

Optimization of Hydrolysis and process Conditions for Bioethanol Production from Spirogyra Biomass

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ABSTRACT

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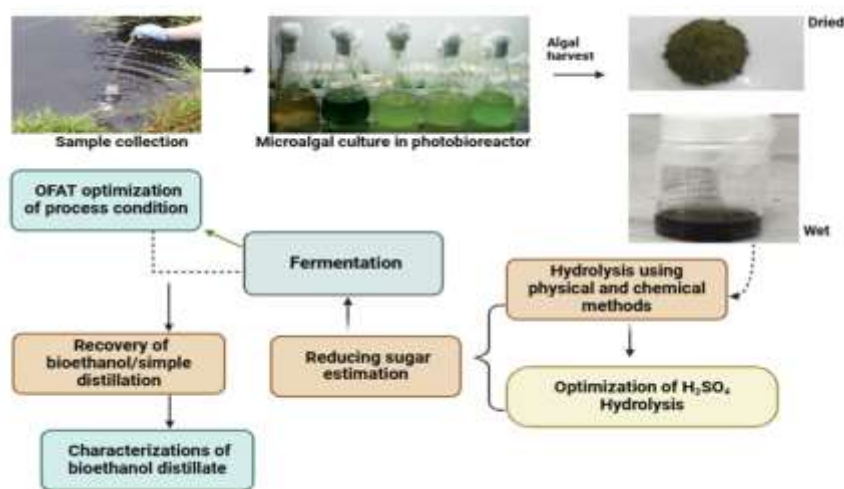
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Background: Bioethanol is gaining prominence as an eco-friendly alternative to fossil fuels, offering the potential to combat global warming and reduce greenhouse gas emissions. This study explores microalgae, specifically *Spirogyra* from Ajiwa Dam of Katsina State, Nigeria, as a third-generation biomass for bioethanol production. **Methodology:** To enable release of fermentable sugars, physical (Sonication and homogenization) and chemical (acid and alkaline) methods of pretreatment were employed. The influence of fermentation conditions (pH, temperature, and fermentation time) on ethanol yield was also evaluated using one-factor-at-a-time (OFAT) optimization. **Results:** Among the physical and chemical hydrolysis methods studied, the best ethanol yield was observed with acid hydrolysis by H₂SO₄ (2 N), at 121 °C for 30 min with dried biomass. The result shows that the ideal parameters for optimum yeast growth and bioethanol yield of *S. cerevisiae* are at pH 5.5, 35 °C temperature, and a harvesting time of 72 hrs. The physicochemical characteristics were found to be comparable to the ASTM standard and those of commercially available ethanol, respectively. **Conclusion:** The promising yield obtained in this study suggests that *spirogyra* biomass can be used as a renewable fermentation feedstock for bioethanol production, as an alternative to lignocellulosic biomass.

Keywords: Bioethanol, *Spirogyra* biomass, Hydrolysis, OFAT optimization, *Saccharomyces cerevisiae*.

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INTRODUCTION

The quest for clean energy alternatives has emerged as one of the most formidable challenges of the twenty-first century. To conserve resources, enhance efficiency in their utilization, and minimize environmental pollution, the shift toward renewable energy sources is both logical and unavoidable (1). While various alternatives are under active research and implementation, it's worth noting that the current supply of petroleum, natural gas, and coal is projected to last for approximately 120 years based on current energy consumption trends (2,3). In this context, biofuels, particularly ethanol, stand out as a significant potential energy resource capable of partially or fully replacing fossil-based fuels (4). Ethanol, derived from plant-based sources, is regarded as a renewable fuel and a potential future energy alternative as petroleum resources become depleted. It constitutes approximately 80% of global biofuel production (5) and is sustainable for large-scale production due to its versatility in utilizing renewable feedstock sources (6). Due to the presence of an oxygen atom in its molecular structure, ethanol requires less additional oxygen for combustion than diesel and gasoline, resulting in lower pollution emissions.

The first generation of ethanol production from sugars and starch-containing materials faces challenges related to competition with human food resources (7). The drawbacks of lignocellulosic substrate have led to the emergence of third-generation biofuel feedstocks, including cyanobacteria, seaweed, algal biomass, and marine macroalgae, as viable alternatives for sustainable biofuel production (8). Recent research has turned its attention towards microalgae as a promising source for biofuels (9). Microalgae, due to their short life cycle and non-reliance on arable land for cultivation, offer substantial potential as algal feedstock for biofuel production. In the future, algal biofuels could become a sustainable energy source for generations, as they utilize CO₂ as a nutrient and offer significantly higher yield potential compared to other terrestrial biomass feedstocks (10).

Furthermore, the biomass demonstrates a low level of lignin, thus providing an additional benefit due to the significant hindrance it presents to the economically viable utilization of feedstock for biofuel production. Additionally, aquatic biomass is being explored to complement terrestrial biomass in the biochemical synthesis of food and energy (11,12).

Certain microalgae species are rich in carbohydrates, making them suitable for bioethanol production. In addition, carbohydrates derived from microalgae primarily exist as starch and cellulose, without lignin, making them more appropriate for conversion into monosaccharides than lignocellulosic materials (13). There exist multiple procedures that are requisite for the generation of bioethanol from microalgae, starting from the cultivation phase. The attainment of bioethanol production is achieved through the preliminary treatment process, which effectively disintegrates the cellular barrier, followed by the hydrolysis of carbohydrates and the fermentation of appropriate sugars.

Nevertheless, challenges associated with algae-based biofuels include the precise identification of efficient strains, productivity enhancement, crop protection, resource management, and the effective handling of byproducts and waste

(14). Despite their immense promise, much work remains to overcome these hurdles (9). The hydrolysis or saccharification of cellulose and hemicellulose components into monomeric sugars represents a crucial step in bioethanol production following pretreatment (5). This phase can be accomplished using various techniques, including enzymatic, acidic, or solvent-based extraction.

Saccharomyces cerevisiae is the most widely utilized microorganism for bioethanol production due to its high efficiency and rapid production rate. Additionally, several filamentous fungi, including specific species of *Trichoderma* and *Aspergillus*, have been documented to directly produce bioethanol as the primary fermentation product from lignocellulosic biomass. For many years, yeast has been the preferred choice for converting sugar-rich biomass into bioethanol. *S. cerevisiae*, owing to its high stress tolerance, is the most commonly used yeast species for bioethanol production (14). However, its fermenting capacity can be compromised under different temperatures, pH, and incubation times (2).

Therefore, optimizing ethanol fermentation parameters known to affect fermenting organisms' metabolic capabilities becomes crucial (11). This research aimed to investigate the impact of different hydrolysis agents and optimize production conditions for improved bioethanol production using freshwater microalgae biomass. For the first time, current studies study algal species of the Ajiwa Dam of Katsina State, Nigeria, and assess the potential of the most common species as a sustainable feedstock in bioethanol production. The results of this work are pivotal in enhancing the development of economically feasible and environmentally sustainable biofuel production techniques, thus assisting in the transition towards a more environmentally friendly energy industry.

METHODOLOGY

1. Sample Collection

Water samples were collected from Ajiwa Dam in Katsina State. Situated within the Batagarawa local government area of Katsina state, Ajiwa Dam lies between latitude 12°30'-13° 00' North and longitude 7 ° 30'-8°00' East, located in the Sudan savannah ecological zone of Nigeria. The site's elevation is approximately 518 meters above sea level. The dam boasts a catchment area of 1678 square kilometers, with a height of 12 m, spillways measuring 60 m, and a lift pump capacity of 1040 km/h. Water samples were collected using 100 mL brown glass bottles following the method outlined by Indabawa (15). These samples were transported in clean, transparent rubber containers with ice to the Department of Plant Science at Bayero University Kano for microscopic examination to identify microalgae.

1.1. Screening and Identification of Microalgae

Microalgae were isolated using the method described by (16). Individual microalgal cells were microscopically identified with the assistance of the microalgae identification guide. *Spirogyra*, being the dominant microalgal species in the water sample, was selected for culture and subsequent bioethanol production.

1.2. (BG11) Preparation for Microalgal Culture (17).

The media was prepared in a 100 mL capacity conical flask by taking exactly 10 mL from the stock solution of NaNO₃ and 1 mL each of K₂HPO₄·3H₂O, MgSO₄·7H₂O, and CaCl₂·2H₂O, citric acid + ferric ammonium citrate, EDTA, and Na₂CO₃ respectively. In addition, 1 mL of trace element solution was also added as illustrated in Table 1. Each of the substances was dissolved separately before being added together. After medium preparation, the flask was plugged with cotton wool and autoclaved at 121°C for 15 minutes. The combined stock was adjusted to pH 7.0 using NaOH and HCl, respectively.

Table1: media composition

Stock	Stock solution	ml/Liter
NaNO ₃	150g/L	10ml
K ₂ HPO ₄ .3.H ₂ O	40g/L	1ml
MgSO ₄ .7H ₂ O	75g/L	1ml
CaCl ₂ . 2.H ₂ O	36g/L	1ml
Citric Acid + Ferric Ammonium Citrate	6g/L	1ml
EDTA	1g/L	1ml
Na ₂ CO ₃	20g/L	1ml

1.3. Microalgae strain activation

The microalga used in this work was *Spirogyra* spp. from the Bayero University Kano Algae Collection (Nigeria). The pre-inoculum was grown on 0.5 L BG 11 medium (pH 7) in a 1 L Erlenmeyer flask, with bubbling filtered air, at a constant temperature of 25 ± 1 °C under low light supply. Approximately 2% (v/v) of the inoculum was transferred into the media. One week after inoculation, the culture was transferred to a bubble column photobioreactor with 50 L of BG11 medium (Figure 1). The temperature was the same as the pre-inoculum. The microalga biomass was harvested by decantation prior to centrifugation at 10,000 rpm, at 4 °C for 15 min. prior to any harvesting step. Also, during the growth phase, the culture purity was checked through microscopic observation (18) with modifications. The culture was allowed to grow and flourish in a photo-bioreactor for 4 weeks within the Department of Biochemistry at Bayero University, Kano, before harvesting for bioethanol production.

**Figure 1:** Algal culture in Photobioreactor

1.4. Harvest and processing of the algal Biomass

The cultured *Spirogyra* spp. were harvested and subjected to hot air oven drying for 24 hours to eliminate moisture content. The dried biomass was then ground and sieved through a 1mm pore size sieve. The resulting 120g fine powder of *Spirogyra* biomass was dissolved in 1 liter of distilled water and agitated to create a homogenous mixture. This mixture was subsequently subjected to a hydrolysis process in accordance with the designated experimental design protocol.

2. Methods for Microalgal cell disruption and reducing sugar (19).

The methods for cellular disruption of *Spirogyra* spp. biomass and sugar extraction were performed using 2.5 g of wet biomass (approximately 0.5 g of dried weight). The biomass was subjected to physical processes such as sonication and homogenization, as well as chemical treatments involving alkaline and acidic hydrolysis using 1 M NaOH and 0.5 M H₂SO₄ in an autoclave for a period of 30 minutes. For biological hydrolysis, the algal biomass was incubated with 3% amylase enzyme solutions (30 U/mL) and placed in a water bath at 55°C for 12 hours. After incubation, the mixture was centrifuged at 3000 rpm at 60°C for 35 minutes and filtered to obtain a clear supernatant for fermentation. During homogenization, the biomass was suspended in 5 mL of distilled water, processed in a homogenizer at 24,000 rpm for 25 minutes, and placed in an ice bath for 10 minutes. This cycle was repeated twice. In the chemical cell disruption method, the biomass was suspended in 5 mL of NaOH and H₂SO₄ within sealed test tubes, autoclaved at 120°C and 1.2 bar pressure for 30 minutes. A similar procedure using 5 mL of water was conducted to assess the combined effects of temperature and pressure. The biological method involved incubating with a 3% amylase enzyme solution in a water bath for 12 hours, followed by centrifugation at 3000 rpm for 35 minutes and filtration to obtain the supernatant for fermentation. All the treatments described were conducted in duplicate. To assess the impact of biomass moisture on cell disruption, the same procedures were also carried out using dried biomass, which had been oven-dried at 80°C until a constant weight was achieved.

2.1. Optimization of Acid Hydrolysis

The temperature, biomass loading, acid concentration, and reaction time were systematically screened and optimized after identifying acid hydrolysis with H₂SO₄ as the most effective method for cell disruption and sugar extraction. The number of extraction cycles required for maximum sugar recovery was also investigated. The impact of temperature was assessed at 55°C and 85°C using a water bath, and at 121°C in an autoclave, each for 30 minutes. Optimal sulfuric acid concentration was determined through various experiments ranging from 0.5 N to 10 N, using an autoclave set at 121°C with 0.5 g of dry and concentrated biomass. Biomass loading effects were tested with 5 mL of 2 N H₂SO₄ and biomass amounts ranging from 0.1 to 2.5 g. To determine the efficiency and optimal number of sugar extraction cycles, residues from acid pretreatment were collected, centrifuged at 12,000 rpm for 10 minutes at room temperature, and the supernatant was removed. The residue was then extracted with 5 mL of distilled water at 50°C using a vortex agitator for 2 minutes, followed by centrifugation. This extraction and centrifugation process was repeated five times for each sample, with the supernatant collected for further analysis.

3. Analytical Methods for Estimation of Reducing Sugars in Hydrolyzed *Spirogyra* Biomass

The quantity of reducing sugars was determined using the dinitrosalicylic acid (DNSA) method as detailed by [18]. In brief, 0.5 mL of both acids, enzyme (30 U/mL amylase), and combined acid-enzyme hydrolyzed *Spirogyra* spp. Biomass was placed in separate test tubes, and the volume was adjusted to 3 mL with distilled water. To each sample, 3 mL of DNSA reagent was added, mixed, and placed in a water bath for 10 min. The absorbance was measured at 510 nm using a UV spectrophotometer. A standard glucose solution was similarly prepared for comparison with a known concentration in mg/mL.

4. Isolation of Yeasts

Approximately 3 kg of sugarcane stalks were collected from Yan Rake market in Kano state, Nigeria. The stalks were crushed using a clean mortar, and the resulting juice was collected in clean petri dishes. After two hours of exposure to air, 1 mL of sugarcane juice was aseptically transferred to test tubes. These samples were then serially diluted tenfold in sterilized distilled water. 1 mL of the serially diluted sediment was inoculated by streaking onto plates containing standard yeast extract potato dextrose agar media (YPD), supplemented with chloramphenicol (0.05 mg/L), and incubated at 28°C for 24 h, following the method outlined by (14).

4.1 Identification of Yeast Isolates

Colonies suspected to be yeast (*Saccharomyces* spp.) were isolated using the spread plates method and purified by streaking on freshly prepared media, followed by incubation for 1 day at 30°C. Isolates from these fresh plates were characterized based on morphology, pseudo-hyphae presence, ascospore formation, vegetative reproduction, and fermentative tests with sugars such as glucose, lactose, and sucrose, as described by (20). The cultured and identified organisms were maintained on potato dextrose agar media (PDA) at 30°C.

4.2 Preparation of Inoculum

A cell suspension (10 mL) of *S. cerevisiae* prepared from a 2-day-old slant culture was inoculated into 100 mL of medium and incubated at 30°C for 48 h on a rotary shaker. The *S. cerevisiae* cells were then collected by centrifugation, and an inoculum concentration of 0.3% (dry weight/volume) was used for the fermentation, following the method described by (17).

4.3 Measurement of Growth Curves

S. cerevisiae yeast, which was previously cultured on potato dextrose agar slants (PDA), was used to inoculate 50 mL Erlenmeyer flasks containing 5 mL of a sterile *Spirogyra* spp., as a substrate, which was adjusted to pH 5 with 0.1 M sodium citrate buffer. These flasks were incubated in a rotary shaker at 15 rpm and 30°C for 24 hours (activation I). Next, 1 mL (10%) from activation I was pipetted into new 50 mL Erlenmeyer flasks with 9 mL fresh *Spirogyra* spp., Substrate,. These were incubated at 15 rpm and 30°C for 24 hours (activation II). From activation II, 5 mL (10%) with OD 600nm = 0.5 was inoculated into 100 mL Erlenmeyer flasks with 45 mL *Spirogyra* spp. substrate for the main fermentation culture. These were incubated in a rotary shaker at 15 rpm and 30°C for 24 hours. To measure the growth curve, the OD600nm of the fermentation culture was measured spectrophotometrically every 3 hours for 24 hours. A growth curve graph was plotted from the OD 600nm values against fermentation time

5. Fermentation

Submerged fermentation was conducted in 250 mL Erlenmeyer flasks. To each hydrolysate, 0.5% (dry weight/volume) of pre-fermentative baker's yeast was inoculated, and fermentation conditions for the ethanol production were carried out at an ambient temperature of 30°C, pH 4.5, with intermittent shaking for 3 days (72 h). The fermentation process was monitored via regular sampling, and samples were filtered using nonsterile 0.22 µm syringe filters before glucose and ethanol assessment.

5.1 Harvesting

After fermentation, the resulting culture broth was centrifuged at 4000 rpm, 60°C for 40 min, followed by distillation at 72-80°C (the boiling point of ethanol). The distilled fraction was collected in containers for subsequent analysis. The fermentation process was monitored via regular sampling, and samples were filtered using nonsterile 0.22 µm syringe filters before glucose and ethanol assessment.

5.2 Evaluation of Fermentation Conditions on Bioethanol Yield

To determine the optimal fermentation conditions for ethanol production, experiments were conducted to assess the effects of pH, temperature, and fermentation time on bioethanol yield.

5.2.1 Effects of pH

Initially, four different pH values, 4.5, 5.0, 5.5, and 6.0, were tested to identify the best pH for ethanol production. For this, 100 mL of biomass was distributed into 250 mL conical flasks, and the pH of each flask was adjusted using a digital pH meter. The flasks were then sterilized at 121°C for 15 minutes. After cooling, 5 mL of pre-fermentative yeast was aseptically added to each sterilized medium, and the flasks were incubated with shaking at 200 rpm at 30°C for 5 days.

5.2.2 Effects of Temperature

Building on the results of the pH experiment, the effect of temperature on ethanol production was then evaluated. Biomass with yeast was incubated at varying temperatures of 30, 35, 40, and 45°C in a shaking incubator set to 200 rpm, using the previously determined optimal pH of 5.0.

5.2.3 Effects of Fermentation Time

To assess the effect of fermentation time on bioethanol production, bioethanol yield was monitored and measured at 24-hour intervals over 5 days, specifically at 24, 48, 72, 96, and 120 hours. The fermentation was carried out at the optimal pH 5.0 and 35°C conditions, as identified in the previous sections.

6 Characterization of Bioethanol

6.1 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier-transform infrared (FTIR) spectroscopy was employed to analyze the functional groups of alcohol bonds in the samples following the distillation process. The chemical structure of bioethanol obtained from the microalgal *spirogyra* was assessed using a PerkinElmer Spectrum 400 FTIR spectrometer, covering a range of 4000–400 cm⁻¹.

6.2 Physicochemical Analysis of the Bioethanol

The evaluated physicochemical characteristics included specific gravity, pH, refractive index, relative viscosity, and boiling point.

Specific gravity

The specific gravity of the bioethanol distillates was calculated using Equation (1)

$$\text{Specific Gravity} = (\text{Density of bioethanol}) / (\text{Density of water}) \quad (21)$$

6.2.2 Density

The density of the bioethanol and water was determined following the method described by (22). Initially, the empty pycnometer weight was noted and labeled as W0. The pycnometer was then filled with the bioethanol, and excess was carefully removed. The weight of the filled pycnometer was recorded as (W1). The density was subsequently calculated using Equation (2)

$$\text{Density } (\rho) = (W1 - W0) / \text{Volume}$$

6.2.3 Relative viscosity

The relative viscosity of the produced ethanol was determined utilizing a conventional Ostwald viscometer maintained at 30°C, following the methodology described by (23). Flow time was measured, and relative viscosity values were calculated as follows:

Relative Viscosity $\mu_r = \mu / \mu_l$ where;

μ_r = relative viscosity, μ = Viscosity of the sample and

μ_l = is the viscosity of deionized water.

$$\mu_r = p \times t_s / p \times t^0$$

Where: t_s = is the falling time (sec.) of the sample at 30°C and

(t^0 sec.) = falling time of deionized water under the same conditions, and

p = Density = mass/volume (kg/m³)

6.2.4 Determination of boiling point

The boiling point of the ethanol was established using the Thiele tube method, as outlined by (24). In this procedure, 0.5 mL of the ethanol sample was placed in a small tube, along with an inverted capillary tube. This setup was connected to a thermometer and heated within a Thiele tube. Subsequently, the tube was allowed to cool, and the boiling point was identified as the temperature at which the liquid first entered the capillary tube, as the bubble formation ceased. All experiments were conducted in triplicate, and the average values were recorded.

7. Statistical Analysis

Each experiment was carried out in triplicate, and the mean standard deviation was calculated. All statistical analyses were carried out using the SPSS statistical package. ANOVA one-way ($p \leq 0.05$) was performed to determine relationships between yield and fermentation conditions.

RESULTS

1 . Isolation and identification of fermentative yeast

Three *S. cerevisiae* isolates (S1, S2, and S3) were isolated from sugarcane molasses using standard morphological and biochemical techniques, and the isolates were characterized. All three isolates were aerobic, exhibited round colonies with a creamy appearance, and were capable of fermenting glucose and fructose, but not lactose, as reported in (Table 2).

Table 2: Fermentation of some simple Sugars by yeast isolate

Isolate	Glucose	Lactose	Fructose
S1	+ Gas	-	+ Gas
S2	+ Gas	-	+ Gas
S3	+ Gas	-	+ Gas

2. Micro Algal cell disruption and Reducing sugar estimation

To evaluate the efficiency of the cell disruption methods, the sugar content of the processed samples was measured. (Figure 2) illustrates the sugars extracted from *Spirogyra* biomass following treatment using physical (homogenization and sonication) and chemical (using 2 N H₂SO₄ and 2 N NaOH at 121°C). The work also illustrates that acid treatments, using H₂SO₄ (2 N) at 121 °C, result in high sugar extraction efficiency, achieving an extraction rate of 9.82% g eq. glu and 7.82% for both dry and wet biomass, respectively. Conversely, the alkaline pre-treatment with NaOH (2 N) yielded a significantly higher sugar output (7.42% g eq. glu in dry and 6.96% g eq. glu in wet biomass) compared to physical treatments, which produced a yield of 5.82% g eq. glu, in wt using homogenization pretreatment.

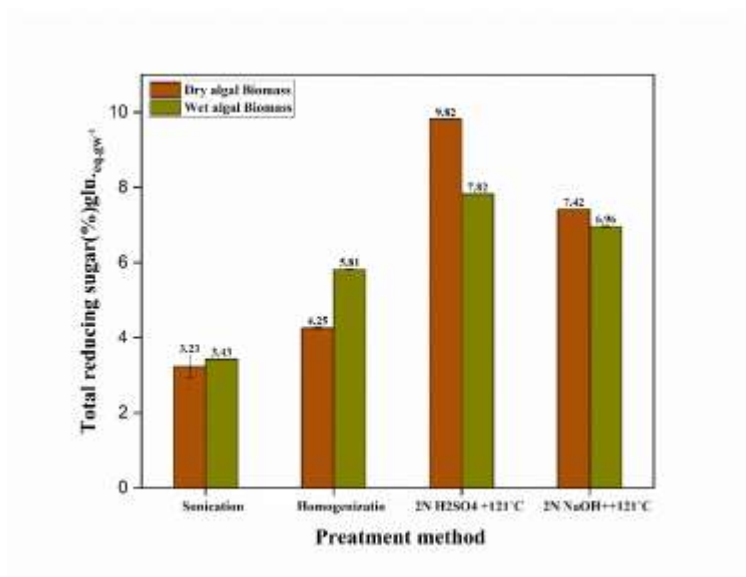


Figure 2: Cell disruption/sugar extraction from *Spirogyra* biomass dried and wet biomass with four different pretreatment methods including standard deviation (SD).

2. Yeast growth in the algal medium

Figure 3 illustrates the growth pattern of *S. cerevisiae* in the algal medium. *S. cerevisiae* growth displays two phases: exponential and stationary. This occurs because the starter and fermentation media are the same. When these media are identical, there is no lag phase. During the exponential phase, microbial cells are in a steady state, with new cells forming consistently and optimal cell division, typically reaching the doubling time midway through this phase. Different fresh water algae were reported to found to contain protein, carbohydrate, and free α -amino acid which is similar to the nutrient content of commercially available yeast extract (25).

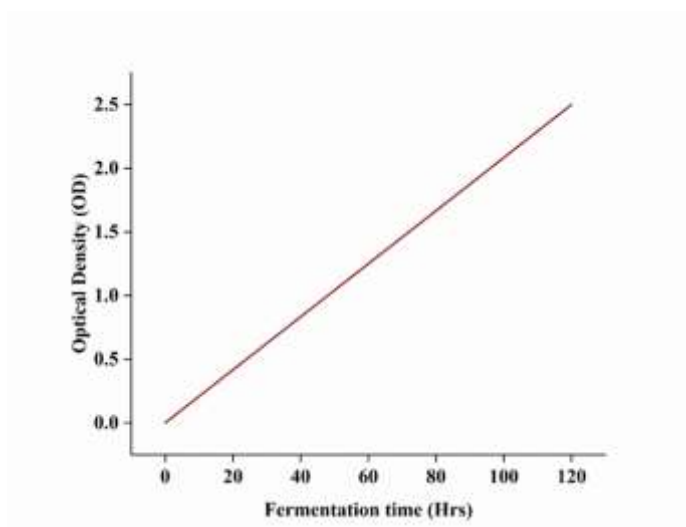


Figure 3: Growth pattern of *S. cerevisiae* in the algal medium

3. Effects of H_2SO_4 concentration on reducing sugar yield

To optimize acid concentration for biomass hydrolysis, various concentrations ranging from 0.5 to 10 N were evaluated using dried biomass in an autoclave at 121 °C for 30 minutes. (Figure 4) depicts the effect of acid concentration on the efficiency of sugar extraction. The highest reducing sugar yield in the supernatants was achieved at acid concentrations above 2 N, while the lowest yield was observed at 0.5 N, likely due to sugar degradation at this concentration. According to (26), the concentration of sugar is primarily affected by the biomass-to-acid ratio, while the reaction time has a negligible effect.

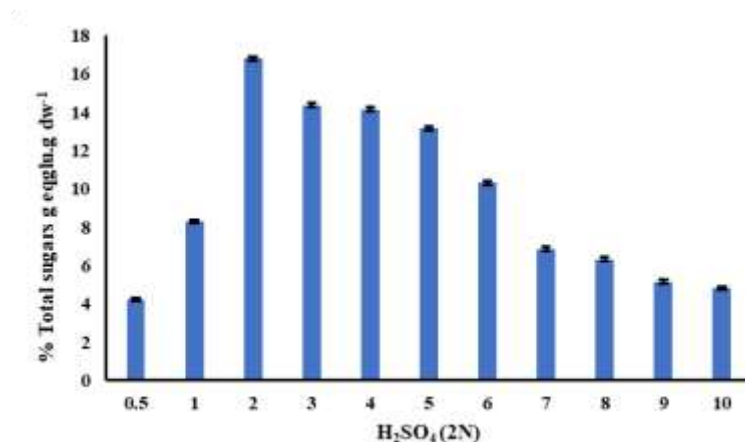


Figure 4: The effect of acid concentration on the efficiency of sugar extraction

4. Effect of biomass loading on sugar yield

Table 3 shows results on the effect of biomass loading on sugar extraction, sugar concentration in the hydrolysate, and glucose content. There were varying trends in the total sugar content of the algal biomass. As the biomass concentration increased from 10 g/L to 100 g/L, sugar extraction efficiency rose, peaking at 16.25±0.04 with 100 g/L biomass. However, further increases in biomass concentration led to a decrease in sugar extraction efficiency, with 16.05% at 300 g/L. But for the sugar concentration in the hydrolysate, an upward trend was observed with increasing biomass loading, reaching a maximum of 34.45 g/L at 250 g/L biomass concentration. Beyond this, the sugar concentration slightly decreased. For glucose content as a percentage of total sugars, the highest glucose content was observed at 100 g/L biomass loading (39.8 ± 2.1%), indicating that this biomass concentration was optimal for extracting glucose. At higher biomass concentrations,

glucose content fluctuated, with lower values observed at 200 g/L (27.9%) and slight increases again at 250 g/L (35.2%) and 300 g/L (36.3%).

Table (3): Effect of biomass loading on the extraction of sugars, on the concentration of sugars within the hydrolysate, and on the glucose content within the overall reducing sugar concentration with SD.

Biomass concentration (g L ⁻¹)	Sugar extraction (% g eqglu g ⁻¹ dw)	Sugar concentration (g L ⁻¹)	Glucose content (%)
10	16.25±0.04	3.25±0.07	33.2 ± 2.6
50	18.1±0.07	12.55±0.07	33.6 ± 2.6
100	18.55±0.04	16.8±0.01	29.8 ± 2.6
150	19.25±0.04	23.75±0.1	29.6 ± 2.2
200	19.15±0.04	32.25±0.07	27.9 ± 1.4
250	17.35±0.04	34.45±0.07	35.2 ± 0.2
300	16.05±0.04	26.4±0.02	36.3± 1.2

5. Studying the effects of fermentation conditions using factor at a time (OFAT) optimization

The ethanol fermentation process is influenced by various factors, with the viability of the fermenting microorganism being a critical factor. Consequently, optimizing the yeast as the fermentation agent in the medium derived from algal hydrolysis is crucial. The objective is to enhance the viability of the microbial cells in the fermentation agent, ensuring optimal ethanol fermentation performance.

5.1 Effect of pH

Figure 5 depicts the effect of different pH levels (4.5, 5.0, 5.5, and 6.0) on bioethanol yield from microalgal biomass at 30 °C for 24 hrs. Initially, an increase in ethanol yield was observed as the pH increased from 4.5 to 5.0, followed by a decline at pH 5.5 to 6.0, as can be observed in Figure 6. The most significant increase was observed at pH 5.0, with a maximum ethanol yield of 7.52%, while the lowest yield was recorded at pH 6.0, with an ethanol yield of 4.36%. Statistical analysis indicated significant differences ($P \leq 0.05$) in ethanol yield at each pH level. Present study observed a decrease in bioethanol yield with increasing pH.

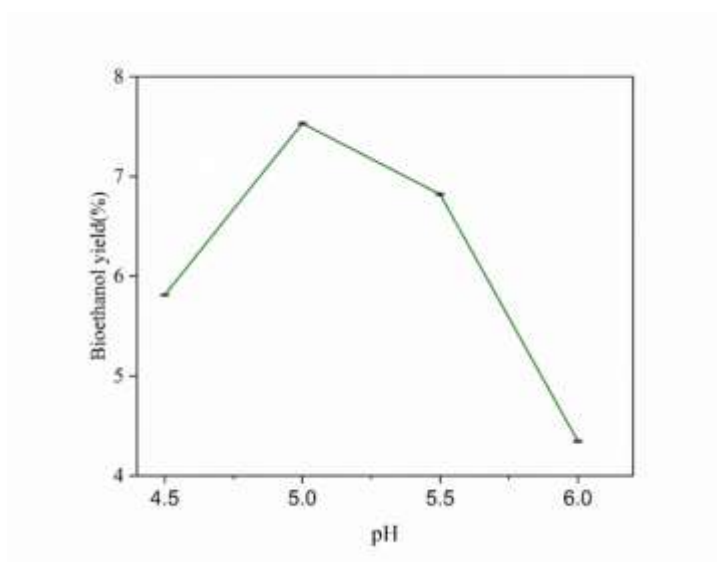


Figure 5: Effect of pH on bioethanol yield

5.2 Effects of fermentation time

The effect of incubation time on ethanol yield was determined by subjecting the flask to incubation at different intervals: 24, 48, 72, 96, and 120 h at 5.0 and 35 °C temperatures. A gradual increase in bioethanol concentration was observed, as shown in Figure 6. The maximum ethanol yield was recorded at 72 h (6.86%), followed by 48 h with 6.22%, and the least ethanol yield of 5.3% was observed at 120 h of incubation. The result of the t-test analysis at a 95% confidence interval indicates that the duration of fermentation significantly ($p \geq 0.05$) influences the ethanol content produced by *S. cerevisiae*.

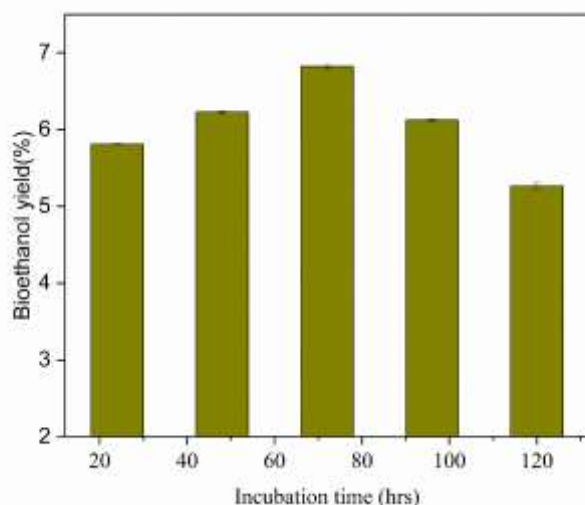


Figure 6: Impact of Fermentation Time on Bioethanol Production by *S. cerevisiae*

5.3 Effects of temperature

The effect of temperature on ethanol yield from microalgal biomass was studied as presented in Figure 8. Initially, an increase in bioethanol yield was observed with an increase in temperature from 30°C to 35°C. However, the yield declined between 40°C and 45°C. The most significant increase was observed at 35°C, with a maximum bioethanol yield of 10.26%. Beyond 35°C, an increase in temperature led to a decrease in bioethanol yield. Statistical analysis indicated a significant difference ($P \geq 0.05$) in ethanol production among the various temperatures Figure 7.

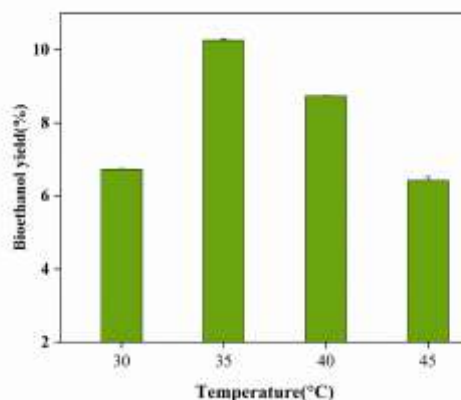


Figure 7: Effect of Temperature on Bioethanol Yield from Microalgal Biomass (Maximum Yield Observed at 35°C)

6. Characterization of Bioethanol

6.1 FTIR spectroscopy analysis

To validate the quality of the produced bioethanol, we employed Fourier transform infrared (FTIR) spectroscopy. FTIR spectroscopy has become a common tool in research laboratories and allows for the quantitative analysis of mixtures (27). The FTIR spectra of the produced bioethanol are presented in (Figure 8). The analysis identified functional groups in the fermentation distillates that are consistent with ethanol spectra data available in the literature. A broad peak at 3313 cm^{-1} corresponds to the O-H stretching vibration, indicating the presence of hydroxyl groups (28). The peak at 2970 cm^{-1} may be due to C-O stretching of sp^3 -hybridized carbon atoms. A narrow peak at 879 cm^{-1} is likely associated with C-C stretching vibrations, commonly observed in organic compounds. Additionally, the peak around 1088 cm^{-1} is attributed to the C-O stretching vibration of the alcohol functional group (29). The absorption band at approximately 1382 cm^{-1} is linked to the bending vibrational modes of the C-H bonds, known as scissoring or deformation vibrations, in the methyl group ($-\text{CH}_3$). Finally, the prominent feature at around 2884 cm^{-1} is associated with the C-H stretching vibrations of the CH_2 group, representing the aliphatic C-H bonds in ethanol and indicating the stretching motion of these hydrogen-carbon bonds (13, 35).

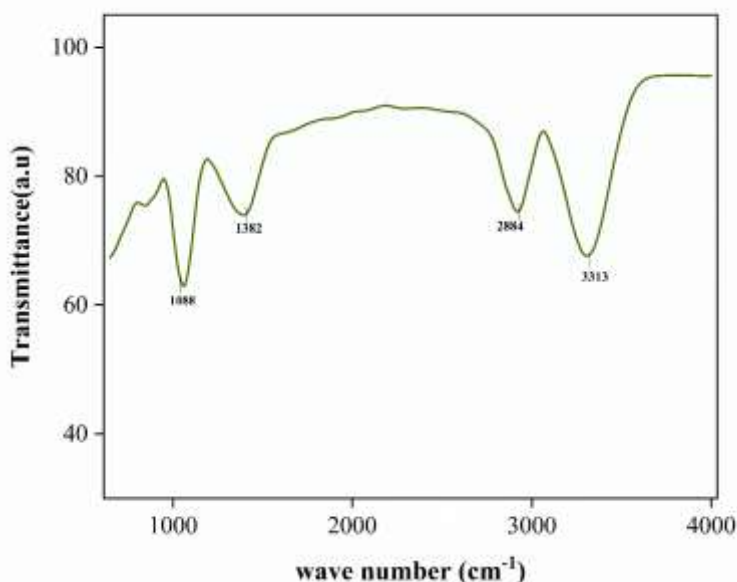


Figure 8: FTIR Result of produced bioethanol

7. Physicochemical properties of the bioethanol

Table 4 shows the physicochemical properties of bioethanol derived from *Spirogyra* biomass. The pH of the bioethanol obtained from this biomass was measured at 7.20, which falls within the acceptable range specified by ASTM D6423 standards. The neutral pH observed indicates that the bioethanol distillate produced was of high quality. As summarized in Table 5, the relative viscosity of the produced bioethanol was $(1.35 \pm 0.02\text{ mPas})$, slightly higher than that of commercially available ethanol ($1.32 \pm 0.02\text{ mPas}$). Similarly, the boiling point (BP) of the produced bioethanol was measured at $(78.81 \pm 0.2\text{ }^{\circ}\text{C})$, which is marginally higher than the boiling point of commercial ethanol ($78.62 \pm 0.2\text{ }^{\circ}\text{C}$). Independent paired T-test analysis revealed no statistically significant differences, with p-values of 0.2 for the boiling point and 0.1 for relative viscosity, both exceeding the significance thresholds of 0.005 and 0.05, respectively. These findings suggest that the viscosity and BP values of the produced bioethanol closely align with those of commercial ethanol.

Table 4: Physicochemical properties of Bioethanol produced from spirogyra biomass.

S/N	Property	Produced bioethanol g/L	ASTM Standard
1	Specific gravity	0.8960 ± 0.007	0.785 to 0.8099
2	pH	7.10±0.09	6.5-9.0
3	Color	Colorless	Clear without particle
4	Refractive index	1.3021±0.0061	1.3568-1.444

Table 5: Relative viscosity and boiling point of bioethanol produced from Spirogyra biomass

Test	Produced bioethanol from <i>Spirogyra</i> biomass	Commercial ethanol
Relative viscosity (mPas)	1.35 ± 0.02	1.32 ± 0.02
Boiling point (°C)	78.81± 0.2	78.62± 0.2

DISCUSSION

According to the results of pretreatment for the release of simple sugars from spirogyra biomass, shown in Figure 2, physical disruption methods tested on wet biomass were the least effective, with extracted sugar yields of less than 4% (glucose equivalents per gram of dry biomass). Thus, physical methods are generally not effective for disrupting the cells of Spirogyra biomass. This finding aligns with previous studies (30), which suggest that chemical pre-treatment disrupts the structural integrity of the fibrous components within the cell wall, enhancing the release of sugars into the aqueous phase. Additionally, low yield recorded in physical treatments it could be linked to the presence of a small amount of lignin in Spirogyra biomass. A study by (31) on saccharification and bioethanol fermentation from carbohydrate-extracted microalgal biomass also reported higher reducing sugar and bioethanol yields in combined acid and enzyme (cellulase) hydrolysis.

The amount of bioethanol yield is mostly dependent on the fermentable reducing sugars available in the given medium. The effect of acid concentration (H_2SO_4) with regard to reducing sugar yield was assessed as shown in (Figure 3). The highest reducing sugar yield in the supernatants was achieved at acid concentrations above 2 N. In comparison, the lowest yield was observed at 0.5 N, likely due to sugar degradation at this concentration. According to (32), the concentration of sugar is primarily affected by the biomass-to-acid ratio, while the reaction time has a negligible effect.

These results, presented in Table 3, show the effect of biomass loading on the bioethanol yield. The findings suggest that while moderate biomass loadings (around 100 g/L) are optimal for maximizing both sugar extraction and glucose yield, excessively high biomass loadings may reduce extraction efficiency and alter the sugar profile in the hydrolysate. However, this contradicts the findings of (33), who reported a high-yield bioethanol production from *Chlorella vulgaris* FSP-E microalgae biomass via fermentation by *S. cerevisiae* FAY-1 at a high-solid loading of 200 g L⁻¹.

Physical factors, such as pH, temperature, and incubation time, are critical fermentation parameters because they affect microorganism growth, fermentation efficiency, and by-product formation. The effects of these fermentation conditions were assessed using one factor at a time (OFAT) optimization. According to the results obtained in Figure 5, the highest ethanol yield is marked at pH 5.5 and beyond, where a gradual decrease was observed. This phenomenon may be due to reduced enzyme activity of the fermenting organism (*S. cerevisiae*) at pH levels > 5.5. This observation aligns with (34), who reported that the activities of bioethanol producers are slightly suppressed at pH levels below 4.5 and above 6.0, but, but contradicts findings of (35) who reported a low effect of pH on bioethanol production and yeast growth using a kinetic model. The result slightly contradicts the work of (36), who reported a maximum ethanol yield of 34.5% (v/v) using waste potato biomass at an optimal temperature of 32.2 °C, pH of 4.07, and incubation time of 72.1 h using *S. cerevisiae* as the fermenting organism, respectively.

The ethanol yield was found to increase spontaneously with increased fermentation time (Figure 6) until 72hrs maximum ethanol yield of about 6.86% was observed. This finding contradicts the results obtained by (37), who obtained maximum ethanol concentration from aqueous carob pod extract after 120 h of incubation. Similarly, (38) produced bioethanol from cashew apple juice using immobilized yeast and reported maximum bioethanol yield after 140 h of incubation at 32°C.

Moreover, results on the effects of temperature on ethanol yield indicate 35 °C as the optimum temperature for bioethanol yield, as illustrated in Figure 7, which reveals that 35°C is the optimal temperature for maximizing yield, achieving a peak of 10.26%. This implies that the *S. cerevisiae* participating in the fermentation process exhibits its highest activity or efficiency at this specific temperature. Conversely, as the temperature exceeded 35°C, a gradual reduction in bioethanol yield was observed. This decrease is likely attributable to the thermal denaturation of enzymes or stress on microbial activity, which diminishes their capability to convert substrates into bioethanol. However, in separate work by (39) an optimum ethanol yield from OPF juice was achieved at 33.03°C. Optimum yield obtained at varying temperatures may be possible due to multiple factors such as the nature of biomass used, the strain of the fermenting yeast, as well as the method of hydrolysis employed.

The results of FTIR analysis, relative viscosity, and boiling point were found to be comparable to those of commercially available ethanol. Specific gravity of the bioethanol derived from *Spirogyra* biomass was measured at (0.8960 ± 0.007) , which falls within the ASTM standard range of 0.785 to 0.8099. This suggests that the bioethanol contains minimal water content, making it appropriate for use as a fuel. As indicated in Table 4, the refractive index of the bioethanol produced from *spirogyra* biomass was slightly below the ASTM standard limit of 1.36 (40). This deviation could be due to the presence of impurities in the feedstocks used. Additionally, bioethanol distillates exhibited a clear and colorless appearance, consistent with the typical visual characteristics of bioethanol.

CONCLUSION

Microalgae offer a sustainable alternative as a biomass biofuel production source without impacting food supplies or utilizing animal feeds and arable land. *Spirogyra* is particularly promising for bioethanol production due to its high sugar content. However, disrupting the cell wall is crucial for releasing the sugars and carbohydrates within the cells. Pre-treatment with 2 N sulphuric acids at 121 °C for 30 minutes, using dried biomass was found to be the most effective method for breaking down complex carbohydrates into simple sugars before fermentation. All physicochemical properties of the bioethanol distillate were found to be comparable to those of the ASTM standard and commercially available ethanol, respectively. Adhering to optimum fermentation conditions is also essential for enhancing bioethanol yields. Moreover, further research is needed to develop improved strains, such as ethanol-tolerant yeast, to enhance fermentation efficiency and reduce harvesting time.

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تحسين التحلل المائي وظروف العملية لإنتاج الإيثانول الحيوي من الكتلة الحيوية للسبيروجيرا

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الخلاصة

خلفية عن الموضوع : يكتسب الإيثانول الحيوي أهمية متزايدة كبديل صديق للبيئة للوقود الأحفوري، مما يتيح إمكانية مكافحة الاحتباس الحراري وتقليل انبعاثات غازات الاحتباس الحراري. تستكشف هذه الدراسة الطحالب الدقيقة، وتحديدًا سبيروجيرا، من سد أجوبة بولاية كاتسينا في نيجيريا، كتلة حيوية من الجيل الثالث لإنتاج الإيثانول الحيوي. **المواد وطرق العمل:** لتمكين إطلاق السكريات القابلة للتخمير، استُخدمت طريقتان فيزيائيتان (الموجات فوق الصوتية والتجانس) وكيميائيتان (حمضية وقلوية) للمعالجة المسبقة. كما تم تقييم تأثير ظروف التخمير (الرقم الهيدروجيني، ودرجة الحرارة، ووقت التخمير) على إنتاج الإيثانول باستخدام أسلوب التحسين بعامل واحد في كل مرة. **الهدف من الدراسة :** إنتاج الإيثانول الحيوي من الكتلة الجافة لطحلب السبايروجير. **النتائج:** من بين طرق التحلل المائي الفيزيائية والكيميائية المدروسة، لوحظ أفضل إنتاج للإيثانول باستخدام التحلل المائي الحمضي باستخدام H_2SO_4 (2 N)، عند درجة حرارة 121 درجة مئوية لمدة 30 دقيقة مع الكتلة الحيوية المجففة. أظهرت النتيجة أن المعايير المثالية لتحقيق النمو الأمثل لخميرة *S. cerevisiae* وإنتاج الإيثانول الحيوي هي عند درجة حموضة (5.5)، ودرجة حرارة 35 درجة مئوية، ومدة حصاد 72 ساعة. وُجد أن الخصائص الفيزيائية والكيميائية تُضاهي معايير ASTM والإيثانول المتوفر تجاريًا، على التوالي. **الاستنتاجات:** تشير الغلة الواعدة التي تم الحصول عليها في هذه الدراسة إلى إمكانية استخدام الكتلة الحيوية من سبيروجيرا كمادة خام متجددة للتخمير لإنتاج الإيثانول الحيوي، كبديل للكتلة الحيوية الليغنوسليلوزية.

الكلمات المفتاحية: الإيثانول الحيوي، الكتلة الحيوية سبيروجيرا، التحلل المائي.