

Production of Oxalic Acid by *Aspergillus niger* using *Chlorella Vulgaris* Grown with an Industrial Effluent as a Potential Feedstock



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Abstract

The potential of *Chlorella vulgaris* as a feedstock for production of oxalic acid through fermentation process was investigated. *Chlorella vulgaris* was obtained by blooming in 60:40 industrial effluents and freshwater under natural illumination by submerged fermentation at a retention period of 14 days. The *Aspergillus niger* spores were standardized and inoculated into the algal formulated medium for oxalic acid production and the oxalic acid produced was assayed using gas chromatography-mass spectrophotometry. Result showed that the total carbon, nitrogen and phosphates content of the algal slurry were 9.85ppm, 18.73ppm and 1.75ppm respectively. The optimum temperature for the production of the acid was 30 °C and pH 6 respectively. The GC-MS result revealed that the algal formulations produced 8.48mg/g of oxalic acid. This work indicates that algal formulations would serve as a cheaper substrate for oxalic acid production and recommended for industrial application.

Keywords: *Aspergillus niger*; *Chlorella vulgaris*; Effluent; Fermentation; Oxalic acid

Introduction

Oxalic acid is a soluble, dicarboxylic acid with the formula, $H_2C_2O_4$ which can also be called ethanedioic acid. Oxalic acid has wide applications in food as a preservative in post-harvest ripening of banana and can serve as anti-browning agent [1], agricultural and textile industries. Its dissociation in bioleaching of iron and different metals has made it important in hydrometallurgy [2]; a major cleansing agent [3] and has been used to remove rust from pipes [4]. Conventionally, most oxalic acids are synthesized by chemical processes which include oxidation of olefins and glycols, oxidation of carbohydrates with trioxonitrate (v) acid, decomposition of formates followed by tetraoxosulphate (vi) acid treatment [5]. Unfortunately, these chemical processes have been reported to be eco-harmful thus, the need for a more sustainable and environment-friendly approach.

Biosynthetically, oxalic acid can be produced by certain microorganisms, plants and animals [6]. Some microorganisms that have produce oxalic acid include: *Aspergillus ficuum* [2]; *Glyphyllum trabeum* [2]; *Paxillus involutus* [7]; *Penicillium oxalicum* [8] and *Aspergillus niger* [4]. Among these microorganisms, *A. niger* has been reported to give the highest

amount of oxalic acid, hence its preference over other isolates. The organism is generally accepted because of ease of handling, rapid growth and ability to ferment versatile cheap raw materials [9,10].

In a bid to produce oxalic acid from cheap substrates, a variety of substrates such as: milk whey [11]; cashew apple juice [5,9]; molasses [12]; sweet potato hydrolysate [13] and corncobs have all been investigated. However, no research on oxalic acid production has been conducted with algal biomass slurry as source of carbon. *Chlorella* sp. is a microscopic cell (<10µm diameter) with physiological characteristics similar to plants [14], an eukaryotic green microalga which replicates, grows optimally in aerated and well humified environment [15]. *Chlorella vulgaris* can be cultivated by photoautotrophic, heterotrophic and mixotrophic methods and can be used as animal feeds [16]; in the pharmaceutical, agricultural and nutraceutical industries as food for man. Hence, it can serve as a good nutrient source for fermentation processes. This study was conducted to assess the potential of *Chlorella vulgaris* biomass for oxalic acid production using *A. niger* in submerged fermentation technique.

Materials and Methods

Sample collection

The effluent and freshwater samples for the algal cultivation were obtained from a food industry within Choba, Port Harcourt, Rivers State Nigeria. The samples were filtered using Whatman filter paper, sterilized and stored in a refrigerator at 4 °C until required.

Microalgae and culture medium

The microalgae used in this study were obtained from the department of microbiology, University of Port Harcourt, Nigeria. A pure culture of the organism was obtained by repeated sub-culturing of the isolate on nutrient agar using spread plate technique, a mixture of chloramphenicol (62.5µg/ml) and nystatin (100µg/ml) was added to the culture medium to ensure free fungal and bacterial cultures. The algal strain was selected after preliminary screening using biochemical and morphological characteristics and was finally maintained on agar slants until when required [17].

Cultivation

Five milliliters of the bloomed culture was aseptically inoculated into flasks containing effluent-and freshwater medium. About 1ml of the bloomed culture was inoculated into a defined synthetic medium (0.132g/L Potassium nitrate, 0.066g/L sodium silicate, 0.066g/L monosodium phosphate and 0.066g/L EDTA. The pH of the medium was adjusted to 6.5 with 4M NaOH prior to autoclaving at 121 °C for 15 minutes [17]. The setups were maintained at 28±2 °C under natural illumination and aerated intermittently by shaking at interval for 14days. Samples were periodically removed every 48h to monitor changes in algal concentration, optical density and biomass as dry weight was determined.

The American and Public Health method were used to determine the chemical composition of the effluent. All experiments were designed in triplicates. The Statistical Software of Statistical Package for Social Sciences (SPSS) was used for the statistical analysis. The Posthoc test was used to test for the significant difference at p-value<0.05 within the group measured at 95% confidence level.

Inoculum preparation for oxalic acid production

The oxalic acid producing strain of *Aspergillus niger* used in the study was locally sourced from the department of Microbiology, University of Port Harcourt, Choba, Nigeria. Spores of *A. Niger* were grown on Potato dextrose agar (PDA) for 5-7days at 30 °C. Afterwards, the spores were aseptically transferred into 100ml sterile distilled water flask [4].

Oxalic acid determination

A slight modification of Emeko et al. [4] was adopted for the medium formulation and fermentation study. The medium consisted of 50g of algal slurry, 1.6g/L of yeast extract, 0.025g/L of yeast extract, 0.025g/L of MgSO₄.7H₂O and 0.5g/L

KH₂PO₄. The medium was adjusted to pH 6.0 using 4M NaOH solutions prior to sterilization. In this work, the oxalic acid produced was measured using the gas chromatography-mass spectrophotometry and the catalytic kinetic spectrophotometry documented by Jiang et al. [18].

Result

The nutrient index observed in this investigation have been shown to be necessary for the growth of micro algae. The dissolved oxygen for industrial effluent was 2.87 0.03ppm while that of the freshwater was shown to be 5.17 0.04ppm and there exists a significant difference between samples. The pH of the industrial effluent and freshwater samples were observed as follows 7.75±0.01 and 6.89±0.03 respectively and the result indicates existence of a significant difference between both samples and regulatory standard (FEPA) of pH 6.0 as shown in Table 1. The salinity for effluent and freshwater samples were reported to be 35.65±0.78ppm and 252.5±3.4ppm and this varied from FEPA standard of 90ppm. The phosphate contents of the effluent and freshwater samples were 18.6c and 2.15, the values of the samples varied from the FEPA standard of 7.0. The optical density of *Chlorella* sp. revealed an increase from 0.145 abs at 620nm to 0.789 abs at 620nm after 14days of culture while the cell dry weight also increased from 0.112mg/10ml to 0.267mg/10ml as shown in Figure 1 & Figure 2 respectively. Table 2 below shows the physico-chemical characteristics of the algal biomass slurry used in this work. The result shows a high concentration of calcium (19.1mg/g) and total nitrogen content (18.73mg/g).

Table 1: Physico chemical characteristics of effluent and fresh water.

| Parameters | Effluent | Fresh Water | FEPA |
|----------------------------------|-------------------------|-------------------------|--------------------|
| pH | 7.75±0.01 ^c | 6.89±0.03 ^b | 6.0 ^a |
| Salinity (mg/L) | 35.65±0.78 ^a | 252.5±3.54 ^c | 90 ^b |
| Electrical conductivity (µS/cm) | 80.15±0.05 ^a | 871.7±2.7 ^c | 120 ^b |
| Dissolved oxygen (mg/L) | 2.87±0.03 ^a | 5.17±0.04 ^c | 4.0 ^b |
| Total hardness (mg/L) | 6.1±0.02 ^b | 165.2±1.01 ^c | 1.25 ^a |
| Total Dissolved solids (mg/L) | 80.0±1.0 ^b | 2.05±0.05 ^a | 500 ^c |
| Total organic carbon (mg/L) | 31.74±0.46 ^c | 4.54±0.46 ^b | 0.05 ^a |
| Chemical oxygen demand (mg/L) | 72±1.0 ^b | 6.45±0.06 ^a | 430 ^c |
| Biochemical Oxygen demand (mg/L) | 6.54±0.06 ^c | 0.16±0.02 ^a | 6.0 ^b |
| Total nitrogen (mg/L) | 16.51±0.49 ^c | 2.65±0.065 ^a | 5.0 ^b |
| Phosphate (mg/L) | 18.60±0.46 ^c | 2.15±0.01 ^a | 7.0 ^b |
| Ammonia (mg/L) | 4.89±0.11 ^b | 0.67±0.08 ^a | 150.0 ^c |
| Sulphate (mg/L) | 0.27±0.06 ^a | 1.06±0.02 ^b | 500 ^c |
| Nitrate (mg/L) | 0.55±0.05 ^b | 0.09±0.01 ^a | 10 ^c |

Means±Standard Error; superscripts with the same alphabet in a given row are statistically insignificant at p <0.05.

Table 2: Chemical composition of algal slurry.

| Parameter | Value |
|----------------------|-------|
| Alcohol content | 0.48% |
| Magnesium (ppm) | 1.83 |
| Manganese (ppm) | 0.48 |
| Zinc (ppm) | 0.31 |
| Calcium (ppm) | 19.1 |
| Total nitrogen (ppm) | 18.73 |
| Phosphorus (ppm) | 1.75 |
| Total carbon (ppm) | 9.85 |
| Iron (ppm) | 1.01 |

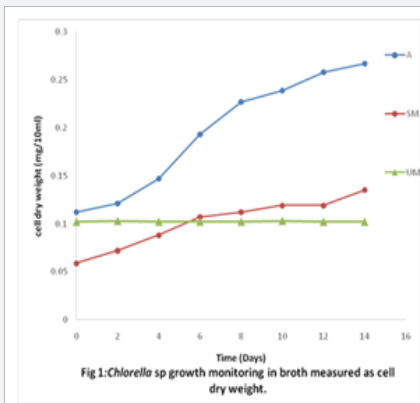


Figure 1: Keys.

A: Algal Biomass in Effluent-Freshwater Medium
 SM: Synthetic Medium
 UM: Uninoculated Medium.

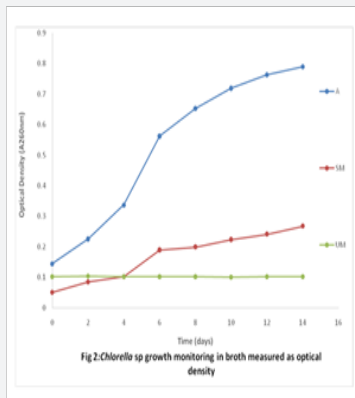


Figure 2: Keys.

A: Algal Biomass in Effluent-Freshwater Medium
 SM: Synthetic Medium
 UM: Uninoculated Medium.

Furthermore, the study revealed that the best conditions for oxalic acid fermentation were pH 6 and 30 °C as both conditions gave the highest oxalate concentrations of 10.55ppm and 8.98ppm respectively after 10 days of fermentation as seen in Figure 3 and Figure 4. The final oxalic acid produced at 30 °C and pH 6.0 was reported to be 8.48mg/g with retention time of 12.621. Other organic acids like pyruvic, citric, malonic acids were also synthesized along the oxalic acid.

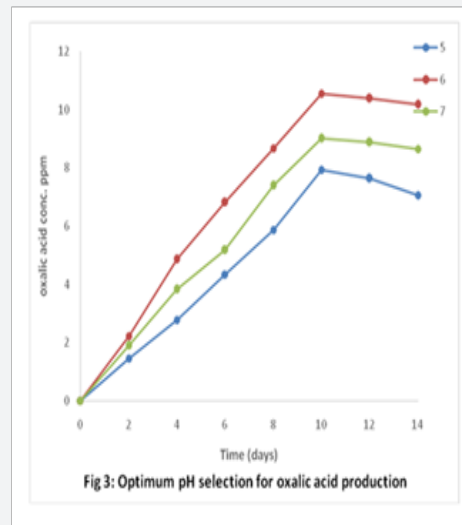


Figure 3

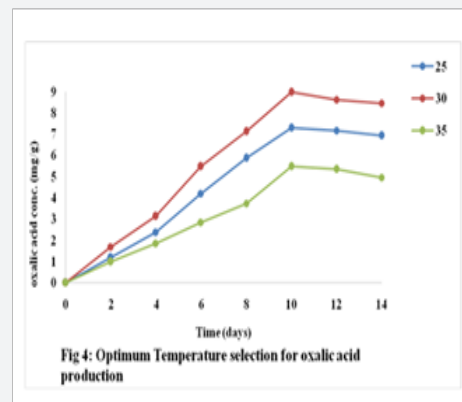


Figure 4

Discussion

Effluent treatment and disposal especially from industrial activities have become a huge challenge to manufacturing processes all around the world. Stiffer limits have been proposed to both safeguard the discharge of harmful materials into ecosystems and possibly reuse them for industrial benefits. The growth of *Chlorella* sp. using industrial effluent medium revealed inherent micronutrients found within the effluent proved its suitability as growth medium. The report concurs with Grima et al. [19] who showed that microalgae can be grown in sea water supplemented with phosphates and nitrates. Amanullah (2007) & Iyoyo et al. (2010) opines that most microalgae can be grown with wastes rich in phosphorus and nitrogen. These nutrients innate within the waste were necessary for the growth of the organism [15]. Poultry waste extracts and industrial dairy waste have also been shown to support the cultivation of microalgae [17,20]. Enitan et al. [21] observed that effluents could be applied to the production of a wide variety of bioactive materials as a cheap source of nutrients. These findings all agree with the need and viability of microalgae cultivation with cheap and inexpensive materials (Figure 5).

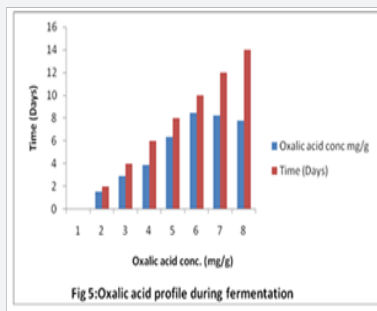


Figure 5

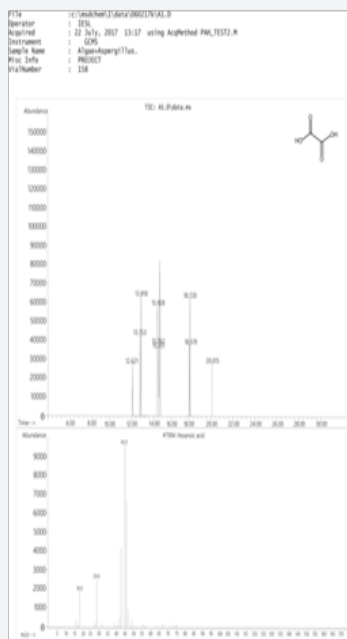


Figure 6

The reportedly low pH levels in the effluent in this study could suggest a lower carbon concentration, in the form of carbonates and bicarbonates. Conversely, Roselin (2015) reported that pH of sewage water could range from 6.3-7.3. The increased nitrate level in the effluent suggests the presence of high nitrogen-containing compounds in the wastewater. The nitrate levels reported in this work also disagrees with Ahmad et al. [22] who reported a higher nitrate content of 1.19ppm. However, the nitrate content reported in this work is similar to that of Nwosu et al. [23] that reported a nitrate concentration of 0.68 3ppm from a food factory during the rainy season and higher than the nitrate content of 0.106 (Figure 6).

It has been well reported that fermentation medium for oxalic acid production must contain carbon and nitrogen sources [5]. Algal starch has been reported to be readily used by yeast via fermentation for the production of ethanol and organic acids [24,25]. The biochemical composition of algae is a function of the species, temperature, light and growth stage. A variation in the biochemical composition due to growth stage is frequently

affected by the stage of the culture and nutrient exhaustion [26]. Basically, algal cultures become depleted in nutrients as they enter stationary stages of growth, while protein content declines, total carbon content increases [27,28].

The study reported a high concentration of total nitrogen content (18.73ppm) in the algal slurry which is in contrast with the report of Dineshkumar et al. [29] on algal biomass slurry. The difference could be attributed to the difference in nature of biomass used in the investigation. Dineshkumar et al. [29] also reported a higher carbon concentration of 20.42±0.33mg/g on dry biomass. This disparity could be as a result of the difference in the algal growth medium. Furthermore, Giselle et al. [30] reports a carbohydrate content of 7.09±0.84 and protein content of 6.07±1.14 in *Chlorella* sp that has attained stationary growth phase. This study also differs with Lum et al. 2013 who reported 12-17% carbohydrates dry weights, 14-22% lipids and 51-58% proteins in *Chlorella* sp. The disparity could be attributed to the fact that the microalgae were possibly in the exponential phase of growth. Also, factors such as environmental factors, nutritional factors and protein production can affect the carbohydrate content and ultimately total carbon content in microalgal species Abdelkhalik et al. [31].

The optimum pH and temperature reported in this work agree with Rujiter et al. [32]; Mandal & Banerjee [4,33]. The yields reported in this work do not corroborate with Betiku et al. [5] who reported an oxalic acid yield of 38mg/g from molasses and sweet potato starch hydrolyzate with oxalic acid yield of 1,038mg/g respectively. This disparity could be attributed to the choice and availability of the feedstock for metabolism by the fungus. Furthermore, the relative reduced yield in this work could also be linked to the non addition of methanol to the fermentation medium. Betiku et al. [5] reported an increased yield of oxalic acid after addition of 1% methanol to the fermentation medium. In addition [34], Cameselle et al. [11] reports that one of the challenges with oxalic acid formation via fermentation is the simultaneous production of gluconic at pH 7, thus, recommends a strict control for the medium environment for optimum production (Figure 7).

EESL Laboratory Quantitation Report (Not Review)

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Data Path      : c:\msdchem\1\data\060217\F
Data File      : 81.D
Operator       : EESL
Acquired       : 22 July 2017 13:17 using AcqMethod PAL_TEST2.M
Instrument      : GCMS
Sample Name    : AlgaeAspergillus
Misc Info     : PROJECT
VialNumber    : 138 Sample Multiplier: 2

Quant Method : C:\MSDCHEM\1\METHODS\PAL_TEST2.M
Quant Title  : ORGANIC ACID CALIBRATION
Class Update : Fri Dec 16 15:48:10 2016
Response Via : External Calibration
    
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| Compound | R.T. | Q Ion Response | Conc (mg/g) | Det (min) |
|------------------|--------|----------------|-------------|-----------|
| Target Compound | | | | |
| 1) Oxalic acid | 12.621 | 8428 | 8.48 | 86 |
| 2) Tartaric acid | 13.751 | 6802 | 0.17 | 45 |
| 3) Glycolic acid | 13.916 | 10163 | 0.09 | 70 |
| 4) Formic acid | 15.151 | 2163 | 0.28 | 33 |
| 5) Acetic acid | 15.702 | 4188 | 0.15 | 92 |
| 6) Citric acid | 15.928 | 3739 | 0.12 | 41 |
| 7) Malonic acid | 18.519 | 7608 | 0.08 | 52 |
| 8) Malic acid | 18.709 | 7698 | 0.08 | 87 |
| 9) Pyruvic acid | 20.015 | 3682 | 0.02 | 61 |

(#) = qualifier out of range (n) = manual integration (*) = signals summed
EESL(*)-Limnema_TEST2.M Sun Jun 11:57:10 2017

Figure 7

Conclusion

The prospect for submerged fermentation using *Chlorella vulgaris* biomass as feedstock for oxalic acid production was explored in this study [35-40]. The results showed that algal biomass had the necessary nutrients for oxalic acid production at pH 6 and temperature of 30 °C for 10 days [41-45]. The oxalic acid concentration reported at the end of the study period was 8.48 mg/g. Algal biomass remains an untapped resource for the production of industry-important substances [46].

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