fermentable sugars in cassava peel (CP) than rice husk (RH) available for bioconversion by fermentation into ethanol – a biofuel. Yosaa et al. (2022) has also previously reported increased carbohydrate levels in cassava peel which aligns with this study. These findings vary differently from that reported by Amaza (2021) on the proximate composition of cassava peel stated as 8.70% moisture, 8.75% crude fiber, 2.15% crude fat, 4.89% crude protein, 8.93% ash and 66.56 % Nitrogen free extract. Nnadiukwu *et al*. (2023) reports the proximate composition of rice husk differently from this study as follows: 37.04% carbohydrate, 7.93% moisture, 3.76% lipid, 25.74% fibre content, 1.85% protein, and 23.39% ash. Although these data differ from that of this study, comparatively they agree that cassava peel does have the highest percentage carbohydrate, moisture and protein and rice husk has the lowest percentage carbohydrate.

Table 1: Proximate Composition of Agrowastes

Composition (%)	Cassava peel	Rice husk
Moisture	6.81 ± 2.022^{b}	2.79 ± 0.141^{a}
Protein	4.77 ± 1.103^{b}	2.53 ± 0.057^{a}
Lipid	4.01 ± 0.226^{b}	2.12 ± 0.028^{a}
Ash	6.54 ± 0.438^{a}	17.3 ± 1.004^{b}
Crude Fibre	7.23 ± 0.212^{a}	28.23 ± 1.089^{b}
Carbohydrate	70.64 ± 1.089^{b}	47.03 ± 1.867^{a}

Values expressed as mean± standard deviation. Values with different superscripts are significantly different ($p<0.05$).

Cellulase production potential of fungal isolates

The cellulase producing potential of different fungal colonies isolated from the different waste dump sites based on zone of clearance is shown in Table 2. Fungal isolate FI_4 with the highest average zone of clearance (11.8 mm) was selected for further identification and used in saccharification of the substrates. Although the CMC agar method is one of the most widely used method of assay, it is sometimes discredited for its low specificity and the fact that zones of clearance can be observed around other enzymes which degrade other polymers (such as amylase or agarose). Apart from Congo red, several other dyes have been introduced, the most commonly known is Gram's iodine. The use of Gram's iodine with CMC agar as the sole method of assay has been considered unreliable due to the presence of minute starch contaminants in commercial CMC agar.

Table 2: Cellulase producing potential of fungal isolates

	ZONE OF CLEARANCE (mm)			AVERAGE ZOC(mm)
ISOLATES	**WD_1**	**WD_2**	**WD_3**	
FI_1	9.0	5.0	8.5	7.5
FI_2	6.0	3.5	7.0	5.5
FI_3	-	11.0	-	3.7
FI_4	11.0	9.5	15.0	11.8
FI_5	5.0	7.5	-	6.3
FI_6	9.5	10.5	8.0	9.3

Key: FI = fungal isolate, WD = waste dumpsite, ZOC = zone of clearance

Cultural and Molecular Identification of Best Cellulase Producing Fungi

Identifying the best cellulase producer involved macroscopic, microscopic, and molecular analyses. The isolate $F1_4$ exhibited cultural characteristics like: dark colonies (brownish-black) having a darker center, evenly distributed growth, smooth yet dense surface texture, and diffuse growth (without clear boundaries around the colonies) (Table 3). Other microscopic characteristics were globose conidia and filamentous soma. Based on these macroscopic and microscopic features, the isolated fungi were presumptively identified as *Aspergillus niger*. These attributes aligned with those described by (Ali et al., 2024).

Molecular analysis by DNA extraction and amplification followed by gel electrophoresis showed that the extracted DNA had a length of 650 base pairs (bp), equivalent to the internal transcribed space (ITS) primer (Appendix 1). After sequencing, the result revealed that isolate FI_4 had 98.00% pairwise identity and adequately aligned with *Aspergillus niger* isolate K4 which has National Center for Biotechnology Information (NCBI) accession number MN180808.1. The e value is 9e-13. The genetic sequence of isolate FI4 is given thus:

ATCGTGGAGTATCGTTTGGCCAACCTCCATCCGT
GTCTATTGTACCCTGTTGCTTCGGCGGGCCGCTT
TTATCGCGCACGGAGAAGTCCATGCCTCGAGCT
CGGACCGTGAACGCTACATTACGGTGCGTCAAC
CGAAGAGGCTAAATTAATTAAACGCAAACGTTA
AAAATTTTTAAAGAAATTTCTTCTTGGTTCCCGC
TTCCCATGAGAAAACCGGGGAAACTGATACCTA
AGGGGAATTGAAGAATCAGTGAATTCTCAGTCT
TTGACGATATCGCTCGCTGGTATTTCGGGGTATG
CTTTCAGAGACAATGTGACATAACACGTATTGA
TTAGTCACTACCTCTCTGGAACGTCAAGAGGTGC
ACGTCAGATCTGAGGAGAGCTTACAAGCCTAAG
ATGCCCCTGACTTACACATCTCAGTGCCGTACAC
GATCCGGATACGGTTAACCGGAGTTATCTT.

Table 3: Macroscopic and Microscopic Characteristics of Fungal Isolate.

Colony Colour	Compact dark mass colony
Type of Soma	Filamentous
Nature of Hyphae	Septate
Special Vegetative structure	Foot cell
Asexual Spore	Globose Conidia
Special Reproductive structure	Smooth walled erect Conidiophore
Conidial Head	Dark globose
Vesicle Shape	Globose
Probable Organism	*Aspergillus niger*

Stress tolerance of fungal isolates

Stress tolerance is a major attribute of yeast cells because stress can lower growth and metabolism rate and cause decreased fermentation efficiency. Hence, cellular tolerance is pivotal for their use in industrial and biotechnological applications. The ability of the yeast cells to tolerate stress conditions such as high temperature and ethanol concentration is shown in Table 4. The results showed that the yeast cells can thrive or remain viable through different concentrations of ethanol and also survive temperatures higher than room temperature.

Table 4: Ethanol tolerance and Thermotolerance of Yeast cells

Ethanol concentration (%)	Optical density (Cells/ml)	Temperature (°C)	THFC (Total Heterotrophic Fungal Count) ($CFUml^{-1}$)
3.0	0.022 ($2.2 x10^6$)	30.0	2.5×10^6
6.0	0.020 (2.0×10^6)	33.0	2.4×10^6
9.0	0.017 (1.7×10^6)	36.0	1.9×10^6
12.0	0.014 (1.4×10^6)	39.0	7.9×10^5
15.0	0.008 (8.0×10^5)	42.0	3.6×10^5

Saccharification of substrate for ethanolic bioconversion

The reducing sugar concentration obtained every 24 h by saccharification of the substrates using a standard fungal inoculum size is shown in Figure 2. The graph shows that cassava peel (CP) gave the higher reducing sugar yield of 0.899 mg/ml compared to rice husk (0.764 mg/ml) after a period of 120 h. Although the reducing sugar concentration for both substrates increased with time until 96 h there was a reduction in yield at 120 h.

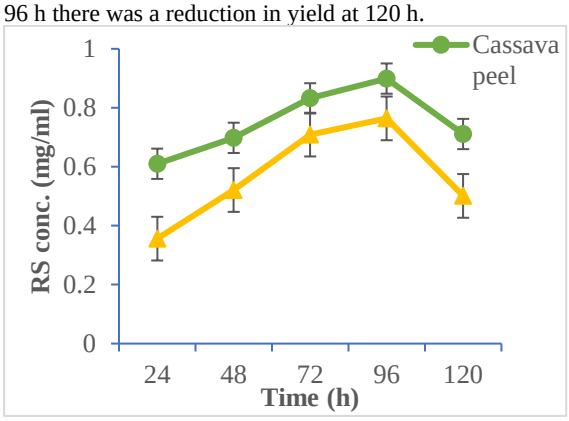


Fig 2: Reducing sugar (RS) concentration of substrates

This was in agreement with Oyeleke and Jibrin (2009) who clearly stated that the amount of sugar released is dependent on type and nature of the utilized substrate, and the concentration or quantity of ethanol produced or obtained consequently depends on the amount of reducing sugar released after hydrolysis. A comparison between studies shows that Adegunloye and Udenze (2017) obtained a similar reduction in the reducing sugar of co-cultured fermented corncobs and cocoyam peel, respectively using *A. niger* and *S. cerevisiae.* This supports that the swift bioconversion of sugar to ethanol during the fermentation process by *S. cerevisiae* could continually lower the sugar concentration to prevent feedback inhibition of the amylolytic activity of certain molds at the onset of the fermentation.

Influence of fermentation time on reducing sugar concentration

The graph (Figure 3) shows the concentration of reducing sugar (mg/ml) over fermentation time (hours) for the two different substrates: cassava peel and rice husk. Though the rate and amount of reduction differed for both cassava peel (0.45 mg/ml at 24 h) and rice husk (0.39 mg/ml at 24 h), the reducing sugar concentration decreased over period of fermentation for both substrates, in line with a previous study (Liu et al., 2021). This suggested enzymatic and/or microbial breakdown of the sugars as the substrates were degraded. The cassava peel retained higher levels of reducing sugars compared to rice husk, probably due to differences in the composition and biomass structure, signifying readily available source of fermentable (reducing) sugar.

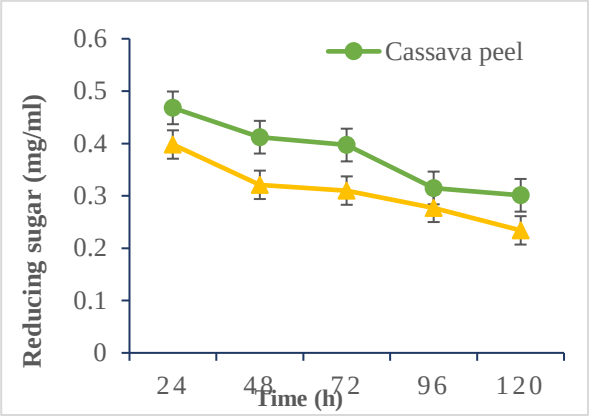


Fig 3: Effect of fermentation time on reducing sugar concentration.

Influence of fermentation time on ethanolic yield

The percentage ethanol yield obtained from each substrate every 24 h interval during simultaneous saccharification and fermentation (SSF) is exemplified in Figure 4. It was observed that highest ethanol production for both substrates was obtained at 120 h interval. Similarly, Fahrizal *et al.* (2013) reported highest ethanol production at 120 h. Although, Shilpa *et al.* (2013) and Zainal *et al.* (2014) also observed a prior increase in the yield which declined after the 4th up to the 7th day. This may be because the organism became starved of nutrients which decreases their metabolic activity and subsequently leads to death thereby reducing ethanol yield.

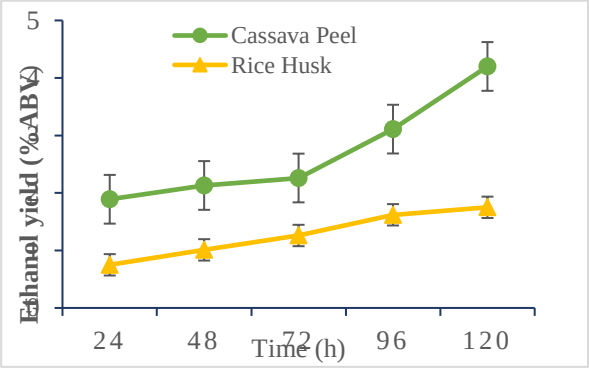


Fig 4: Effect of fermentation period on ethanol yield of substrates

Effect of pH on ethanol production

The variation in ethanol yield at different fermentation pH using a standard SC and YC at ambient temperature is

depicted in Figure 5. It was observed that the optimum pH of fermentation for ethanol production from each substrate is pH 5 for both substrates. The maximum ethanol yield (which was found to be statistically different) obtained from both substrates were 8.87% and 5.08% for CP and RH, respectively. There was a continuous decrease in the yield at pH 5.5 to 6.0 indicating that as pH increased ethanol production decreased – suggesting a proportionally indirect relationship

This proved that pH does have a significant effect on the production of ethanol as supported by the works of Salihu *et al*. (2022) and Tenkolu *et al*. (2022). The lowest ethanol yield was obtained at pH 6.0 for all two (2) substrates studied. Elsewhere, Saini et al. (2018) reported that the decrease in ethanol yield may be due to the fact that the yeast preferred a mildly acidic environment hence, with the increase in pH, the yeast tends to produce more acid than alcohol thereby causing a decrease in yield.

Also, a decrease in the ethanol yield at high pH may be because enzymes are more active in mildly acidic medium and hence amylase activity of *A. niger* are sensitive to pH changes (El-Gendi et al., 2021). This was in agreement with the work of Ado *et al*. (2009) who reported maximum yield of 4.3g/100ml at pH of 5.0, as well as Thippareddy *et al.* (2010) who reported a yield of 1.60% at pH of 5.0 in a two-step fermentation using *Aspergillus niger* and *Zymomonas mobilis*.

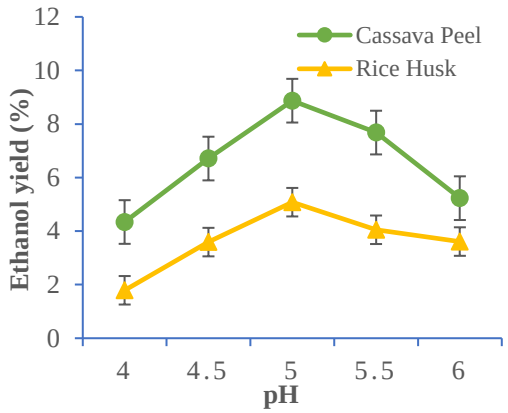


Fig. 5: Optimization of pH and its effect on Ethanol yield

Effect of temperature on ethanol production

The effect of varying temperature on ethanol yield of substrates at optimum pH and a constant SC and YC is illustrated in Fig 6. This showed that the optimum temperatures for ethanol production by SSF from CP and RH are 30 ˚C and 32 ˚C, respectively. Unal et al. (2020) reported the optimum temperature for maximum ethanol production from starch using co-cultures of amylolytic yeast and *Saccharomyces cerevisisae* at 30 ˚C. Misra et al. (2023) also reported maximum ethanol yield from beet molasses by *S. cerevisiae* Y-7 after 72 hours of incubation at 30 ˚C. Tse et al. (2021) reported that the decrease in ethanol yield may also be attributed to the fact that increasing temperatures beyond the optimal level might dispel the available oxygen or increase the toxic effect of ethanol in the medium due to dissociation of the molecules as well as decreased enzyme activity due to denaturation of the enzymes giving no significant impact on ethanol yield.

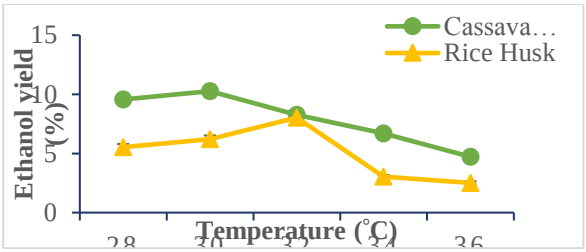


Fig. 6: Effect of Temperature on Ethanol yield of substrates

Effect of Substrate concentration on Ethanol yield of substrates

The graph (Figure 7) represents ethanol yield (%) as a function of substrate concentration (%) for cassava peel and rice husk. Cassava peel consistently produced higher ethanol yields compared to rice husk, regardless of substrate concentration. This aligned with the observation that cassava peel has higher reducing sugar content, making it more readily fermentable for ethanol production. For cassava peel, the optimal ethanol yield occurred at 25% concentration, while rice husk required higher substrate concentrations to reach its maximum potential. The slight drop in ethanol yield at 30% for cassava peel could be due to substrate inhibition or limitations in fermentation capacity at higher concentrations.

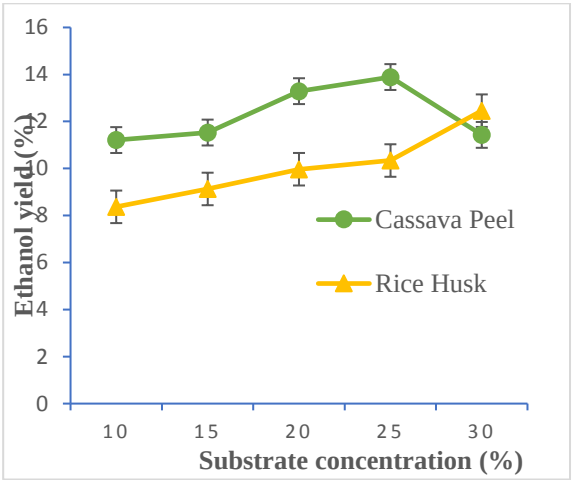


Fig. 7: Effect of Substrate concentration on Ethanol yield of substrates

Wen *et al.*, (2004) reported that an increase in the animal manure (substrate) concentration to 50g/l favoured glucose yield and consequently increased cellulose conversion to 40%. Ado *et al.* (2009) carried out similar research using cassava starch and observed the increase in ethanol yield as cassava starch concentration is increased from 1% to an optimum of 8%. Mardawati *et al*. (2019) also obtained a corresponding increase in ethanol yield with subsequent decrease as the substrate concentration increased.

The effect of varying yeast concentration on the yield of ethanol is depicted in Figure 8. It was observed that the maximum ethanol yields of 15.77% and 13.22% were obtained from CP and RH respectively at optimum YC of 20% for both substrates. There was a statistically significant difference ($p < 0.05$) between the yield of both substrates. The increase in ethanol yield with increase in immobilized yeast concentration could be due to fact that

the increase in the number of yeast cells led to a better or higher bioconversion efficiency of the substrates. The decrease in ethanol yield with further increase in yeast concentration as stated by Nandal et al. (2020) could be due to the fact that the cells deep inside a carrier particle can become inactive due either to deprivation of some essential nutrients or to accumulation of product due to inhibiting concentrations (feedback inhibition). The research carried out by Lamido *et al.* (2021) also agrees that the increase in yeast concentration increases yield until an optimum is reached.

4.9 Effect of Yeast concentration on Ethanol yield of substrates

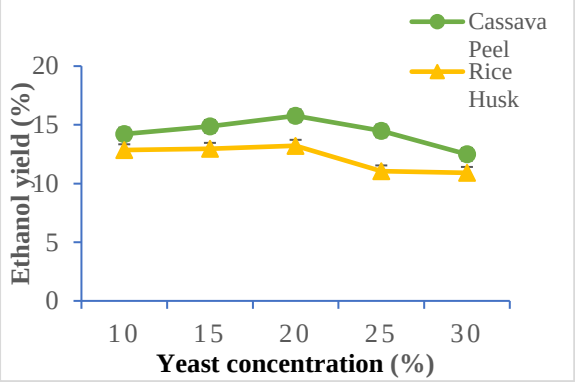


Fig. 8: Effect of Yeast concentration on Ethanol yield of substrates

The maximum yield of ethanol obtained from each substrate after a fermentation period of 120 h under optimal pH, temperature, SC and YC is illustrated in Table 5. Cassava peel (CP) was seen to have a higher yield of 23.02% while RH had a yield of 15.92%. There was a statistically significant difference in the yield ($p < 0.05$) obtained from both substrates.

Table 5: Ethanol yield of substrates at optimal conditions and fermentation period of 120 h (5 days).

Cassava Peel					
RS(mg/ml)	SG	EY(%ABV)	RS(r		(%ABV)
0.288	0.937	23.02±1.05^{b}	0.592		2±1.23^{a}

Values expressed as Means± SD. Different letters represent significant difference (P≤0.05).

The yield of ethanol obtained from CP and RH by SSF using free yeast cells under optimal fermentation conditions was lesser compared to the ethanol yield from the substrates using immobilized yeast under same conditions (Table 6). These data strongly suggested the remarkable potential which lies in the use of efficiently immobilized microorganisms for increased productivity in making future alternative biofuels which can be used both industrially and domestically. The calcium-alginate gel is one of the commonest immobilizing agents and an efficient matrix for the entrapment of yeast cells as far as enhancing ethanol yield is concerned.

Senthilraja *et al.* (2011) who undertook a comparative analysis of bioethanol production by different strains of immobilized marine yeast, obtained a maximum yield of 47.3g/l using immobilized *Candida albicans* as against 28.12g/l using free cells of the same fungus. Also, Ramaraj *et al*. (2021) reported that the free yeast cell during the 1st day of fermentation afforded an ethanol production of 57.574 g/L, while the yield for immobilized yeast was 60.714 g/L. Consequently, the ethanol yield on the second day of fermentation from the directly injected immobilized yeast was 60.088 g/L.

Table 6: Ethanol Yield of Substrates using Free Yeast Cells at Optimal Condition

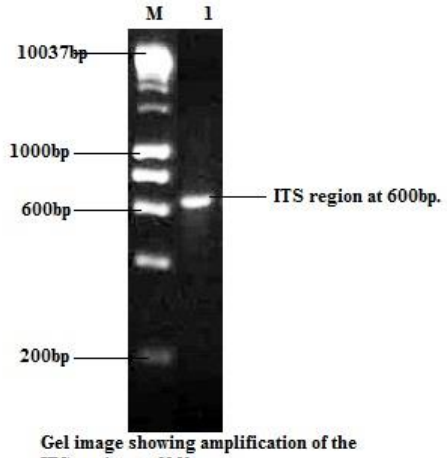
Cassava Peel			Rice Husk		
RS (mg/ml)	SG	EY(%ABV)	RS (mg/ml)	SG	EY(%ABV)
0.335	0.967	19.92	0.603	0.997	12.96

Conclusion

The result of the investigation showed that a significant amount of ethanol was obtained by fungal fermentation of cassava peels and rice husks using immobilized yeasts by simultaneous saccharification and fermentation. The yield fluctuated significantly favouring cassava peel though with variations in pH, temperature, substrate concentration, yeast concentration and fermentation period. The study is cost-effective and shows promise and potential for scale-up as demonstrated in the high yield of ethanol obtained from optimization studies. The use of immobilization technology and the several advantages associated with it is one that should be integrated fully into the bioenergy industry.

APPENDIX 1

Gel image showing genomic DNA extracted from the fungal isolate.



Gel image showing amplification of the ITS region at 600bp.
M is HyperLadder 1kb Bioline.

Gel images of fungal isolate

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