

Bioethanol Production from *Chlorella vulgaris* Biomass Cultivated with Plantain (*Musa paradisiaca*) Peels Extract

Obioma Kenechukwu Agwa, Ifeyinwa Geraldine Nwosu, Gideon Orkwagh Abu

Department of Microbiology, University of Port Harcourt, Port Harcourt, Nigeria

Email: obioma.agwa@uniport.edu.ng, ifyje@yahoo.com

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Abstract

The feasibility of nutrient uptake by *Chlorella vulgaris* using a cheap carbon source such as plantain peel extract was studied and its biomass utilized for bioethanol production. Unripe plantain peels were obtained, processed, infused for 48 hrs, extracted and cultivated with the *Chlorella* species for a period of fourteen days. The microalgal carbohydrate content was hydrolyzed with acid and enzyme while the hydrolysate fermented with 10% concentration of *Saccharomyces* sp. and *Aspergillus* sp. at 30°C and pH 4.5 using Separate Hydrolysis and Fermentation (SHF) and Separate Hydrolysis and Co-culture Fermentation (SHCF) methods. Results show that maximum cell growth of 1.56 (OD) and biomass concentration of 19 g/l were obtained with 48 hrs infusion. The result indicated that *C. vulgaris* utilized PPE medium as a sole carbon substrate and stimulated the secretion of biomass. The highest reducing sugar of 0.63 mg/ml was obtained after hydrolysis of the biomass, while the ethanol production yield of 0.33 g/l was obtained after fermentation. The ethanol production yield increased with the increase in fermentation time, while the reducing sugar was reduced after five days of fermentation. The highest ethanol percentage of 10.82% v/v was obtained from the distillate. This study showed that plantain peel can be utilized by *C. vulgaris* which provides a feasible route of reducing production cost of bioethanol from a cheap carbon substrate for biomass and bioenergy production.

Keywords

Bioethanol, *Chlorella vulgaris*, Fermentation, Hydrolysis, Plantain Peel

1. Introduction

80% of the world's economy is highly dependent on fossil energy source which is

gradually depleting with industrialization, constant energy consumption and rising world's population. This has consequently led to a rapid decline of fossil fuel and environmental instability which stimulated various investigators for the quest and search for alternative, renewable cost-effective and industrial efficiency energy sources [1]. Biofuel production emerged as an alternative sustainable energy source competing to meet the world's energy demand [2]. Among the existing biofuel, bioethanol and biodiesel are regarded as the promising alternative energy source replacing fossil fuel due to their advantages of low toxicity and biodegradability [3]. Bioethanol is the most widely acceptable liquid biofuel product because of its use as a combination of gasoline in various percentages without any engine modification [4].

The world's biggest bioethanol producers, Brazil and USA use grain base feedstock such as cane sugar, corn and cassava, first generation biofuel production feedstock which raise competition on food or feed versus fuel production. The second generation biofuel considered avoiding existing competition and focus on green gold feedstock, lignocellulosic waste such as switch grass, rice straw and agricultural fruit peels for its bioethanol production. This feedstock is renewable, low-cost and abundantly available but its limitation of crystalline nature of the material and difficulty to hydrolyze lignin component created gap in bioethanol production [5]. Hence the challenges of the grain base and green gold fuel feedstocks led to the emergence of the world's trending feedstock, algal biomass. Microalgal biomass feedstock is the most attractive, alternative renewable feedstock recently studied for bioethanol production [6] [7]. Microalgae generally consist of 40% - 70% carbohydrate, 10% - 20% protein and residual low molecular weight compounds such as amino acid, amines and fatty acids. Microalga is considered as a potential feedstock for bioethanol production due to its high biomass carbohydrate content [8], fast growth rate, high photosynthetic efficiency, ability to fix greenhouse effect, non-competitive nature with food production, easily cultivated on non-agriculture site and lack of lignin in the cell wall [9] [10]. Microalgae are microscopic unicellular and photosynthetic organism commonly found in aquatic environment. In nature, they generate up to 50% of atmospheric oxygen as well as trapping emitted greenhouse CO₂. Microalgae possessing increase carbohydrate contents are excellent feedstocks for biofuel production. *C. vulgaris* constitutes 37% - 55% dry weight carbohydrate content which comes from the cellulose and hemicelluloses on the cell wall and starch in the chloroplast [6]. They serve various commercial and industrial applications in food, agricultural medical and even nutraceutical production. They can be cultivated autotrophically, heterotrophically or mixotrophically. Mixotrophic cultivation of *C. vulgaris* using organic carbon source produced improved higher carbohydrate biomass than its autotrophic cultivation [11] [12]. When cultivated in limited nitrogen and phosphorus medium, lipid and carbohydrate are simultaneously synthesized utilizing the carbon substrate [13]. Inexpensive carbon substrate like residual crude glycerol got after biodiesel production, agricultural waste converted sugars and cellulosic materials, trapped

CO₂ emitted from industries and molasses of sugar cane paved way to successful biomass production from microalgae. Plantain peels are produced as byproducts of plantain processing, which is high in mineral contents such as potassium and phosphorus. It is a major source of polyphenols, carotenoids, dietary fibre and other bioactive compounds [14]. Potential biotechnology applications include sources of ruminant livestock [15], energy source in biogas production [16], production of soap [17] and in the management of various disease conditions due to its ulcerogenic, antimicrobial, analgesic, anti-uro-littatic properties [18].

The exploitation of *Chlorella* species feedstock using animal waste such as poultry waste as a growth medium resulted to high biomass yield of 2.5 g/l under mixotrophic condition [19]. Most researchers divert the exploitation of algal biomass to biodiesel production rather than bioethanol due to the low yield of sugar after hydrolysis of intracellular starch granules. Due to high cost of carbohydrate enzyme mixture which necessitated developing a cost-effective alternative for creation of multiple carbohydrases. This necessitates developing simple technologies such as combination of dilute acid or alkaline hydrolysis with enzyme saccharification [20].

The green algae, *C. vulgaris* used for this study was cultivated in a low-cost plantain peel extract medium and the biomass extracted was exploited as a carbon source for bioethanol production using Separate Hydrolysis Fermentation (SHF) and Separate Hydrolysis Co-culture Fermentation (SHCF) technologies.

2. Materials and Methods

Sample collection and processing:

Soil sample used for this study was obtained from refuse dump site within Abuja campus, Choba in Uniport was used for the isolation of *Bacillus cereus*. The soil sample was collected with a sterile labeled screw capped bottle using a soil auger of about 30 cm depth. The sample was transported to the Industrial Microbiology Research Laboratory for analysis. Fresh palm wine was purchased from the University Teaching Hospital bush bar gardens and transported to the laboratory and used for the isolation of *Saccharomyces cerevisiae*. Spoilt food sample used for the isolation of *Aspergillus niger* was picked from a refuse dumpsite of Abuja campus, Uniport. This was collected with a sterile nylon bag and immediately transported to the laboratory for further analysis. The enzymes used in the study were amylase and cellulase. Amylase was obtained from *Aspergillus niger* isolated from spoilt food sample and identified in the Microbiology Laboratory following standard procedures by [21] [22]. Cellulase was obtained from *Bacillus cereus* isolated from soil sample and cultured using method of [23]. Unripe plantain peels were collected from different markets within Port Harcourt metropolis, Rivers State, Nigeria, sun-dried, ground into fine powdery form using a mechanical blender (Corna model, 562), infused in distilled water for 48 h, extracted and stored in a sterile bottle for further use. The plantain peel extract was sterilized by autoclaving at 121°C for 20 mins and cooled to room temperature. The harvested alga was inoculated in duplicates to 250 ml conical

flask containing 100 ml of the extract in the ratio of 1:10. They were incubated at 30°C under natural illumination for 14 days with intermittent shaken at regular intervals to ensure homogenous growth [19].

Isolation and characterization of microorganisms:

Aspergillus niger was cultured on a PDA medium incubated at 30°C for 72 h. Pure spores of *A. niger* were dislodged from their agar plates, inoculated in pre-sterilized 10 ml Potato Dextrose Broth and incubated at 30°C for three days. *Saccharomyces cerevisiae* was cultured using a solid medium containing the following in g/l: yeast extract 10, peptone 20, glucose 20, agar 15 and pH 7.5. About 1ml of the palm wine was serially diluted in sterile physiological saline and aliquots of the dilution were aseptically plated onto the medium in duplicate by spread plate method to previously prepared modified yeast synthetic agar, inoculated in sterilized 10 ml yeast extract peptone dextrose broth (YEPD) and incubated at 30°C for 12 h. The medium was supplemented with 1% lactic acid to prevent bacterial growth; they were incubated at 30°C for 24 - 48 h [24]. *C. vulgaris* was cultivated in a novel synthetic agar medium [25] with tetracycline and nystatin (antibiotics) to eliminate bacteria and fungi growth respectively. The medium consists of the following salt in g/l: potassium nitrate 0.132, sodium silicate 0.666, monosodium phosphate 0.666 and EDTA 0.666. The pH was adjusted to 7.5 prior to autoclaving at 121°C for 15 mins. The plates were incubated for about three to five days under natural illumination (sunlight) for monoculture development. The colonies were harvested, aseptically transferred to a sterile 250 ml conical flask, stored in a refrigerator at 4°C until further use. These growth media were used for the fermentation.

Bioethanol production: Various methods used for the production of bioethanol from plantain peel involves growth monitoring and biomass extraction, enzyme hydrolysis, fermentation and distillation

Growth monitoring and biomass extraction:

The growth conditions were monitored at three day intervals for the algal cell growth and biomass quantifications. Samples were collected and subsequently analyzed for optical density (abs) determination using UV spectrophotometer (model 721 UV-VIS) [26]. The cell dry weight was measured using a centrifuge (model spin ACL270) at 12,000 rpm for 15 mins. The residue was washed twice with physiological saline (0.85% w/v, NaCl), dried in an oven at 60°C in a pre-weighed filter paper until constant weight. The amount of dried microalgae was measured as cell dry weight [27]. Using a 250 ml conical flask, about 100 ml of the *C. vulgaris* biomass was heated to concentrate the algal biomass at a temperature of 100°C, and then cooled to 45°C in a water bath. The cell dry weight of the heated biomass was also determined [28].

Hydrolysis by Acid and Saccharification by Enzyme:

The concentrated algal biomass was subjected to dilute acid pretreatment using 10% 2 N H₂SO₄, autoclaved at 121°C for 45 mins and neutralized to pH 4.8 using citrate buffer. The solution was incubated with 3% each of already prepared crude enzyme cocktail and kept in a water bath for 12 h at 45°C. The solu-

tion was centrifuged at 9000 rpm for 15 mins, analyzed for total reducing sugar and stored as sugar hydrolysate for the fermentation [29] [30].

Fermentation:

The algal sugar hydrolysate was supplemented with the following nutrients: Ammonium sulphate (2 g/l), potassium monophosphate (1 g/l), potassium dihydrogen phosphate (1 g/l), zinc sulphate (0.2 g/l), magnesium sulphate (0.2 g/l) and yeast extract (2 g/l), sterilized at 121 °C for 25 minutes [31]. The pH of the medium was adjusted to 4.5 and each conical flask containing 100 ml of the sample was inoculated with 10% growth medium preparations of *S. cerevisiae*, *A. niger* and or combination of both in duplicate. They were incubated at 30 °C for 120 hrs [28].

Analytical methods:

Estimation of reducing sugars

For reducing sugar determination 3,5-dinitosalicylic acid method was used. The concentration of the reducing sugar present in the sample was estimated by adding 1 ml of DNS reagent to 1 ml of each of the sample then boiled for 5 mins and diluted with 10 ml of distilled water. The absorbance was determined at 540 nm using UV-VIS spectrophotometer. The concentration value was extrapolated from the glucose standard curve [32].

Estimation of ethanol concentration

Dubios chromate method was adopted for estimation of ethanol [33]. 5 ml of each sample was treated with 2 ml of chromate reagent. The mixture was allowed to stand for an hour and the absorbance measured at 588 nm using UV-VIS spectrophotometer [34].

Distillation process

Method according to [35] was adopted for distillation of the fermentation medium to ethanol. The distillation apparatus with around-bottom flask containing the fermentation broth was attached at one end and a receiving flask at another end to collect the distillate. A heating mantle with the temperature of 78 °C was used to heat the round-bottom flask until completely distilled. The ethanol percentage of the distillate was estimated and extrapolated from ethanol standard curve.

3. Results

After 48 h infusion in distilled water, the extract inoculated with the algal cell was monitored using optical density and cell dry weight. The culture had an obvious growth from the initial to final day. After the lag phase (about two days), the algal cells went into their logarithmic growth phase. Then, the cell growths entered the stationary phase after day twelve (**Figure 1**).

The carbohydrate extracted biomass had initial sugar composition of 0.39, 0.36, 0.40 and 0.63 in mg/ml after acid, enzyme, steam-pretreated and combined acid-enzyme hydrolysis processes respectively (**Figure 2**).

During the fermentation, ethanol concentration yields of the fermentation broths inoculated with *S. cerevisiae*, *A. niger* separately and their co-culture

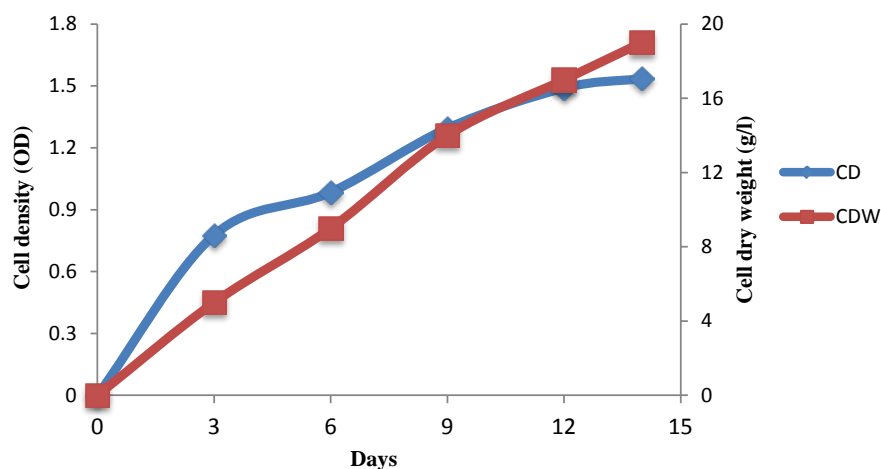


Figure 1. Growth curve of *Chlorella vulgaris* in plantain peel extract.

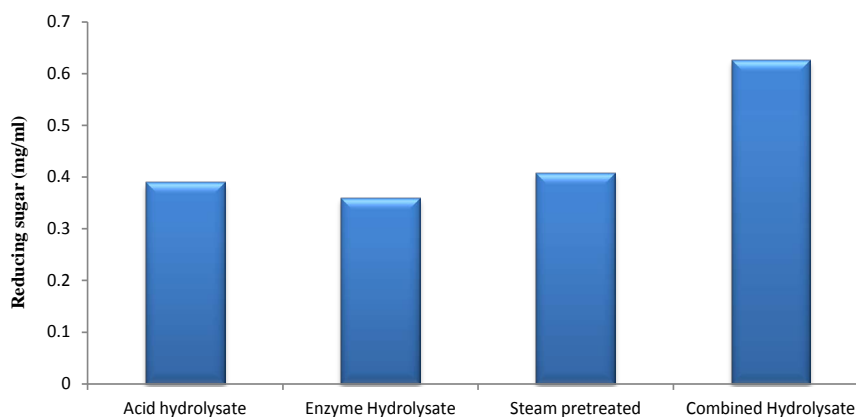


Figure 2. Reducing sugar concentration after hydrolysis.

resulted to steadily increase from the beginning of the fermentation to the final day. The fermentation broth inoculated with co-culture *S. cerevisiae* and *A. niger* recorded the highest yield of ethanol (0.33 g/l), directly followed by fermentation broth inoculated with mould (0.25 g/l), which was finally trailed by fermentation broth inoculated with yeast (0.23 g/l) (**Figure 3**).

After five days fermentation at 30°C and pH 4.5, the reducing sugar was consumed highly by fermentation broth inoculated with *A. niger* from 0.63 - 0.09 in mg/ml, followed by broth inoculated with co-culture of the two organisms from 0.63 - 0.10 in mg/ml while the least consumed sugar was recorded with fermentation broth of *S. cerevisiae* from 0.63 - 0.13 in mg/ml (**Figure 4**).

The cell density obtained from the fermentation broths indicated that the highest increase was recorded by the fermentation broth inoculated with mould which increased from 0.08 (OD) at the first day of the fermentation to 1.23 (OD). The fermentation broth inoculated with co-culture of yeast and mould showed rapid increased from 0.03 (OD) on the first day to 1.18 (OD) on the last day of the fermentation. The yeast biomass yield of the fermentation increased gradually from 0.07 (OD) on the first day of the fermentation until the third day

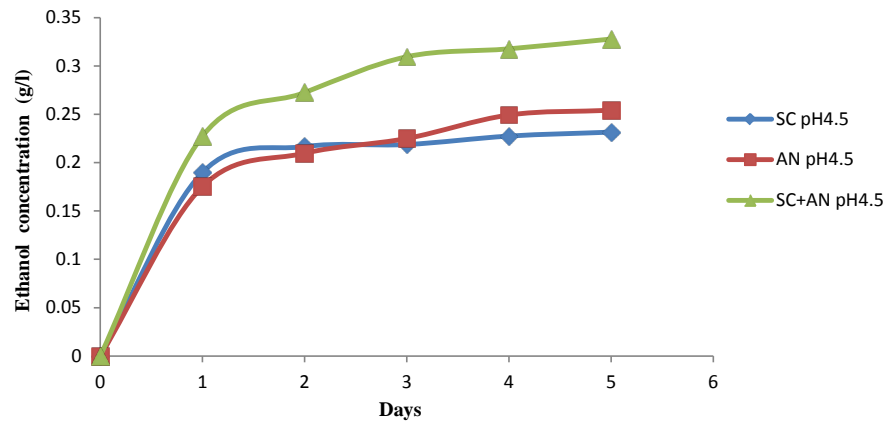


Figure 3. Ethanol concentration during fermentation.

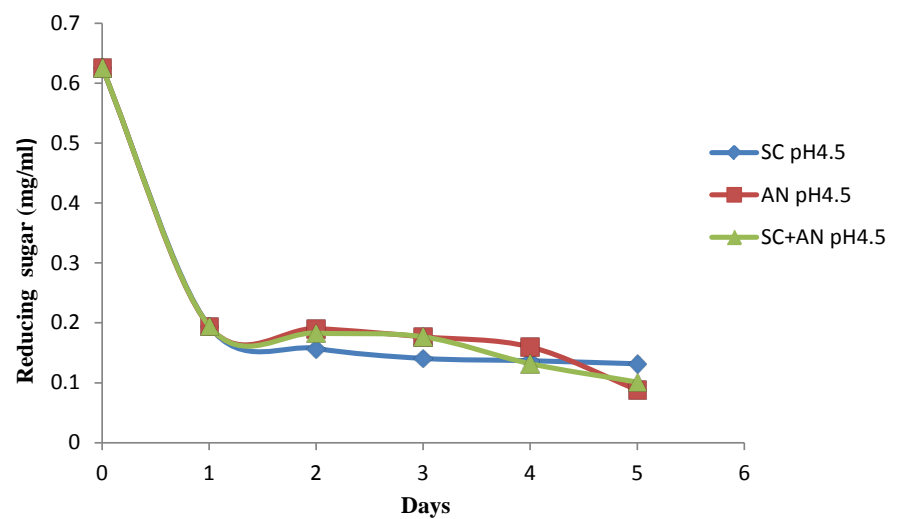


Figure 4. Reducing sugar concentration during fermentation.

where growth gradually decreased to 0.75 (OD) on the fifth day of the fermentation (Figure 5).

The co-culture fermentation broth showed highest percentage yield of ethanol than separate fermentation broths. The percentage of the ethanol yield of the distillate from the co-culture of *S. cerevisiae* and *A. niger* was observed to be highest (10.82% v/v), followed by mould fermentation broth (9.13% v/v) while the least was recorded to be yeast fermentation broth (7.90% v/v) (Figure 6).

4. Discussion

Currently, significant attention has been directed toward maximal microalgal biomass production by using cost effective strategies. It has been observed that about 80% of the entire cost of production in microalgae cultivation is carbon substrate [3]. Recent studies are now focusing on more inexpensive microalgal biomass production methods such as decreasing carbon substrate cost by using alternative or cheap carbon sources like poultry waste [19], waste water applications [36], microalgal cultivation using corn powder hydrolysate [37]. [38]

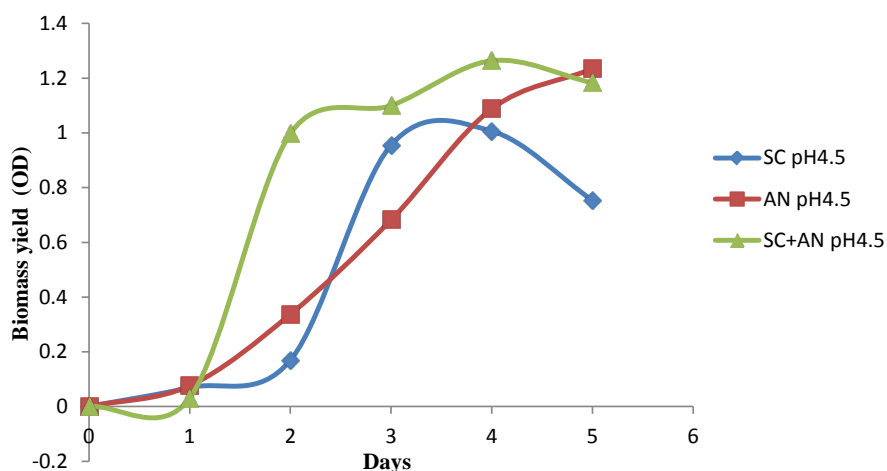


Figure 5. Biomass yield during fermentation.

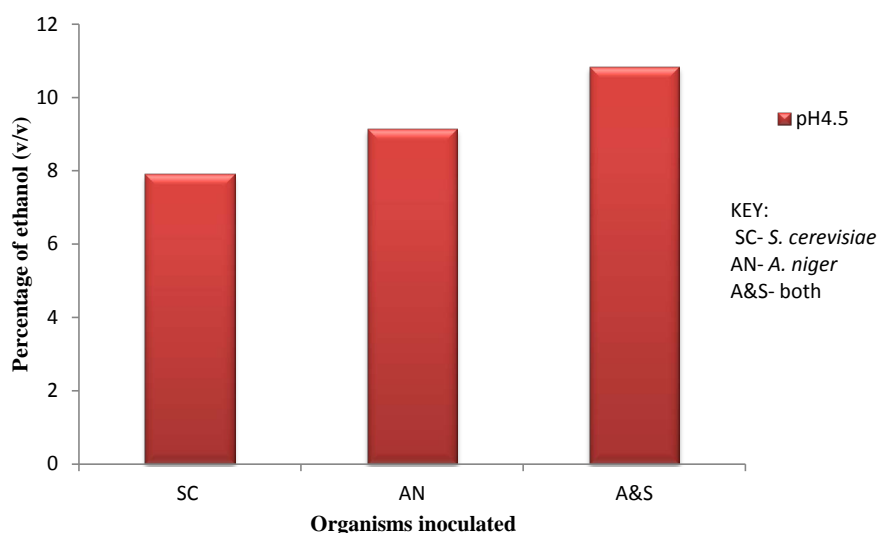


Figure 6. Ethanol yield after distillation.

observed the assimilation of organic carbon source by microalgae depend on the strain of the cell and culture conditions. The size of inoculum available in the culture media as well as the amount of carbon substrate added to the culture affects the increase in algal growth. In this study, plantain peel extracts were chosen for algal cultivation due to their high carbohydrate content and presence of other micronutrient contents which are one of the basic requirements for cell cultivation growth. The results show the effects of *Musa paradisiaca* peel extract on the algal cell growth and cell production of biomass under mixotrophic condition for fourteen days.

After inoculation of microalgae to plantain peel extracts, initially, the cell density was remained dormant and the cells have to adapt to the new environment. At three days lag phase was observed in which the microalgal cultures showed slight growth as the cell allocate most resources to the physiological adaptation induced by the new environment [39]. This lag phase was preceded by a rapid

exponential growth phase at day five because the initial small population is not nutrient limited. As the nutrients become depleted, the growth phase decreased and the cell biomass experienced linear increase. The growth phase remains station until the fourteen day, when all the nutrients were depleted and the biomass concentration decreased drastically.

After fourteen days of cultivation, the biomass obtained was 2.4 g/l, which was higher than those reported by [31] following two months cultivation of *A. platensis* about 2.0 - 2.2 g/l biomass concentration was produced. [40] work agreed with this study which illustrated that the high cell density in the *C. vulgaris* mixotrophic cultivation was because of the invigorating growth consequence of light and carbon source (CO₂) in the culture, the outcome is equivalent to when cultivated with glucose. Earlier reports have shown that batch system of mixotrophic cultivation possess the ability to significantly raise the concentration of the cell and production of biomass of the microalgal [41]. Based on these findings, plantain peel extract can as a medium for culturing and enriching microalgal biomass for large scale production.

After carbohydrate extraction, the biomass quantification increased to 13 g/l which when compared closely to the values of maximum biomass quantification accounted by [42] and [43]. Integration of dilute acid and crude enzymes in the hydrolysis of *Chlorella* biomass exhibited the best saccharification efficiency and highest yield of ethanol. This is as a result the combined process has the capacity to decompose undisrupted cell wall carbohydrates of microalgal biomass.

Microalgae encompass a potential attractive and sustainable energy basis for bioethanol production with the prospective of a renewable transportation fuel which can substitute gasoline. The gradual increase in the cell density from day one to the fifth day of fermentation suggested that sugars in the hydrolysates were utilized as carbon sources for ethanol production rather than cell production and this is as a result of the ability of the mould and yeast to hydrolyze and convert them to bioethanol. The microalgal biomass hydrolysates in this work were fermented by *Aspergillus niger*, *Saccharomyces cerevisiae* and co-culture of both. Co-culture fermentation by *A. niger* and *S. cerevisiae* yielded highest ethanol during the fermentation period than with their SHF process. Also highest percentage ethanol in the distillates was obtained using SHCF process. This may be due to various mixtures of sugars such as hexoses and pentoses released into the hydrolysates which cannot be utilized by *S. cerevisiae* but were hydrolysed to fermentable sugars by *A. niger*. This organism is capable of producing carbohydrate hydrolases and certain enzymes like amylases, cellobiases, xylanases which degrade the non-starch polysaccharides resulting to related increase in the amount of soluble sugars available in the fermentation broth [44] [45] [46]. The susceptibility of some sugars obtained after hydrolysis by *A. niger* during the fermentation process to the fermentable activity of *S. cerevisiae* became higher causing corresponding increase in ethanol yield. The result obtained from this study agrees with the study on ethanol production by simultaneous saccharification and co-culture of *A. niger* and *S. cerevisiae* fermentation of yam peel

which stated that most substrates were utilized for ethanol production in co-culture fermentation [47].

5. Conclusion

The result of this study shows that *Chlorella vulgaris* can utilize plantain peel extract as sole carbon substrate for biomass production providing feasible, sustainable and waste management process to reduce the cost of bioenergy production. Likewise, co-culture fermentation technique involving *S. cerevisiae* and *A. niger* gave much better ethanol yield than their monoculture counterpart.

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