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Bio-butanol Production from Hydrolysates of Napier Grass Using Clostridium acetobutylicum: A Comparison of Separate and Semi-Simultaneous Fermentation Processes

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Abstract

The quest for sustainable and economically viable biofuels has intensified the focus on lignocellulosic biomass as a renewable feedstock. This study aimed to investigate the valorization of Ethiopian Napier grass (Pennisetum purpureum) for biobutanol production via Acetone-Butanol-Ethanol (ABE) fermentation. The biomass was characterized, subjected to optimized alkaline pretreatment (2.5 wt% NaOH, 1.5 wt% urea, 121°C, 40 min), and enzymatically hydrolyzed. Fermentation using Clostridium acetobutylicum was optimized via Response Surface Methodology (RSM) with a Box-Behnken Design (BBD), and two bioprocessing strategies Separate Hydrolysis and Fermentation (SHF) and Semi-Simultaneous Saccharification and Fermentation (sSSF)-were compared. Compositional analysis revealed that Napier grass contains 35.2% cellulose, 16.9% hemicellulose, and 22.4% lignin, offering 52.1% total fermentable carbohydrates. Alkaline pretreatment effectively delignified the biomass, yielding 28.74 g/L of glucose upon enzymatic hydrolysis. The optimized SHF process (37.5°C, pH 6.0, 64.7 h, 6.5% w/w substrate) produced 15.65 g/L butanol with 0.077 g L^{-1} h^{-1} productivity and 29.5% efficiency. Notably, the sSSF strategy achieved superior results: 17.6 g/L butanol, 0.083 g L^{-1} h^{-1} productivity, and 33.6% efficiency. The distilled biobutanol exhibited favorable fuel properties, including a Higher Heating Value (HHV) of 33,050 kJ/L. This study conclusively demonstrates that Ethiopian Napier grass is a promising feedstock for second-generation biobutanol, with the sSSF strategy offering a more efficient and integrated bioprocessing approach.

1. Introduction

The 21st century is defined by a dual crisis: the relentless depletion of finite fossil fuel reserves and the escalating environmental catastrophe driven by greenhouse gas (GHG) emissions from their combustion [1]. The global energy sector remains predominantly dependent on petroleum, coal, and natural gas, creating profound economic volatility and geopolitical tension [2]. More critically, the combustion of fossil fuels is the primary contributor to global warming, leading to a higher frequency of extreme weather events and biodiversity loss [3]. This pressing context has triggered an urgent, global quest for sustainable, renewable, and environmentally benign alternatives. Among the various options, biofuels derived from biomass have emerged as a particularly promising pathway for decarbonizing the transportation sector, a major source of CO_2 emissions [4]. Unlike other renewables, biofuels offer the unique advantage of being storable and compatible with existing internal combustion engine infrastructure and liquid fuel distribution networks, enabling a more seamless transition.

First-generation biofuels, primarily bioethanol and biodiesel produced from food crops like corn and sugarcane, initially offered hope. However, the "food-versus-fuel" debate, along with concerns over land-use changes and limited GHG reduction potential, severely undermined their sustainability [5]. This led to a strategic pivot towards second-generation, or advanced, biofuels produced from non-edible lignocellulosic biomass, which avoids competition with the food supply and utilizes waste materials [6]. Among the various biofuel candidates, biobutanol (C_4H_9OH), produced via the Acetone-Butanol-Ethanol (ABE) fermentation process has regained significant attention as a next-generation biofuel with properties far superior to the more established bioethanol [7]. The ABE fermentation, one of the oldest known industrial bioprocesses, utilizes solventogenic clostridia bacteria, such as Clostridium acetobutylicum, to convert sugars into a solvent mix [8]. Butanol, as the primary product, possesses several compelling advantages. Butanol has an energy content of approximately 29.2 MJ/L, which is close to gasoline (32.5 MJ/L) and about 30% higher than ethanol (21.2 MJ/L). This translates directly to better fuel economy [9]. In addition, butanol is less prone to absorbing water, which prevents phase separation in gasoline blends and allows it to be blended at higher ratios without requiring vehicle modifications [7]. Moreover, due to its

lower corrosivity, butanol can be distributed through existing petroleum pipelines, unlike ethanol, significantly reducing distribution costs [8].

Despite these advantages, the commercial viability of ABE fermentation has been historically challenged by high substrate costs and low final product titers, which make recovery and purification energy-intensive [8]. The key to overcoming these economic hurdles lies in utilizing inexpensive, abundant feedstocks and optimizing efficient bioprocessing strategies. Lignocellulosic biomass represents the most abundant renewable organic resource on Earth. It is primarily composed of three structural polymers: cellulose, hemicellulose, and lignin, which form a recalcitrant structure that is resistant to microbial and enzymatic deconstruction [10]. To access the valuable sugar polymers, a pretreatment step is essential. Alkaline pretreatment has proven particularly effective for herbaceous biomass like grasses, as it efficiently removes lignin with minimal production of fermentation inhibitors like furfural that are common in acid-based pretreatments [11]. For a biofuel process to be sustainable and economically viable at a regional level, it must leverage locally abundant and low-cost feedstocks. Napier grass (Pennisetum purpureum Schumach), also known as elephant grass, is a tall, perennial tropical grass native to Sub-Saharan Africa. It is characterized by high biomass yield, rapid growth, strong adaptability to marginal lands, and environmental benefits such as soil erosion prevention [12]. Compositionally, Napier grass typically contains 35-45% cellulose, 20-30% hemicellulose, and 15-25% lignin, making it a rich source of fermentable sugars [13].

The conventional process for converting lignocellulosic biomass to biofuels is Separate Hydrolysis and Fermentation (SHF), where the enzymatic hydrolysis and the fermentation are performed in two distinct, sequential steps. While SHF allows each step to be performed at its optimal conditions, it suffers from end-product inhibition of cellulase enzymes by sugars, which can slow down the hydrolysis rate [13]. To address this limitation, integrated processes such as Semi-Simultaneous Saccharification and Fermentation (sSSF) have been developed. In sSSF, a dedicated enzymatic hydrolysis step is initiated first, but before completion, the fermenting microorganism is inoculated into the same bioreactor. This allows the initial hydrolysis to proceed at a higher temperature, after which the fermentation begins, and the microorganisms immediately consume the released sugars, alleviating end-product inhibition and potentially leading to higher overall solvent productivity [14]

To address Ethiopia's reliance on fossil fuel imports, this research explores the untapped potential of local Napier grass for producing biobutanol, a superior biofuel. The study aims to determine the feasibility of its conversion using Clostridium acetobutylicum and to establish whether a simultaneous saccharification and fermentation (sSSF) process offers a significant advantage over the conventional separate hydrolysis and fermentation (SHF) method.

2. Materials and methods

2.1. Experimental Overview

Most of the experimental work was done in the laboratories of Addis Ababa institute of Technology at School of Chemical and Bioengineering and School of Civil and Environmental Engineering waste water laboratory.

For the production and characterization of bio butanol the following equipment and chemical were utilized.

2.2. Materials and equipment

The required materials and equipment lists are presented in below Table 1

Table 1: List of Equipment's and Chemicals

No	Equipment's	Model	Chemicals
1	Polyethene bag		Distilled Water
2	Cooler		Acetone
3	Digital Weighing Balance	OHAUS Explorer Pro	Sulfuric acid
4	pH meter,	Denver Instrument Model 250	Sodium hydroxide
8	Water bath	Grant TXF200	Yeast extract
9	Incubator, oven, autoclave		Potassium hydrogen phosphate
10	Desiccators		Ammonium chloride
11	Laminar flow		Potato Dextrose Agar
13	Spectrophotometer	Lambda 950 PerkinElmer UV-Vis spectrometer	
14	Fourier Transform Infrared Spectrometer	Thermo Fisher Nicolet iS5OR	
15	High Performance Liquid Chromatogram (HPLC)	Waters 600	
16	Centrifuge	Universal 320R	
17	Bomb Calorimeter	Cussons	

2.3. Feedstock Collection and Preparation

Napier grass (Pennisetum purpureum Schumach) was manually harvested from the Menagesha Mushroom Farm, Oromia Special Zone, Ethiopia (Figure 1). The biomass was washed with tap and deionized water, then sun-dried for five days to approximately 10-12% moisture content. The dried biomass was chopped and pulverized using a laboratory-scale cutting mill (Retsch SM 200). The milled biomass was sieved to obtain a uniform particle size of less than 0.84 mm to maximize the surface area for

subsequent processing. The powder was further dried in a hot-air oven at 60°C for 24 hours to achieve a constant weight and stored in sealed zip-lock bags at room temperature



Figure 1: Napier Grass from Menagesha Mushroom Farm

2.4. Chemical Reagents and Microorganisms

All chemicals used were of analytical grade from Sigma-Aldrich, including NaOH, urea, H_2SO_4, and citric acid. The commercial cellulase enzyme complex, Novozymes Cellic® CTec2, was a donated sample. The solventogenic strain Clostridium acetobutylicum was obtained from a commercial culture collection as lyophilized spores. Culture medium components (yeast extract, peptone, minerals) were of microbiological grade from Himedia Laboratories.

2.5. Compositional Analysis of Raw Biomass

The proximate and structural composition was determined using standard methods. Moisture content was determined by drying at 105±3°C for 24 hours to constant weight (15; 16). Ash content was measured by incineration in a muffle furnace at 525±25°C for 4 hours [17]. The chemical composition was determined using a sequential gravimetric method [18]. Extractives were removed via Soxhlet extraction with acetone for 8 hours. Hemicellulose was solubilized by boiling in 0.5M NaOH for 3.5 hours. Acid-insoluble lignin (Klason lignin) was determined by a two-stage acid hydrolysis (72% H_2SO_4 followed by dilution and autoclaving). Cellulose content was calculated by difference.

2.6. Pretreatment and Enzymatic Hydrolysis

The pretreatment of the biomass was conducted using an alkaline-urea solution comprising 2.5% (w/v) NaOH and 1.5% (w/v) urea at a solid loading of 8% (w/v). The mixture was homogenized and subsequently subjected to autoclaving at 121°C. Following this, the slurry was centrifuged, and the resulting solid residue was neutralized via repeated washing with deionized water and then dried. The enzymatic saccharification of the pretreated substrate was performed at 5% (w/v) solids in a 0.05 M citrate buffer (pH 5.0), employing Novozymes Cellic® CTec2 at a loading of 2 mL per 5 grams of biomass, with 0.02% (w/v) sodium azide added to prevent microbial contamination. The hydrolysis was carried out in a controlled environment at 50°C and 50 rpm for 40 minutes, after which the reaction was quenched by boiling. The resulting hydrolysate was centrifuged, and the supernatant was collected and stored at -20°C for subsequent analytical procedures.

The concentration of total reducing sugars was determined using the DNS method [18]. A standard curve was prepared. Absorbance was measured at 540 nm using a UV-Vis spectrophotometer (Lambda 950, PerkinElmer).

2.6. Microorganism and Inoculum Preparation

Lyophilized spores of C. acetobutylicum were reactivated under strict anaerobic conditions in Reinforced Clostridial Medium, with a heat shock at 80°C for 10 minutes. For inoculum development, a modified LB-s medium was used [13], containing (per liter): 10 g NaCl, 5 g yeast extract, 0.11 g $FeSO_4 \cdot 7H_2O$, 0.6 g $MgSO_4 \cdot 7H_2O$, and 0.008 g $CaCl_2$ (pH 6.8). Media were pre-reduced by boiling and sparging with nitrogen before autoclaving. The active pre-culture, incubated at 37°C for 18-24 hours, was used as the inoculum (Figure 2).

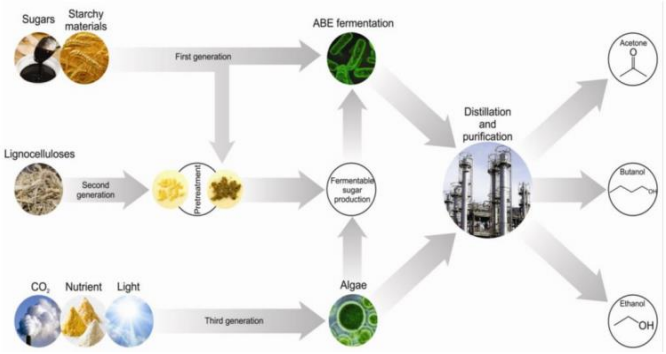


Figure 2: Schematic Illustration for Solvent Production

2.7. ABE Fermentation Processes

Separate Hydrolysis and Fermentation (SHF): The sugar-rich hydrolysate was used as the carbon source, supplemented with LB-s nutrients. The pH was adjusted to predefined levels (5.0-7.0). Batch fermentations were conducted in 500 mL stirred-tank bioreactors with a 250 mL working volume. Anaerobic conditions were maintained by CO_2 sparging. The fermenters were inoculated with 10% (v/v) active pre-culture and run at controlled temperatures (34-40°C) with 150 rpm agitation for 48-96 hours. Semi-Simultaneous Saccharification and Fermentation (sSSF): Alkaline-pretreated biomass (12 g/L) was added directly to the fermentation medium with nutrients and a cellulase enzyme cocktail (3 mL). An initial hydrolysis phase was carried out for 24 hours at 50°C and 200 rpm. Without separation, the temperature was shifted to 37°C, and the pre-culture was inoculated for a 96-hour fermentation phase.

2.8. Analytical Methods for Product Characterization

Concentrations of ABE solvents and metabolic acids were quantified using a Waters 600 HPLC system with a UV-Vis detector and a reverse-phase C18 column. The mobile phase was 0.005 M H_2SO_4 at a flow rate of 0.5 mL/min [19]. Samples were centrifuged and filtered (0.22 µm) before injection. Fourier Transform Infrared (FTIR) Spectroscopy: The chemical identity of distilled biobutanol was confirmed using a PerkinElmer Spectrum 65 FT-IR spectrometer. A thin film was deposited on a NaCl plate, and the spectrum was recorded from 4000 to 400 cm^{-1}.

The Higher Heating Value (HHV) was determined using a Part Cussons

bomb calorimeter according to the ASTM D2015 standard method, with benzoic acid calibration [13].

2.9. Experimental Design and Statistical Analysis for SHF Optimization

A four-factor, three-level Box-Behnken Design (BBD) was employed using Design-Expert® software (Version 12, Stat-Ease Inc.). The independent variables were: Substrate Concentration (2, 6, 10% w/w), Fermentation Time (48, 72, 96 h), pH (5, 6, 7), and Temperature (34, 37, 40°C). The response variable was Butanol Yield (g/L). The BBD generated 29 randomized runs, including five center points. The data were fitted to a second-order polynomial model. The significance of the model and its terms was evaluated using Analysis of Variance (ANOVA). The model's adequacy was assessed using the coefficient of determination (R^2), adjusted R^2, and lack-of-fit test.

3. Results And Discussion

3.1. Characterization of Raw and Pretreated Napier Grass

3.1.1. Proximate and Chemical Composition of Raw Biomass

The initial characterization of the native Napier grass was crucial to evaluate its inherent potential as a fermentation feedstock. The results of the proximate and chemical composition analyses are summarized in Table 2.

Table 2. Compositional Analysis of Native Ethiopian Napier Grass (Dry Basis).

Component	Weight Percent
Proximate	
Moisture	9.0 ± 0.5
Ash	7.8 ± 0.3
Chemical	
Extractives	8.7 ± 0.4
Hemicellulose	16.9 ± 0.6
Acid Insoluble lignin	22.4 ± 0.7
Cellulose	35.2 ± 0.8
Total Structural Carbohydrate	52.1

The high total carbohydrate content of 52.1% (comprising 35.2% cellulose and 16.9% hemicellulose) firmly established the suitability of this Ethiopian Napier grass for sugar-platform biorefining. This value is consistent with, and in some cases superior to, other lignocellulosic feedstocks commonly investigated for biofuel production, such as rice straw (~40-50% carbohydrates) and wheat straw (~50-60%) [20]. The lignin content of 22.4% is significant and typical for perennial grasses, confirming the necessity of a robust pretreatment step to disrupt the lignocellulosic matrix and expose the polysaccharides for enzymatic attack [10]. The ash content of 7.8% was slightly higher than values reported for Napier grass from other regions (e.g., 7.3% [13] a variation likely attributable to differences in soil mineral composition and plant age at the time of harvest [20].

3.1.2. Impact of Alkaline-Urea Pretreatment

The alkaline-urea pretreatment resulted in a mass loss of 32.5% in the solid fraction. This substantial reduction is indicative of the effective solubilization and removal of lignin and a portion of the hemicellulose. Visually, the color of the biomass changed from deep green to a light brown, a characteristic sign of delignification. The pretreated solids became more porous and less fibrous, which is a highly desirable outcome for increasing the accessible surface area for cellulase enzymes.

This pretreatment efficacy is critical. Lignin acts as a physical barrier, non-productively binding cellulases and preventing their access to cellulose fibers. By dissolving a large fraction of the lignin, the alkaline pretreatment drastically reduces this recalcitrance. Furthermore, the use of a mild alkali-urea mixture, as opposed to a strong acid, successfully minimized the formation of degradation products like furfural and 5-hydroxymethylfurfural (HMF), which are potent inhibitors of microbial growth during subsequent fermentation (18). This provides a cleaner hydrolysate, reducing the need for costly detoxification steps.

3.2. Sugar Yield from Enzymatic Hydrolysis

The effectiveness of the combined pretreatment and enzymatic hydrolysis was quantified by measuring the total reducing sugars (TRS) released into the hydrolysate. The DNS assay, calibrated with a highly linear glucose standard curve ($y = 143.45x + 17$, $R^2 = 0.9822$), was used for this purpose.

The concentration of TRS in the hydrolysate was determined to be 28.74 g/L. Given the 5% (w/v) solid loading during hydrolysis, this translates to a yield of 0.574 g of TRS per gram of pretreated biomass, or a sugar recovery of 3.2 g from the initial 5 g load. This represents a 78% conversion efficiency of the available cellulose in the pretreated biomass into fermentable sugars.

When compared to the study by [7] which recovered 3.9 g of sugar from a similar setup, the yield in this work is approximately 18% lower. This discrepancy can be attributed to several factors inherent to biomass variability, including the specific cultivar of Napier grass, its maturity at harvest, and the distinct climatic and soil conditions in Ethiopia compared to Thailand. Nevertheless, a conversion efficiency of 78% confirms that the pretreatment was successful and that the hydrolytic enzymes were highly effective, providing a rich sugar solution for the subsequent fermentation stage.

3.3. Optimization of SHF Process Using Response Surface Methodology

3.3.1. Model Fitting and Statistical Analysis

A Box-Behnken Design (BBD) with 29 experimental runs was executed to investigate the effects of four critical process parameters on butanol yield. The experimental design and the corresponding butanol yields are presented in Table 3. A wide range of butanol production, from 5.6 g/L to 16.8 g/L, was observed, unequivocally demonstrating the profound impact of optimizing the fermentation conditions.

Table 3: Selected Experimental Runs and Butanol Yields from the BBD.

Run	Substrate (%w/w)	Time (hr)	pH	Temp(oC)	Butanol (g/L)

1	2	48	6	37	5.6
7	10	48	6	37	9.8
13	2	96	6	37	9.7
4	10	96	6	37	10.6
26	6	72	6	37	16.3
29	6	72	6	37	16.8

3.4. Analysis of Individual Factor Effects

The optimization of fermentation parameters is crucial for enhancing process efficiency and economic viability. The response surface methodology analysis provided clear insights into the individual and interactive effects of four critical parameters on butanol production. The model predicted maximum butanol production under the following optimized conditions: substrate concentration of 6.51% w/w, fermentation time of 64.65 hours, pH of 6.00, and temperature of 37.46°C. Validation experiments confirmed these predictions, yielding an average butanol concentration of 15.3 g/L, which demonstrated the robustness and accuracy of the optimization model.

3.4.1. Effect of Substrate Concentration

The concentration of fermentable sugars in the hydrolysate significantly influenced butanol production, showing a clear optimum at approximately 6.5% w/w. When substrate concentration increased from 2% to 6% w/w, butanol yield showed a substantial increase from 5.6 g/L to 16.8 g/L. This positive correlation can be attributed to enhanced microbial metabolism and increased solvent production under sufficient nutrient availability. However, further increase in substrate concentration beyond 6.5% w/w resulted in a marked decrease in butanol yield, with the yield dropping to 10.6 g/L at 10% w/w substrate concentration (Figure 3).

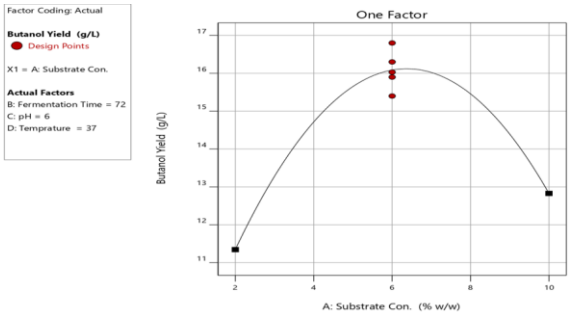


Figure 3: Effect of Substrate on butanol yield

This phenomenon represents a classic case of substrate inhibition, where elevated sugar concentrations become toxic to Clostridium acetobutylicum cells. The inhibition mechanism involves osmotic stress and disruption of cellular metabolic pathways, ultimately leading to reduced solvent production. Similar substrate inhibition patterns have been reported in previous studies using lignocellulosic hydrolysates, where optimal sugar concentrations typically range between 5-7% w/w for efficient ABE fermentation. This finding is particularly important for process economics as it defines the maximum substrate loading that can be efficiently utilized without triggering inhibitory effects.

3.4.2. Effect of Temperature

Temperature optimization revealed a clear optimum at 37.5°C for butanol production. The butanol yield increased steadily from 7.5 g/L at 34°C to a maximum of 16.8 g/L at 37.5°C, followed by a gradual decline to 10.1 g/L at 40°C. This temperature profile aligns perfectly with the mesophilic nature of Clostridium acetobutylicum, where enzymatic activities and membrane fluidity are optimized within this temperature range (Figure 4).

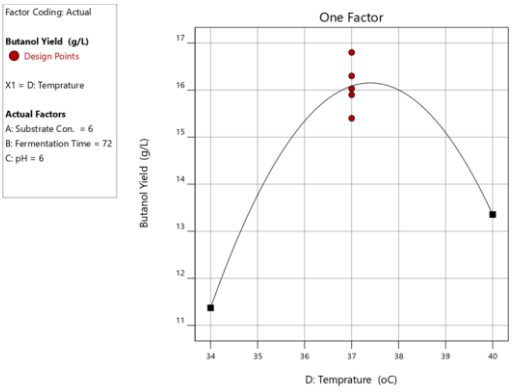


Figure 4: Effect of Temperature on Butanol yield

The decrease in butanol yield at lower temperatures (34°C) can be attributed to reduced microbial metabolic activity and slower reaction rates of key enzymes involved in the solventogenesis pathway. At elevated temperatures (40°C), the observed decline in productivity likely results from partial denaturation of critical enzymes, disruption of cellular membrane integrity, and increased energy requirements for cellular maintenance. The narrow temperature optimum highlights the sensitivity of solventogenic clostridia to temperature variations and emphasizes the importance of precise temperature control in industrial ABE fermentation processes.

3.4.3. Effect of Fermentation Time

The time course of ABE fermentation exhibited characteristic biphasic behavior, with butanol production showing a strong dependence on fermentation duration. Butanol accumulation increased progressively during the first 72 hours of fermentation, reaching maximum concentrations of 16.3-16.8 g/L. Beyond this point, extended fermentation time up to 96 hours resulted in either a plateau or slight decrease in butanol concentration (Figure 5).

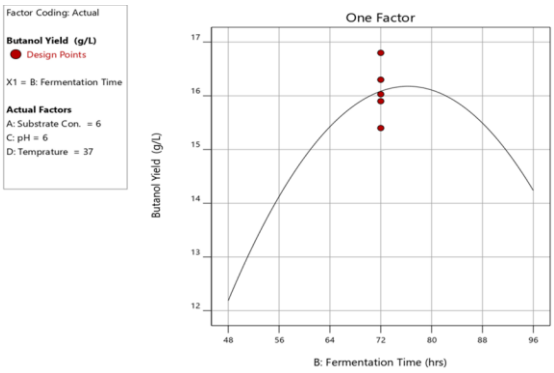


Figure 5: Effect of Fermentation Time on Butanol yield

The initial increase corresponds to the active solventogenic phase, where the culture shifts from acid production to solvent generation, accompanied by the reassimilation of previously accumulated acetic and butyric acids. The subsequent decline after 72 hours can be attributed to multiple factors: butanol toxicity to the producing microorganisms, nutrient depletion in the fermentation medium, and possible degradation of solvents or conversion to other metabolites. The identification of optimal fermentation time at 64.65 hours represents a balance between maximizing product yield and minimizing process time, which is crucial for enhancing volumetric productivity in industrial applications.

3.4.4. Effect of pH

The pH of the fermentation medium exerted a profound influence on the metabolic behavior of Clostridium acetobutylicum, directly affecting the transition from acidogenesis to solventogenesis. Butanol production showed a distinct optimum at pH 6.0, with yields decreasing significantly at both lower (pH 5.0) and higher (pH 7.0) pH values. At pH 5.0, the culture predominantly remained in the acidogenic phase, accumulating acetic and butyric acids with minimal solvent production (Figure 6)**.**

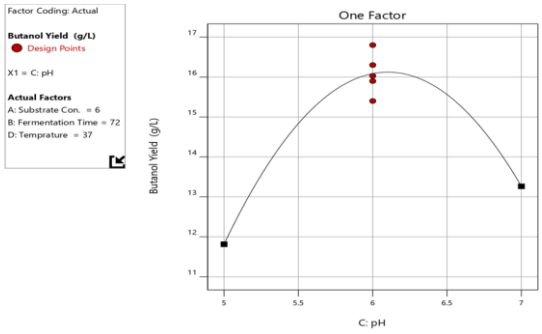


Figure 6: Effect of pH on Butanol yield

The optimal pH of 6.0 effectively triggered the metabolic shift toward solventogenesis, enhancing the expression of genes encoding solvent-forming enzymes and facilitating the reassimilation of organic acids. The reduced performance at pH 7.0 was primarily attributed to the accumulation of sodium chloride formed during pH adjustment using NaOH, which is a known inhibitor of clostridial growth and solvent production. This finding underscores the critical importance of pH control strategy in ABE fermentation and suggests that alternative buffering systems or neutralizing agents might be beneficial for maintaining optimal pH without introducing inhibitory compounds.

3.4.5. Interaction Effect of Sugar Concentration and Fermentation Time

The interaction between substrate concentration and fermentation time revealed complex, non-linear effects on butanol production. At shorter fermentation times (48 hours), systems with higher substrate concentration (10% w/w) produced significantly more butanol (9.8 g/L) compared to systems with lower substrate concentration (2% w/w), which yielded only 5.6 g/L. This relationship, however, changed dramatically with extended fermentation time.

At 96 hours of fermentation, the difference in butanol yield between high (10% w/w) and low (2% w/w) substrate concentrations diminished considerably. The low-substrate system showed substantial improvement, reaching 9.7 g/L, while the high-substrate system showed only marginal improvement to 10.6 g/L. This convergence indicates that given sufficient time, even low substrate concentrations can be efficiently converted, whereas high substrate concentrations, while providing initial productivity advantages, ultimately lead to inhibition that limits complete substrate utilization.

The most favorable combination was observed at intermediate substrate concentration (6% w/w) and fermentation time (72 hours), which yielded the maximum butanol production of 16.8 g/L. This optimal point represents a balance between providing adequate substrate for microbial growth and solvent production while avoiding the inhibitory effects associated with high substrate concentrations. The interaction analysis suggests that fed-batch strategies, where substrate is added gradually rather than all at once, could potentially overcome the inhibition limitations and further improve process performance (Figure 7 and Figure 8).

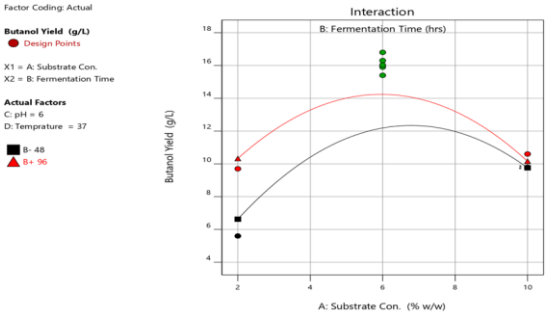


Figure 7: Interaction Effect of Sugar Concentration and Fermentation Time

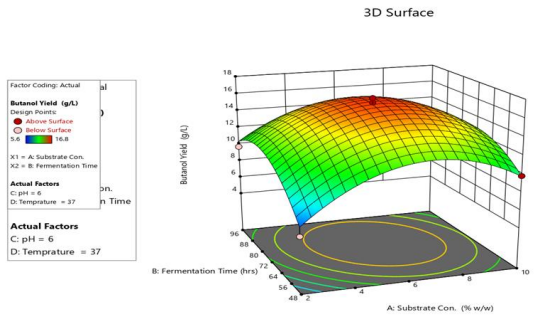


Figure 8: 3D surface interaction between Sugar concentration and Fermentation time

3.5. Comparative Performance: SHF vs. sSSF

The performance of the optimized SHF process was compared with the Semi-Simultaneous Saccharification and Fermentation (sSSF) process. The results, summarized in Table 4, reveal a clear advantage for the integrated process.

Table 4: Comparison of SHF and sSSF Fermentation Performance.

Parameter	SHF Process	sSSF Process
Final Butanol Titer (g/L)	15.3 ± 0.3	17.6 ± 0.4
Total Fermentation Time (h	64.7	120 (96 h fermentation + 24 h hydrolysis)
Butanol Productivity (g/L/h)	0.077	0.083
Process Efficiency (%)*	29.5	33.6

*Efficiency = (Mass of butanol produced / Theoretical mass of butanol from sugar consumed) × 100

The sSSF process achieved a 15% higher final butanol titer (17.6 g/L vs. 15.3 g/L) and a 7.8% higher productivity compared to the optimized SHF process. The primary reason for this enhancement is the alleviation of end-product inhibition during the enzymatic hydrolysis phase. In SHF, the released sugars (cellobiose and glucose) accumulate and inhibit the cellulase enzymes, slowing down the overall hydrolysis rate. In sSSF, the fermenting cells immediately consume these sugars as they are released, maintaining a low sugar concentration in the broth. This keeps the hydrolysis rate high and results in a greater total amount of sugar being converted to solvents over the course of the process [14].

Furthermore, the sSSF process, while having a longer total process time (120 h vs. 64.7 h), consolidates two-unit operations into a single vessel, which can lead to reductions in capital and operational costs at a larger scale by eliminating the need for a separate hydrolysis reactor and associated transfer steps.

The process efficiencies of 29.5% for SHF and 33.6% for sSSF are within the range reported for other lignocellulosic feedstocks. For instance, [15] reported 36% efficiency from barley straw, [17] achieved 19% from pine wood. The slightly lower than theoretical maximum efficiency (~40%) is expected due to carbon diversion towards cell mass, the production of other solvents (acetone, ethanol), and maintenance energy.

3.6. Comparative Performance of SHF and sSSF Processes

The validation experiment at the predicted optimum conditions for SHF (6.5% w/w, 64.7 h, pH 6.0, 37.5°C) yielded an average of 15.3 g/L of butanol, closely matching the model's prediction of 15.65 g/L. This corresponds to a productivity of 0.077 g L^{-1} h^{-1} and a process efficiency of 29.5% (based on the mass of butanol produced per mass of theoretical sugar available).

In contrast, the sSSF process, under similar fermentation conditions, achieved a significantly higher butanol titer of 17.6 g/L. This resulted in a higher productivity of 0.083 g L^{-1} h^{-1} and an improved process efficiency of 33.6%. The superior performance of sSSF can be attributed to the continuous release of glucose during hydrolysis, which immediately becomes available for fermentation. This reduces the accumulation of sugars that can cause catabolite repression or substrate inhibition and minimizes the feedback inhibition of cellulases by their end-products (cellobiose and glucose), leading to a more efficient and rapid overall conversion.

3.7. Comprehensive Characterization of Biobutanol Product

The successful production of biobutanol was confirmed and quantified through a multi-faceted characterization approach. The analyses not only verified the chemical identity and purity of the product but also critically assessed its key properties as a potential fuel.

3.7.1. Fuel Property Assessment: Higher Heating Value (HHV)

The energy content of a biofuel is a paramount property determining its viability as a gasoline substitute. The Higher Heating Value (HHV) of the distilled biobutanol produced in this study was determined to be 33,050 kJ/L (6,757.86 kcal/L) using a bomb calorimeter.

Significance and Comparison: This value is in close agreement with the HHV reported for commercial Petro-butanol, which is approximately 33,170 kJ/L (13), and is substantially higher than that of bioethanol (~23,322 kJ/L). This 30% higher energy density than ethanol is a critical advantage, as it translates directly to a longer driving range for vehicles using butanol-blended or pure butanol fuel. When compared to gasoline (~31,929 kJ/L), the biobutanol's HHV is remarkably similar, confirming its capability to be a "drop-in" fuel that can replace gasoline liter-for-liter without sacrificing engine performance or fuel economy, unlike lower-energy-density alternatives.

Implication for Blending: The high HHV underscores one of the principal benefits of biobutanol over ethanol. While ethanol blending typically reduces the energy content of the fuel mix, leading to a 2-5% decrease in fuel economy for a 10% blend, butanol's comparable energy content minimizes this penalty. This makes the biobutanol derived from Napier grass a technically superior and more economically attractive biofuel for blending mandates and future biofuel adoption.

3.7.2. Molecular Fingerprinting: Fourier Transform Infrared (FTIR) Spectroscopy

FTIR analysis was employed to confirm the molecular structure and identify the functional groups present in the purified fermentation product. The resulting spectrum (Figure 9) displayed all the characteristic absorption bands of a primary alcohol, unequivocally confirming the product as butanol.

Detailed Spectral Analysis:

O-H Stretch: A broad and intense band centered at ~3320 cm^{-1} is indicative of the O-H stretching vibration. The breadth of this band is characteristic of intermolecular hydrogen bonding, which is typical for liquid alcohols and confirms the presence of the hydroxyl (-OH) group.

C-H Stretch: The spectrum showed distinct peaks in the 2950-2850 cm^{-1} region. Specifically, the peaks at ~2950 cm^{-1} and ~2870 cm^{-1} are attributed to the asymmetric and symmetric C-H stretches of methyl ($-CH_3$) groups. The peaks at ~2925 cm^{-1} and ~2855 cm^{-1} correspond to the asymmetric and symmetric C-H stretches of methylene ($-CH_2-$) groups. This pattern is a definitive signature of the four-carbon alkyl chain of butanol.

C-O Stretch: A strong and prominent absorption band at ~1055 cm^{-1} is a definitive marker for the C-O stretching vibration in primary alcohols. This band is crucial for distinguishing butanol from secondary or tertiary alcohols and confirms the structure of 1-butanol.

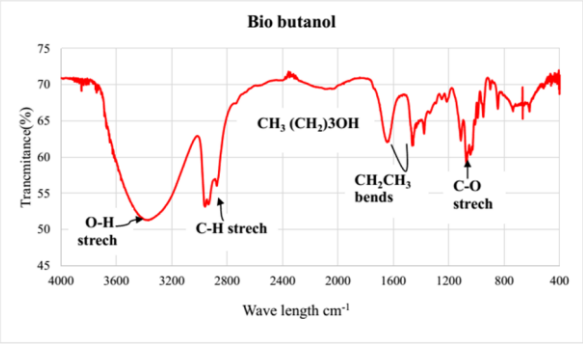


Figure 9: FTIR spectrum of the purified biobutanol product, showing characteristic O-H, C-H, and C-O stretching vibrations.

Conclusion from FTIR: The simultaneous presence of these three key vibrational modes—O-H, C-H (of a C_4 chain), and C-O (primary)—provides conclusive evidence that the target product, biobutanol, was successfully synthesized and recovered from the ABE fermentation broth. This analysis rules out significant contamination from other solvents or residual water at a molecular level.

3.7.3. Process Monitoring and Quantification: High-Performance Liquid Chromatography (HPLC)

HPLC analysis served a dual purpose: it provided a quantitative profile of all major metabolites in the fermentation broth and confirmed the identity of butanol based on its retention time.

Chromatographic Profile: A representative HPLC chromatogram of the fermentation broth at the end of the optimized batch is presented in Figure 10. The identification of compounds was based on the retention times of authentic standards.

Peak Identification and Quantification:

Residual Glucose (~23 min): A small peak indicated the presence of unconsumed glucose, confirming that the fermentation was not entirely substrate-limited and could potentially be optimized further with a higher microbial load or different feeding strategy.

Acetic Acid (~40 min) and Butyric Acid (~58 min): The presence of these organic acids is a hallmark of the ABE fermentation pathway. Their concentrations were relatively low at the time of sampling, which is expected during the solventogenic phase, as C. acetobutylicum reassimilates these acids to produce solvents.

ABE Solvents: The chromatogram clearly shows the peaks for the target solvents: Ethanol (~24 min), Acetone (~59 min), and Butanol (~95 min). The butanol peak was the largest among the solvents, which aligns with the typical product ratio for C. acetobutylicum.

Yield and Ratio Calculation: The area under each peak was integrated and converted to concentration using calibration curves. This allowed for the precise calculation of the final butanol titer (17.6 g/L in sSSF), as well as the acetone and ethanol titers. The typical ABE ratio observed was approximately 3:6:1 (Acetone:Butanol:Ethanol), which is consistent with reported literature for this strain. The HPLC data was therefore critical for calculating the precise yield, productivity, and mass balance of the fermentation process.

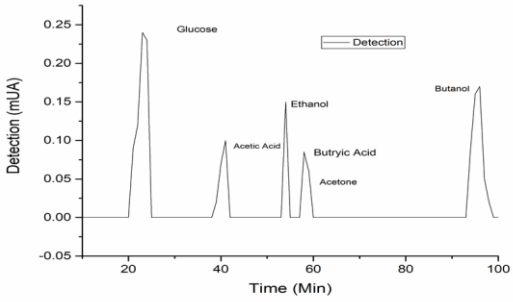


Figure 10: HPLC chromatogram of the ABE fermentation broth, showing the separation and identification of residual glucose, metabolic acids (acetic, butyric), and solvent products (ethanol, acetone, butanol).

The collective data from HHV, FTIR, and HPLC provides a robust and conclusive characterization of the biofuel product. HPLC confirmed that the target process produced butanol as the major solvent and provided quantitative data on the entire fermentation profile. FTIR verified the molecular structure of the distilled product as 1-butanol, ensuring its chemical identity. HHV validated that the produced biobutanol possesses the essential fuel property of high energy density, making it a functionally

equivalent substitute for fossil-derived fuels. This multi-technique approach leaves no doubt about the success of the bioconversion process and the quality of the final product, firmly establishing the potential of Napier grass as a feedstock for high-quality biobutanol production.

4. Conclusion

This study examined the possibility of producing bio-butanol from Napier grass. Napier grass has 52.1 % carbohydrates which makes it a potential biomass for ABE fermentation to produce bio-butanol. 78 % of reducing sugar yield was obtained by alkaline pretreatment jointly with enzymatic hydrolysis that showed the potential of Napier grass to be efficiently transferred from polysaccharides to its monomer structure. In addition, this study showed production of butanol by using C. acetobutylicum bacteria and ABE fermentation.

Experimental design was conducted by BBD to study the effect of four operational parameters, pH, temperature, sugar substrate concentration, and fermentation time. The optimum operating condition was found at temperature of 37.459 °C, pH of 6, fermentation time of 64.65 hrs, and 6.51 % w/w of reducing sugar concentration. At these optimum operating conditions, the yield of butanol was 15.649 g L-1

The maximum observed value of butanol productivity recorded was 0.077 g L-1hr-1 and 29.5 % efficiency for separated enzyme hydrolysis and ABE fermentation process while 0.083 g L-1hr-1 productivity and 33.6 % efficiency was obtained by sSSF process.

Based on this study it is evident that the chosen method of optimization was efficient and sSSF process has high productivity and also it minimizes the process. From this it can be conclude that the selected model was adequately fit the data of response variable and Napier grass is excellent potential biomass for production of bio-butanol.

Chemical characterization of bio-butanol was performed and its higher heating value of 6,757.86 kcal L-1 fulfills the desired range to be used as fuel. In addition, FTIR spectroscopy result of the optimized bio butanol product exhibited O-H, C-O, C-H, – CH2, and –CH3 functional groups on the specified wavenumbers and confirms the presence of butanol in the product. This study successfully establishes a complete and optimized pathway for biobutanol production from underutilized Ethiopian Napier grass.

Declaration of competing interest

The authors declare that they have no competing interests.

References

1. International Energy Agency. World energy outlook 2022. Paris: IEA Publications; 2022.
2. Naik SN, Goud VV, Rout PK, Dalai AK. Production of first and second generation biofuels: a comprehensive review. Renew Sustain Energy Rev. 2010;14:578-97.
3. Green EM. Fermentative production of butanol—the industrial perspective. Curr Opin Biotechnol. 2011;22:337-43.
4. Jang YS, Malaviya A, Cho C, Lee J, Lee SY. Biobutanol production from renewable resources: metabolic engineering and process development. Biotechnol Bioeng. 2012;109:2437-59.
5. Zhen X, Wang Y, Liu D. Bio-butanol as a new generation of clean alternative fuel for SI (spark ignition) and CI (compression ignition) engines. Renew Energy. 2020;147:2494-521.
6. Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, et al. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol. 2005;96:673-86.
7. Cardona E, Rios J, Peña J, Rios L. Effects of the pretreatment method on enzymatic hydrolysis and ethanol fermentability of the cellulosic fraction from elephant grass. Fuel. 2014;118:41-47. doi:10.1016/j.fuel.2013.10.055..
8. He CR, Kuo YY, Li SY. Lignocellulosic butanol production from Napier grass using semi-simultaneous saccharification fermentation. Bioresour Technol. 2017;231:101-8.
9. Shen J, Agblevor FA. Modeling semi-simultaneous saccharification and fermentation of ethanol production from cellulose. Biomass Bioenergy. 2010;34:1098-107.
10. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, et al. Determination of structural carbohydrates and lignin in biomass. Golden (CO): National Renewable Energy Laboratory; 2008. Technical Report.
11. Wyman CE, Dale BE, Elander RT, Holtzapple M, Ladisch MR, Lee YY. Coordinated development of leading biomass pretreatment technologies. Bioresour Technol. 2005;96(18):1959-1966. doi:10.1016/j.biortech.2005.01.010.
12. Production, characterization and nutritional quality of Napier grass (*Pennisetum purpureum (Schum*.) cultivars in Western Kenya. Afr J Plant Sci. 2010;4(12):496-502. doi:10.5897/AJPS.9000194.
13. Kuszewski H. Experimental study of the effect of 1-butanol addition on the physicochemical properties of diesel fuel and emissions of a compression ignition engine. Energy Fuels. 2018;32:619-31.
14. Al-Shorgani NKN, Kalil MS, Yusoff WMW, Hamid AA. Impact of pH and butyric acid on butanol production during batch fermentation using a new local isolate of Clostridium acetobutylicum. Saudi J Biol Sci. 2018;25:339-48.
15. Yang M, Zhang J, Kuittinen S, Vepsäläinen J, Keinänen M, Pappinen A. Enhanced sugar production from pretreated barley straw by additive xylanase and surfactants in enzymatic hydrolysis for acetone-butanol-ethanol fermentation. Bioresour Technol. 2015;189:131-7.
16. ASTM International. ASTM D1102-84: standard test method for ash in wood. West Conshohocken (PA): ASTM International; 2014.
17. Amiri H, Karimi K. Autohydrolysis: a promising pretreatment for the improvement of acetone, butanol, and ethanol production from woody materials. Chem Eng Sci. 2015;137:722-9.
18. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem. 1959;31:426-8.
19. Kumar M, Saini S, Gayen K. Acetone-butanol-ethanol

fermentation analysis using only high performance liquid chromatography. Anal Methods. 2014;6:774-81.

20. Mohammed IY, Abakr YA, Kazi FK, Yusup S, Alshareef I. Comprehensive characterization of Napier grass as a feedstock for thermochemical conversion. Energies. 2015;8:3403-17.