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Effect of variation of process parameters on bioethanol production from sorghum straw using sequential co-cultures of *Aspergillus niger* and *Scheffersomyces stipitis*

Christopher Osazuwa *

Department of Microbiology, Faculty of Science, Adekunle Ajasin University, Akungba-Akoko, Ondo State, Nigeria.

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Abstract

The global quest for sustainable and environmentally friendly alternatives to fossil fuels has accelerated research into second-generation bioethanol production from agricultural residues. In this study, sorghum straw a lignocellulosic waste abundant in sub-saharan Africa was valorized for bioethanol production using a co-culture system of *Aspergillus niger* and *Scheffersomyces stipitis*. *A. niger* facilitated the saccharification of pretreated biomass by producing cellulases, while *S. stipitis* efficiently fermented the released hexose and pentose sugars into ethanol. Process parameters such as pH, temperature, inoculum size, substrate concentration, and fermentation time were systematically optimized. Optimal fermentation conditions (pH 4.5, 25°C, 10% inoculum, and 15% substrate concentration) yielded a maximum bioethanol concentration of 17.05 g/L with a conversion efficiency of 52.12% over 120 hours. Notably, a substrate concentration beyond 15% inhibited sugar release and ethanol yield, likely due to increased viscosity and mass transfer limitations. The study confirms that indigenous microbial strains, coupled with simple process optimization, can significantly enhance bioethanol yields from underutilized agro-wastes. Despite modest yields compared to integrated fermentation systems, the approach remains cost-effective and accessible for low-resource settings. The results hold promise for decentralized bioethanol production in rural communities, potentially transforming agricultural waste into a valuable energy source. This work not only advances local biofuel technologies but also contributes to global efforts in achieving renewable energy goals.

Keywords: Fermentation; Bioethanol; Optimization; Sorghum Straw; *Scheffersomyces stipitis*; *Aspergillus niger*

1. Introduction

The increasing demand for sustainable and renewable energy sources has intensified global interest in bioethanol production as an alternative to fossil fuels. Bioethanol, a clean-burning and biodegradable alcohol, is primarily produced through the fermentation of sugars derived from various biomass sources. First-generation bioethanol production, which utilizes food crops like corn and sugarcane, has sparked significant concern due to its competition with food supply and arable land use [1]. This has shifted research focus towards second-generation bioethanol production from lignocellulosic biomass, including agricultural residues such as sorghum straw, which is both abundant and underutilized in many developing countries [2,3]. Lignocellulose (LC) is the most cost-effective and abundant renewable feedstock for sustainably manufacturing bioethanol. Nigeria's need for ethanol may be readily satisfied if cellulosic ethanol, which produces 22 billion liters of ethanol per year, is made from all of the burnt agricultural leftovers now in use [4].

Bioethanol is mostly obtained from sweet and starchy feedstocks and is known as first-generation ethanol. During the last two decades, the United States and Brazil have been leaders in bioethanol production, employing mostly corn and sugarcane, respectively. The food-related feedstock is substituted in underdeveloped economies by inedible raw

* Corresponding author: Osazuwa C. O.

resources such as cassava or sweet sorghum [5]. The selection of appropriate feedstocks and efficient microorganisms that help in fermentation is a continuous topic of research. In this situation, it is anticipated that lignocellulosic agricultural leftovers will make the best feedstocks [6].

Sorghum (*Sorghum bicolor*) is a drought-tolerant cereal crop widely cultivated in sub-Saharan Africa, Asia, and parts of the Americas. Its by-product, sorghum straw, consists mainly of lignocellulosic components cellulose, hemicellulose, and lignin making it a promising feedstock for bioethanol production [7]. This crop can boost bioethanol production in regions where sugarcane or crop varieties are not doing well since it can be grown in drier conditions. It usually contains >70 % complex carbohydrates which contain C4 and C5 sugar units [8]. The percentages of cellulose, hemicellulose, and lignin vary between 30.48 % and 42.75 %, 19.20 and 27.30 %, and 6.40–28.10 %, respectively [9,10]. Cellulose is a linear polymer formed of D-glucose units connected by β-1,4-glycosidic bonds. At the same time, hemicellulose is a complex heterogeneous polysaccharide mostly composed of pentoses (xylose and arabinose) and hexoses (mannose, glucose, and galactose). Conversely, lignin is an amorphous heteropolymer made up of several phenylpropane units. It covers the cellulose-hemicellulose complex, giving the plant stiffness and defense against microbes [11]. Therefore, while creating effective methods for manufacturing bioethanol, it is important to consider the complexity and varied composition of lignocellulosic materials [12].

However, the structural complexity and recalcitrance of lignocellulosic materials mainly due to the tight bonding between cellulose, hemicellulose, and lignin necessitate pretreatment and hydrolysis before fermentation [13]. Biological pretreatment using enzyme-producing microbes has emerged as an eco-friendly and cost-effective method. In particular, co-culturing cellulolytic and fermentative microorganisms offers a synergistic approach that enhances the conversion efficiency of lignocellulosic biomass to ethanol [14].

Aspergillus niger is a filamentous fungus widely recognized for its potent cellulase and hemicellulase production capabilities. It efficiently hydrolyzes complex polysaccharides into fermentable sugars [15]. Meanwhile, *Scheffersomyces stipitis* (formerly *Pichia stipitis*) is a well-known xylose-fermenting yeast, capable of converting both hexose and pentose sugars into ethanol, thereby complementing the enzymatic action of *A. niger* [16]. Co-cultivation of these two microorganisms integrates saccharification and fermentation processes, minimizing costs and reducing the need for commercial enzyme supplementation [17].

The efficiency of bioethanol production from lignocellulosic biomass depends on several critical process parameters, including substrate concentration, pH, temperature, inoculum size, and fermentation time [18]. These parameters significantly influence microbial growth, enzyme activity, and sugar utilization, ultimately affecting ethanol yield and productivity. Optimization of these parameters is essential to improve the economic feasibility and scalability of lignocellulosic bioethanol production processes. Previous studies have demonstrated that even minor variations in operational conditions can lead to significant differences in ethanol yield [19]. However, limited research has been conducted on the systematic evaluation of these parameters when using *S. stipitis* with sorghum straw as feedstock.

Therefore, this study investigates the effect of process parameter variation on bioethanol production from sorghum straw using co-cultures of *A. niger* and *S. stipitis*. This work aims to contribute to the development of cost-effective, scalable, and sustainable bioethanol production technologies using locally available agricultural residues. By systematically assessing the influence of conditions such as pH, temperature, inoculum size, substrate concentration and fermentation time, this research seeks to identify optimal conditions for maximum ethanol yield. The findings of this study are expected to contribute to the development of a cost-effective and efficient second-generation bioethanol production process, particularly relevant for regions rich in sorghum cultivation and agro-waste.

2. Materials and Method

2.1. Sample Collection

Five hundred (500) grams of sorghum straw was collected from farms within Nigeria. The sample were aseptically bagged and transported to the laboratory for microbial and chemical analysis.

2.2. Microbial Cultures

Aspergillus niger and *Scheffersomyces stipitis* were obtained from the Department of Microbiology, Federal University of Technology, Akure. They were transferred to appropriate culture media (Potato dextrose ager (PDA) and Yeast peptone Dextrose agar (YPD) and refrigerated at 4^{o}C until further use.

2.3. Sample Preparation

The straws were washed, cut and oven-dried at 60°C for 48 hours to a moisture content of 10 % dry basis. The first part was milled with a Retsch mill to 0.1 - 0.5mm particle size. The milled particles were then sieved to obtain a uniform particle size between 0.200 – 0.250 mm. They were then stored in sealed plastic jar at room temperature until required for further analysis [20].

2.4. Chemical pretreatment

A two-stage procedure which combines the dilute acid pre-hydrolysis (DAPH-100-121) and alkaline delignification using NaOH as described by Olugbenga and Ibileke [21] was employed. Dry sample was treated with dilute sulfuric acid which required the use of 1.25% (w/v) H_2SO_4 solution in a 1:8 g:g solid: liquid ratio. The one step dilute acid pre-hydrolysis (DAPH-121) was carried out in an autoclave for 17 minutes at 121°C, following which the particles were collected and drained. The solid was subsequently treated with 2% (w/v) sodium hydroxide solution in a solid: liquid ratio of 1:20 g: g, at 120°C for 90 min. Thereafter, the leftover solid material (cellulose pulp) which was separated by filtration was washed with distilled water to remove the residual alkali, and dried at 50 ± 5°C for 24 hours and tested.

2.5. Production of Cellulases by Promising Fungal Isolates

Aspergillus niger was evaluated for cellulase production. The isolates were preserved on Sabouraud Dextrose Agar (SDA) plates at 4°C. For cellulase production, 100 mL of minimal salt medium containing (L^{-1}): $(NH_4)_2SO_4$, 1.4g; urea, 0.3 g; KH_2PO_4, 2.0g; $MgSO_4.7H_2O$, 0.3g; $CaCl_2$, 0.3g; Tween 80, (0.2%); pretreated substrate, 20g; CMC, 8g; 1 ml trace element solution was added in 250 mL conical flask. Each flask was inoculated with 150ul (2×10^8 sfu/ml) spore suspension. The flask was incubated in a rotary shaker at 30°C with a speed of 130 rpm. After 4 days' cultivation, the culture media was harvested by centrifugation at 8000 rpm for 10 minutes at 4°C. Clarified supernatant was used as the source of cellulases [22].

Enzyme activity of the supernatant was assessed. Filter paper activity (FPase activity) was assessed using Whatman No.1 filter paper (1 × 6 cm, 50 mg) for 60 min. Endoglucanase activity (CMCase activity) was tested using 1% (w / v) carboxymethyl cellulose for 30 min; the amount of reducing sugar produced from the enzyme reaction was determined by the DNS technique with glucose as the standard. One unit of Enzyme activity was defined as the quantity of enzyme necessary to liberate 1 µmol glucose or xylose equivalents per minute under standard test conditions [23, 24].

2.6. Enzymatic hydrolysis

Enzymatic saccharification of pretreated substrates was done using the procedures of El-Shishtawy *et al.* [25]. The pretreatment substrates were combined with an adequate amount of enzyme (30 FPU/g of pretreated substrate slurry) in a 250ml Erlenmeyer flask containing 0.05 M acetate buffer (pH 5.0). The enzymatic hydrolysis was carried out at 50°C for 72 hours using a rotary shaker (100 rpm). After the saccharification time, enzyme activity was stopped by heating the mixture for 15 minutes. The reaction mixture was centrifuged at 4000 rpm for 30 minutes to remove the residue and the supernatant was used for reducing sugar determination [26].

2.7. Determination of Reducing Sugar

The approach of Olugbenga and Ibileke [21] was used. Two (2) mls of the substrate hydrolsate was placed in a test-tube and 1g of activated charcoal was added. The mixture was thoroughly shaken. The mixture was then filtered using a filter paper until a colourless liquid was obtained. One (1) ml of filtrate was placed in a test-tube and two drops of alkaline DNS reagent were added and the tube was immersed in boiling water for 5 minutes. The mixture was left to cool and the absorbance was measured at 540 nm. This measurement was carried out after three days. A standard curve of glucose was created and used to calculate the percentage of reducing sugar.

2.8. Optimization of Fermentation Parameters for Bioethanol and Organic Acid Production

The various parameters such as pH, temperature, substrate concentration and incubation temperature were evaluated for bioethanol production [27].

2.9. Effect of different initial pH

The influence of Initial pH (3.5 - 6.5) was studied by altering the initial pH of the fermentation medium in order to discover the optimum initial pH for bioethanol production. All other factors were kept constant [27].

2.10. Effect of different fermentation temperature

The effect of different fermentation temperatures (25°C to 37°C) on bioethanol production were evaluated. Bioethanol fermentation was carried while varying the incubation temperature, maximum product yield obtained under the varied conditions were noted [27].

2.11. Effect of different inoculum concentration

The effect of different inoculum concentrations (2 - 15%) on bioethanol was evaluated. Bioethanol fermentation was carried while varying the inoculum concentration in the medium, maximum product yield obtained under the varied conditions were noted [27].

2.12. Effect of different substrate concentrations

The effect of different substrate concentrations (5, 10, 15 and 20%) on bioethanol and organic acid production by selected organisms were evaluated. Bioethanol fermentation was carried out while varying the substrate concentration in the medium and maximum product yield obtained by each organism under the varied conditions were noted [27].

2.13. Effect of fermentation Time

The effect of fermentation time was observed by measuring the bioethanol yield at 24 hour intervals for 7 days. All other parameters were kept constant [28].

2.14. Submerged Fermentation for Bioethanol Production

2.14.1. Preparation of inoculum

A 25 ml MYPG medium containing (g/l): dextrose 10, peptone 5, yeast extract 3, malt extract 3 [29] was dispensed in 250 ml conical flasks, sterilized and inoculated with a loop full of *Scheffersomyces stipitis* from yeast agar slants and incubated at 28°C, 120 rpm for 24 hrs.

2.14.2. Fermentation Process

The fermentation media used was composed of 10 % substrate hydrolysate. The hydrolysates were supplemented with (g/L); yeast extract, 2; $(NH_4)_2SO_4$, 1; K_2HPO_4, 0.5; peptone, 1; $MgSO_4$, 0.5; $MnSO_4$, 0.5 and distilled water [30].

One hundred milliliter of the hydrolysate was transferred into 250-ml flasks with various nutrients added as described above. The medium was then sterilized by autoclaving at 121°C for 15 minutes, left to cool at room temperature after which 1 ml vitamin solution was added [31]. The pH of each medium was regulated using 1 N of HCl and 2 M of NaOH according to the experimental design. Thereafter, purified inoculum was aseptically added and then plugged with cotton wool. The inoculated flasks were afterwards shaken constantly on an environment-controlled incubator shaker (model G25-R, New Brunswick Scientific, Edison, N.J., USA) at 180 rpm for 7 days. Samples were withdrawn at interval, centrifuged and tested for glucose and organic acid concentrations according to the experimental design [32].

2.15. Determination of Bioethanol Concentration in Fermentation Medium

Bioethanol quantification was conducted utilizing a set up distillation device. The fermented medium was transferred into round bottom flask and set on a heating mantle linked to a distillation column encased in a running tap water. Another flask was mounted at the other end of the distillation column to collect the distillate at 78^{o}C (standard temperature for ethanol production). Ethanol yield was then obtained by determining the mass of the distillate in grams. Percentage ethanol was then estimated by collecting the specific gravity of the ethanol produced and utilizing it to calculate the percentage (v/v) ethanol produced [33].

2.16. Statistical analysis

All data generated were analyzed using Statistical Package for Social Sciences (SPSS) version 22.0 (IBM Corp. Armonk, NY, USA); data were subjected to one way ANOVA and difference between means were determined by Duncan's New Multiple Range Test at ($p \leq 0.05$).

3. Result

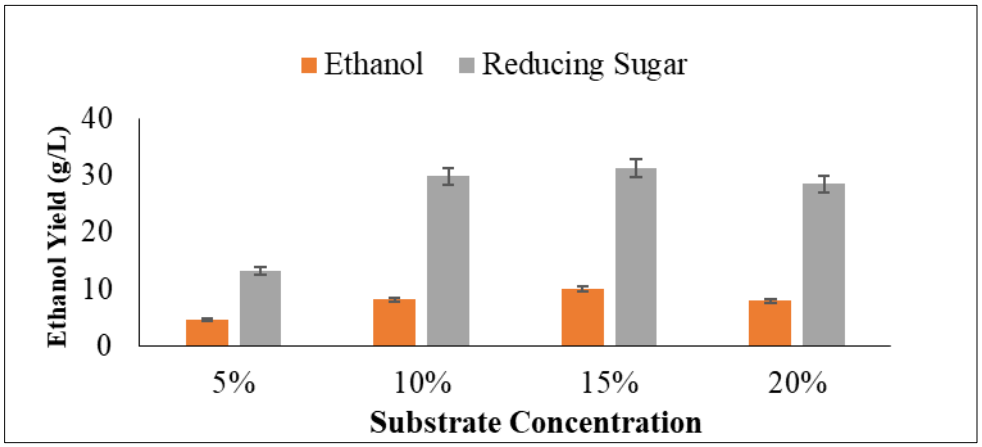
3.1. Effect of different substrate concentration on bioethanol and reducing sugar generated by the selected isolates

Table 1 Shows the cellulase activity of crude enzyme obtained from *A. niger,* FPase was 5.1 U mL-1, Endoglucanase activity was 7.2 U mL-1 and β-glucosidase activity was 4.0 U mL-1. On-site celluloses produced by *A. niger* was found to effectively hydrolyze available cellulose fractions in Sorghum straw

Table 1 Cellulase activity of crude enzyme obtained from *A. niger*

Parameter	Activity (U/ml)
Filter paper activity	5.1
Endoglucanase activitty	7.2
b-glucosidase activvity	4.0

Figure 1 reveals the effect of different substrate concentrations on reducing sugar and bioethanol yield. Reducing sugar and bioethanol increased from 5% and reached optimal at 15% substrate concentration (31.26g/L and 10.03g/L respectively). Beyond 15%, a decline in reducing sugar and bioethanol were observed.



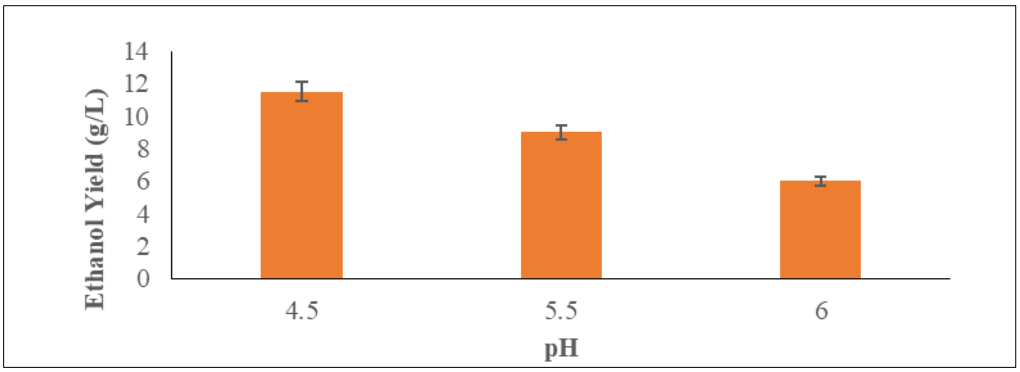
($p<0.05$)

Figure 1 Effect of different substrate concentrations of sorghum straw on reducing sugar and bioethanol produced by *S. stipitis*

3.2. Effect of different pH and Temperature on bioethanol production by the selected isolates

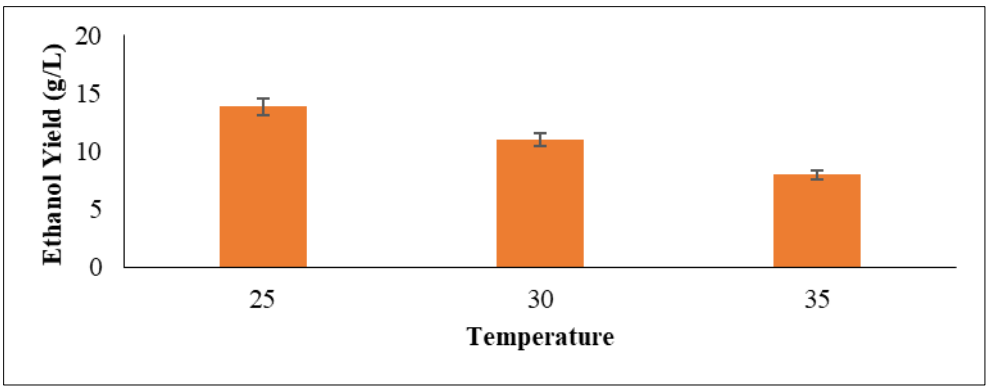
The effect of different pH on bioethanol production by *Scheffersomyces stipitis* is shown in Figure 2. Highest bioethanol yield by *S. stipitis* was 11.55 g/l at pH 4.5.

Figure 3 shows the effect of different temperatures on the bioethanol yield of *S. stipitis.* Highest bioethanol yield by *S. stipitis* was 13.86 g/l at 25°C.



($p<0.05$)

Figure 2 Effect of different pH on bioethanol production by *S. stipitis* in 15% substrate concentration of saccharified sorghum straw

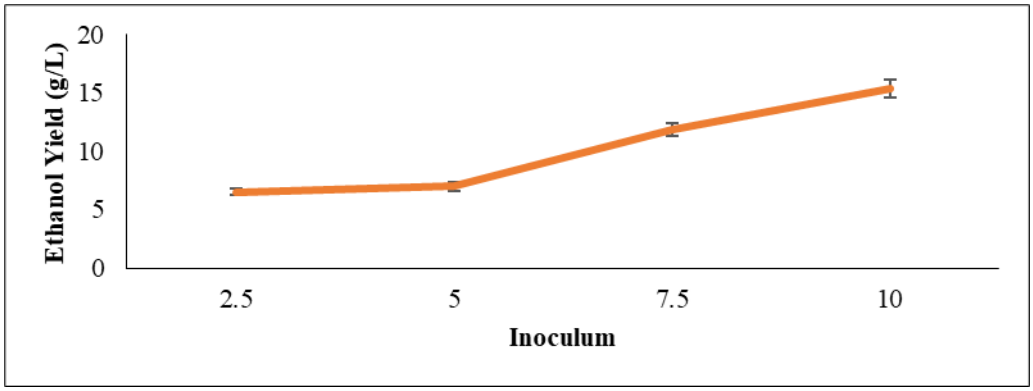


($p<0.05$)

Figure 3 Effect of different incubation temperatures on bioethanol production by *S. stipitis* in 15% substrate concentration of saccharified sorghum straw

3.3. Effect of different inoculum size on bioethanol production by the selected isolates

Figure 4 shows the effect of different inocula sizes on bioethanol production by yeast isolates. Increase in bioethanol yield was directly proportional to increase in inoculum size up to 10% (v/v) for *S. stipitis*. Highest bioethanol by *S. stipitis* was 15.37 g/l at 10% inoculum size.

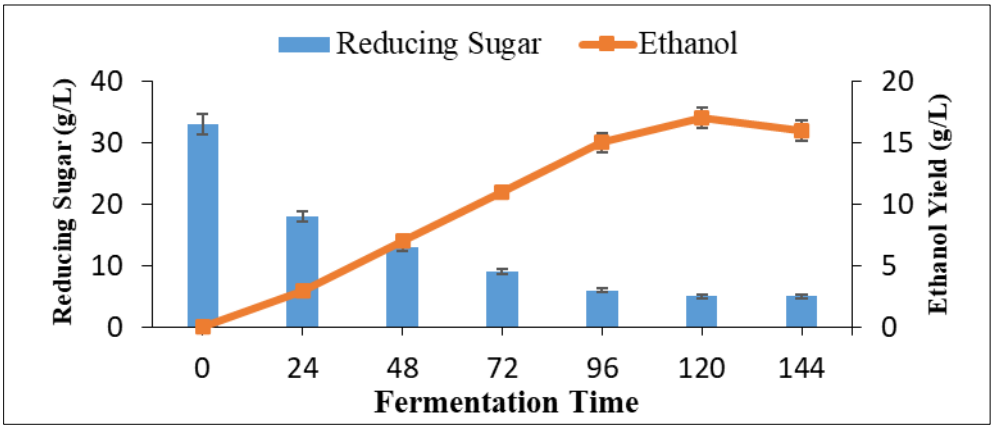


($p<0.05$)

Figure 4 Effect of different inoculum size on bioethanol production by *S. stipitis* in 15% substrate concentration of saccharified sorghum straw

3.4. Bioethanol Production by *Scheffersomyces stipitis* on the Hydrolysates Obtained from Saccharified Substrates using Optimum Parameters

Figure 5 shows the bioethanol produced by the selected yeast isolates on sorghum straw hydrolysate. Highest bioethanol yield by *S. stipitis* was 17.05g/L on sorghum straw after 120 hours of fermentation. Reducing sugar content in sorghum straw fermentation media after 144 hours was 2.50g/L. Bioethanol conversion rate by *S. stipitis* on Sorghum straw using optimal environmental conditions was 52.12% as shown in Table 2.



($p<0.05$)

Figure 5 Bioethanol production by *Scheffersomyces stipitis* on sorghum straw using optimized conditions

Table 2 Bioethanol conversion rates of *Scheffersomyces stipitis* on Sorghum straw

Parameters	Sorghum straw
Total Reducing Sugar	33.00 ± 0.20[a]
Highest Ethanol yield	17.20 ± 0.00[a]
Bioethanol Conversion Rate	52.12 ± 1.50%

Data are represented as mean ± standard deviation, n=3 with the same superscript down the column are not significantly different ($p<0.05$)

4. Discussion

This study investigated the optimization of bioethanol production from sorghum straw, a lignocellulosic agricultural residue, using a sequential co-culture approach. *Aspergillus niger* facilitated saccharification, while *Scheffersomyces*

stipitis performed fermentation, owing to its observed ability to ferment glucose and withstand extreme environmental conditions. The use of sorghum straw is particularly relevant in sub-Saharan Africa, where such biomass is abundant yet underutilized.

4.1. Influence of Substrate Concentration

Substrate concentration significantly influenced both the release of reducing sugars and the subsequent ethanol yield. The highest bioethanol yield (10.03 g/L) and reducing sugar content (31.26 g/L) were obtained at a 15% substrate concentration. Beyond this threshold, a decline was observed, likely due to substrate inhibition effects. High solid loadings increase the viscosity of the medium and limit mass transfer, which hampers enzymatic hydrolysis and fermentation efficiency. These findings corroborate earlier observations by Onoghwarite *et al.* [20], who reported reduced saccharification efficiency at elevated biomass concentrations.

4.2. Effect of pH

Fermentation pH played a crucial role in optimizing ethanol yield, with the optimum found at pH 4.5, resulting in a maximum yield of 11.55 g/L. This acidic condition likely improved enzyme stability and enhanced the metabolic activity of *S. stipitis*. Pramanik [34] previously reported similar optimal pH conditions for ethanologenic yeasts, noting that deviations can disrupt enzymatic processes and promote the accumulation of inhibitory metabolites, such as acetic acid and glycerol. Hence, precise pH control is essential for efficient lignocellulosic ethanol fermentation.

4.3. Temperature Optimization

Temperature also had a marked impact on fermentation kinetics. The optimal temperature for ethanol production was 25 °C, yielding 13.86 g/L. This result aligns with the mesophilic profile of *S. stipitis*, which exhibits maximum metabolic activity in the range of 25–30 °C [35]. Determination of the appropriate temperature of fermentation is a crucial factor that affects the productivity of bioethanol. Fermentation temperature has a direct effect on the biochemical reactions and metabolism of yeast, which stems from reduced enzyme activities. Elevated temperatures may induce thermal stress and impair membrane integrity, while lower temperatures can slow enzymatic and cellular functions, ultimately reducing fermentation efficiency.

4.4. Inoculum Size

Inoculum volume affected ethanol production, with an optimal yield of 15.37 g/L achieved at a 10% (v/v) inoculum size. An appropriate cell density ensures rapid fermentation initiation and minimizes the risk of contamination. However, excessive inoculum levels can lead to premature nutrient depletion, increased biomass accumulation at the expense of ethanol production, and oxygen limitations. These findings are consistent with those of Hashem *et al.* [36] and Eskicioglu and Ghorbani [37], who reported 5 – 7.5% optimal inoculum size for bioethanol production by yeast strains. It was suggested that increase in inoculum size up to 7% induces the rate of consumption of sugars; however, further increase beyond 10% results in exhaustion of substrate [37]. The study shows that *S. stipitis* was not a very effective glucose fermenter under optimized conditions. As fermentation progressed, reduction in sugar concentration in the medium was directly proportional to bioethanol production. This was a direct implication of the sugar fermentation by the yeast strains Öhgren *et al.* [38]

4.5. Fermentation Kinetics and Conversion Efficiency

Under optimized conditions, the maximum ethanol yield was 17.05 g/L after 120 hours of fermentation, with a conversion efficiency of 52.12%. Although satisfactory, this yield is lower than those reported using simultaneous saccharification and fermentation (SSF) processes. For instance, Öhgren *et al.* [38] achieved ethanol yields of 41.28 g/L using *Saccharomyces cerevisiae* in SSF mode. The possible production of inhibitory compounds such as fufural and acetic acid during pre-treatment has also been found to result in low bioethanol yield. Various researchers' have proposed the incorporation of an additional pre-treatment step in a bid to eliminate these inhibitory compounds; however, the implementation of this suggestion is cost intensive and is not industrially feasible [38].

Furthermore, while *S. stipitis* is known for its ability to ferment pentose sugars like xylose, its ethanol production under anaerobic or microaerobic conditions is often constrained due to its partial oxygen requirement. This limitation may hinder the complete fermentation in tightly sealed fermentation setups, leading to reduced overall ethanol yields compared to hexose-fermenting yeasts such as *S. cerevisiae* [39].

5. Conclusion

This study has demonstrated that sorghum straw, a widely available and underutilized agricultural residue in sub-Saharan Africa, can serve as a viable feedstock for second generation bioethanol production. By optimizing fermentation parameters particularly substrate concentration, pH, temperature, and inoculum size the yield of bioethanol by *Scheffersomyces stipitis* was significantly improved. The highest ethanol yield of 17.05 g/L and a conversion rate of 52.12% under optimized conditions provide a promising foundation for developing economically feasible and environmentally sustainable biofuel technologies. Although this yield is modest compared to more advanced integrated fermentation systems, the process remains accessible, especially in resource-limited settings where simplicity and cost-effectiveness are critical.

Importantly, the study confirms that a sequential approach using *A. niger* for enzymatic saccharification followed by fermentation with *S. stipitis* can effectively convert lignocellulosic biomass into bioethanol. However, the limitations observed particularly the moderate conversion rate and possible presence of fermentation inhibitors highlight the need for further process refinement.

In conclusion, the findings contribute valuable data to the growing field of lignocellulosic ethanol production and demonstrate the potential of using indigenous agro-wastes and microbial strains for sustainable bioenergy generation in Nigeria and similar environments. Future studies should focus on pilot-scale validation, economic modeling, and life cycle assessment to assess the industrial viability of the process.

Compliance with ethical standards

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Disclosure of conflict of interest

The author declare no conflict of interest.

Statement of ethical approval

The present research work does not contain any studies performed on animals/human subject by the author.

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