

CaCO₃ Supplementation of Low-Carbon Wastewaters for the Cultivation of Microalgae: A Study with *Desmodesmus multivariabilis*

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Microalgal biomass cultivated in wastewater has the potential for refining to energy products such as biodiesel and biohydrogen with the additional benefit of also treating the wastewater. As many species of microalgae can employ both Heterotrophic and Autotrophic modes of carbon synthesis, low-carbon waters benefit from the addition of inorganic carbon to the water. Capital and operational costs are a deterrent to using CO₂ and other alternatives may be more attractive. NaHCO₃ is a popular alternative but as a result of its solubility and alkalinity quickly raises the pH of the water which inhibits algal growth due the presence of free ammonia at high pH values. In this experiment, the growth of a South African strain of *Desmodesmus multivariabilis* was studied in the presence of solid calcium carbonate. Calcium carbonate was chosen for its low solubility, which would potentially allow for its dissolution to be driven by the inorganic carbon uptake in the media. The performance was compared to that of aerated wastewater and wastewater with solid CaSO₄ as a non-carbon-containing substitute. It was found that there were significant differences in the growth and metabolism of all three experiments. Growth in the presence of solid calcium carbonate and calcium sulphate showed a preference for attached growth in the vicinity of solids, while suspended growth was preferred when just air was supplied. Furthermore, the experiment with air showed the highest growth rate, nitrogen uptake and a biomass yield that was more than an order of magnitude higher than with CaCO₃. The experiment with CaSO₄ showed low yields and growth rates, possibility indicating and inhibitory effect of the CaSO₄. In the presence of CaCO₃, a very high yield of extracellular organic metabolites was observed. The presence of these metabolites, as well as the stability of the pH and low growth, is a possible indication that the organism was controlling the pH as a defence mechanism. Despite not being a favourable substrate for growing *D. multivariabilis*, the high yield of extracellular metabolites may have a commercial potential, and the nature and use these metabolites deserve further investigation.

1. Introduction

Cultivation of microalgae in wastewater (WW) is attracting a wide variety of interest due to the dual benefits of water treatment and the generation of valuable biomass that can be valorized into a variety of energy products, such as biodiesel and biohydrogen (Wang et al., 2010). As many different varieties of microalgae can employ both autotrophic and heterotrophic methods of carbon synthesis, they benefit from the introduction of inorganic carbon, especially in low-carbon WW (Kandimalla et al., 2016). Microalgae can absorb inorganic carbon in the dissolved CO₂ as well as the HCO₃⁻ forms (Thomas et al., 2016). Introduction of CO₂ as an inorganic carbon substrate through bubbling is well-attested in literature, with different forms of photobioreactors available that vary in the methods of CO₂ introduction (Razzak et al., 2013). In industry however, gas transportation and handling requires significant energy investment (Moheimani, 2016). NaHCO₃ has been studied in literature as an alternative form of inorganic carbon substrate. NaHCO₃ is readily soluble and chemically and biologically available inside the solution. The major problem with NaHCO₃ is that while it supports strong initial growth, the rapid uptake of inorganic carbon from HCO₃⁻ leads to a rapid increase in the pH (Ebrahimian et al., 2014). This is accompanied by the conversion of NH₄⁺ to NH₃, which is toxic to some

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microalgae and leads to cell death and a drop in cell concentration (Vasconcelos Fernandes et al., 2015). The potential advantage of using CaCO₃, which has a very low solubility in water ($K_{sp} = 3.3 \times 10^{-9}$ as reported by the American Water Works Association, 2011) is that the slow release into the aqueous medium can be driven by the biological uptake while the remainder of the substrate remains in a chemically inactive solid form. In this study, blocks of solid Calcium Carbonate are tested as an alternative carbon source to augment the growth of the microalgae in WW in the presence of atmospheric air. As part of the search for a local, novel, potentially high-yielding strain in terms of the South African Bioeconomy Strategy (Department of Science and Technology (South Africa), 2013), *D. multivariabilis* was selected from the various strains sampled due to the scant availability of literature for this specific strain in relation to biofuel production and domestic wastewater treatment.

2. Materials and methods

2.1 Algal culture identification and preparation

The culture used consisted of purified strains of microalgae isolated from samples collected from the Hartebeespoort dam, a reservoir in the North West Province, South Africa (Birungi & Chirwa, 2017). Purification was performed by using the Streak Plating technique. Verification of sample identity as *D. multivariabilis* was performed by 18s rRNA and ITS sequencing (Roestorff & Chirwa, 2018). Algal starter cultures were cultivated in a Triple Nitrogen, Bold Basal Medium with Vitamins (3N-BBM+V) to a cell density of 200–450 mg/l before inoculation (Roestorff & Chirwa, 2018).

2.2 Culture media

WW media was obtained from the feed to a small activated sludge WW treatment works on a private housing estate. The WW underwent primary sedimentation and flow reduction (by means of a septic tank and French drain system) prior to entering the head of the works.

The media was autoclaved at 121 °C and 2 bar for 20 minutes and stored at 4 °C to slow the rate of biodegradation. Chemical Oxygen Demand (COD) analysis (Lovibond 2420720), Total Carbon (TC) and Total Organic Carbon (TOC) analysis (Shimadzu TOC-Vwp), Total Nitrogen analysis (Spectroquant 14763/14537 kit with Spectroquant Nova 60), and PO₄³⁻ analysis (Hanna Instruments HI 93717) was performed to characterize the effluent prior to usage.

2.3 Experimental Setup.

Algal cultures were all cultivated in 500 ml culture flasks. Flasks were equipped with specially designed airlocks equipped with a filter which allowed the throughput of air but reduced contact with airborne contaminants. All equipment, media and reagents were autoclaved at 121 °C and 2 bar for 20 minutes prior to use. Flasks were mounted on Velp Am4 magnetic stirrers with stirrer setting at 6.

A set of experiments as well as three sets of control experiments were performed. In Experiment 1, 5.0 g of Calcium Carbonate chunks (obtained from commercially available chalk) were suspended in nylon mesh bags inside the media. In the first control experiment (Experiment 2), 5.0 g of Calcium Sulfate chunks were suspended in nylon mesh bags. In the second control experiment, Experiment 3, air with a background CO₂ concentration of 410 ppm was bubbled through the flasks at an average rate of 0.12 Vessel Volumes per Minute (VVM). The blank control experiment (Experiment 4) used uninoculated WW media with 5.0 g of Calcium Carbonate suspended in nylon mesh bags inside the culture flasks.

The algae was cultivated at 25–28 °C at the required lighting conditions (Osram L 36W/77 Flouora x 2) (Roestorff & Chirwa, 2018). The light/dark cycle was adjusted to replicate peak summer lighting conditions in Pretoria, South Africa (14 h light/10 h dark).

2.4 Sampling and analysis

Sampling of the media was conducted on a daily basis. Samples were harvested in 10 ml centrifuge tubes and were analyzed for TOC as well as biomass concentration. As only the liquid portion of the media was sampled while monitoring growth, the mass of the attached growth was only assessed at the termination of the experiment for Experiments 1 and 2. Biomass was separated by centrifuging at 6500 rpm for 10 min (Hettich Universal 320R Centrifuge), repeating with the supernatant until all cells were removed. The biomass was dried at 50 °C for 24 h before being weighed (Mettler-Toledo XS205 Dual Range). Samples were filtered using Whatman No. 1 filter paper and diluted to a ratio of 1:30 with de-ionized, distilled water prior to TOC analysis.

The absorption spectra of the samples were measured at 680 nm, 660 nm and 600 nm using UV-Visible light spectroscopy (WPA, Light wave II, Labotech, South Africa). The experiments were conducted until the growth rate decreased and cultures exhibited characteristics of maintenance growth, i.e. metabolically active yet non-reproducing. This was approximately on day 5 for Experiment 3 and day 14 for Experiments 1, 2 and 4. As the

cells forming the attached growth in Experiments 1 and 2 were not strongly bonded to the nylon mesh bags, agitating the flasks by hand for no longer than 10 seconds was sufficient to detach all of the attached cells and harvest them together with any suspended growth.

At the termination of each experiment, the attached growth and mixed liquor were harvested, and the cellular material separated by means of centrifuging at 6500 RPM for 10 min in 50 ml tubes. The resulting supernatant from the experiments were repeatedly centrifuged until it was completely cell free. The cell-free media was analyzed for TOC, COD, Total N, pH and PO_4^{3-} concentration. The mass of solid CaCO_3 and CaSO_4 remaining was weighed after drying for 24 h at 70 °C. The cellular mass produced from Experiment 1 was treated with 2M sulfuric acid to remove any undissolved CaCO_3 , oven-dried for 24 h at 50 °C and weighed. Transesterification was performed on the algal mass of Experiment 1 according to Indarti et al., (2005) and a qualitative indication of the FAME constituents was obtained via GCMS (Perkin-Elmer Clarus 600), using the analysis method described in (Li, 2012). Total carbon dioxide utilization was calculated by means a carbon balance. Carbon content of the algal biomass was determined using a Total Carbon SSM module (Shimadzu SSM-5000A)

3. Results

3.1 Definition of metrics

The starting media parameters, experimental outputs and metrics are provided in Table 2. For the purpose of evaluating the performance and to enable comparison of the experiments, the following metrics and parameters are defined (Table 1):

Table 1: Definition of growth metrics and parameters for performance evaluation

Description	Parameter	Unit	Equation/Definition
Effective growth rate	\bar{r}_{EFF}	mg/d	$\bar{r}_{EFF} = \frac{M_f}{T}$ (1)
Specific Biomass Yield, time adjusted	\bar{Y}_{BM}	1/d	$\bar{Y}_{BM} = \frac{M_f - M_0}{M_0 \cdot T}$ (2)
Biomass Yield, time adjusted	Y_{BM}	1/d	$Y_{BM} = \frac{M_f - M_0}{T \cdot \sum_0^T S_t}$ (4)
Organics Yield, time adjusted	Y_C	1/d	$Y_C = \frac{C_{E,f}V_f - C_{E,0}V_0}{T \cdot \sum_0^T S_t}$ (5)
Parameters	$M_f;$	mg	Total mass of cells produced in the experiment
	$M_0;$	mg	Initial mass of cells inoculated
	$C_{E,f}; C_{E,0}$	mg/l	Final and initial concentrations of organics in the media, respectively
	T	d	Duration of the experiment
	S_t	mg	The inorganic substrate utilized at time, t (Calcium Carbonate and Carbon Dioxide)

3.2 Algal growth & Substrate depletion

The most notable difference between the first two experiments and the third experiment was the preference for attached growth on the nylon mesh bags in the first two experiments. Only the first iteration of Experiment 1 showed some suspended growth (Figure 1). In Experiment 3 the microalgae were fully suspended in solution (Figure 2).

Within 1-2 days of initiating Experiment 1 and 2 the WW media started to clear and on day 6 the growth rate of the cultures started increasing. The increase in turbidity of the blank control experiment (Experiment 4) can be

attributed to the agitation resulting in the disintegration and suspension of the solid CaCO_3 . The process was also driven by the decrease in pH of the solution due to infection that occurred with an opportunistic phosphate-depleting atmospheric bacterium (possibly *Serratia marcescens*), which became apparent around days 4 – 6.

Due to the mixed liquor in Experiments 1 and 2 not being representative of overall cellular content, growth curves could not be conclusively determined via UV-VIS spectroscopy, and the effective growth rate (\bar{r}_{EFF}) was calculated instead. The effective growth rate provides an overall estimate of the rate of growth where other data is unavailable, allowing comparison between the experiments. The algae *D. multivariabilis* displays an effective growth rate nearly 5.5 times higher in Experiment 3 than in Experiment 1 and 8 times higher than in Experiment 2 (Figure 3). The specific biomass yield compares the mass of cells produced to the original mass inoculated, therefore factoring for differences in starting biomass concentration. The specific biomass yield, adjusted for experimental run length, was 2.5 and 3.6 times higher in Experiment 3 than in Experiment 1 and 2 respectively (Figure 3). A similar trend can be seen for Nitrogen utilization (Figure 4). Nitrogen utilization displays a similar pattern, while Phosphate usage seems to be constant for all experiments (Figure 4). The qualitative GC analysis indicated that the major FAME constituent after transesterification of biomass produced in Experiment 1 was Methyl Dodecanoate. The other biomass was not evaluated.

Table 2: Characterization of WW media

Experiment	1	2	3	4
Experiment Description	WW + CaCO_3	WW + CaSO_4	WW + Air	Uninoculated WW + CaCO_3
Pre-Experimental Parameters				
Initial Volume, V_0 (ml)	500	500	500	500
Initial CaCO_3 Mass (mg)	5000	0	0	5000
Initial TOC Mass (mg)	28.2	28.2	29.7	28.2
Initial N Mass (mg)	22.8	22.8	27.5	22.8
Initial PO_4 (mg)	10.0	10.0	9.2	10.0
Initial pH	9.4	9.4	8.9	9.4
Post-Experimental Parameters				
Final pH	9.6	9.8	10.3	-
Biomass Produced (mg)	39.6	27.2	76.9	-
Carbon dioxide utilized (mg)	62.3	28.7	64.3	-
Nitrogen Utilized (mg)	5.1	2.5	12	-
PO_4^{3-} Utilized (mg)	4.6	4.5	4.5	-
Air feed rate (ml/min/vessel)	-	-	61	-
Net TOC production (mg)	208	27	0	-
CaCO_3 utilized (mg)	124	-	-	-
WW TOC Removed (mg)	-	-	1.33	-
Growth Metrics				
\bar{r}_{EFF} (mg/day)	2.89	1.99	16.14	-
\bar{Y}_{BM} (1/day)	1.45	0.99	3.58	-
Y_{BM} (1/day)	0.07	0.25	0.92	-
Y_C (1/day)	0.40	0.26	0.00	-

3.3 Production of organics, carbon source preference

An unexpected result was the high concentration of organic metabolites that was observed in the experiments with CaCO_3 . Due to this reason the utilization of WW TOC as a substrate could not be determined. In contrast, Experiment 2 with CaSO_4 and WW produced a small amount of excreted metabolites and Experiment 3 with WW and air bubbling produced no metabolites; instead cellular growth was promoted and WW was utilized as a Carbon source. According to Shipin et al. (1999) the metabolites may be Extracellular Polysaccharides/Extracellular Polymeric Substances (EPS) which get released under conditions of stress, such as when the organisms undergo a shift in metabolism. Wang et al. (2010) found that microalgae released small organic compounds (e.g. glycolic acid) as a byproduct of photosynthesis if organic substrate is not available.

The contrast of the high organic metabolite yield in Experiment 1 and the promotion of Cellular growth in Experiment 3 confirms the existence of two completely different metabolic pathways for this strain of *D. multivariabilis*. The extracellular metabolite is acidic in nature, as witnessed by the fact that the pH of the solution increased only slightly, despite the increase that was predicted due to the utilization of CaCO_3 as a carbon source. This correlates with the findings of Shipin et al. (1999).

The utilization ratio of $\text{CO}_2:\text{CaCO}_3$ is $0.74 \frac{\text{g of CO}_2 \text{ as C}}{\text{g of CaCO}_3 \text{ as C}}$. An interesting result of the experiment is that the mass of Carbon Dioxide utilized calculated for Experiment 1 and Experiment 3 are of a similar value, indicating that the uptake of CO_2 is not limited by mass transfer. The acidification caused by the release of metabolite would also drive the dissolution of CaCO_3 in water. A possible reason for the lower growth may be that the organism is actively controlling the pH of the water as a defense mechanism rather than expending most of its energy on growth. This is supported by the fact that the time adjusted biomass yield (Figure 5) is more than an order of magnitude higher in Experiment 1 than in Experiment 3. The time adjusted Organics yield (normalized), is also included in Figure 5. The lower growth rate of Experiment 2 coupled with the low uptake of CO_2 and no detectable organic Carbon uptake may indicate an inhibitory effect of the Ca^{2+} or SO_4^{2-} ions.

Exp 1:
Repeat 1: Repeat 1: Repeat 1: Repeat 2: Repeat 2: Repeat 2:
Day 5 Day 7 Day 14 Day 5 Day 7 Day 14

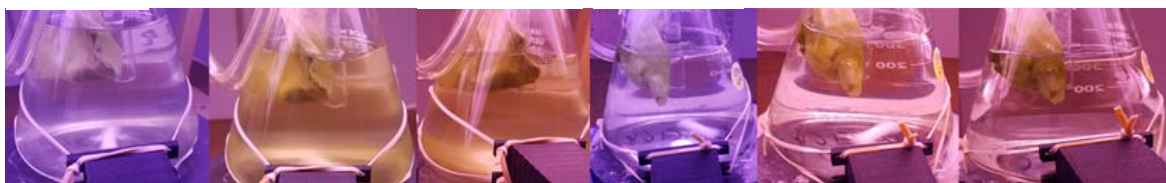
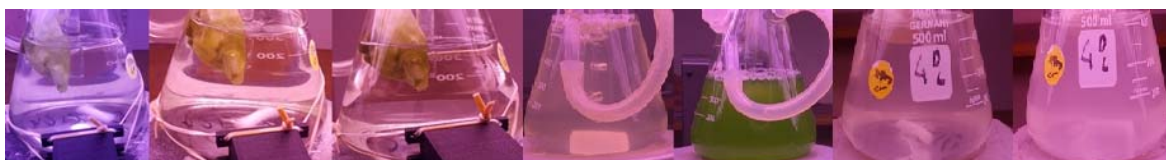


Figure 1: A composite image illustrating the experimental progression of Experiment 1 on Days 5, 7 and 14. The first three images are of the iteration that experienced suspended growth. The last three images are of another iteration.

Exp 2: Exp 2: Exp 2: Exp 3: Exp 3: Exp 4: Exp 4:
Day 5 Day 7 Day 14 Day 0 Day 5 Day 5 Day 14



(first three images), of Experiment 3 on Days 0 and 5 (next two images) and of Experiment 4 on days 5 and 14 (last two images).

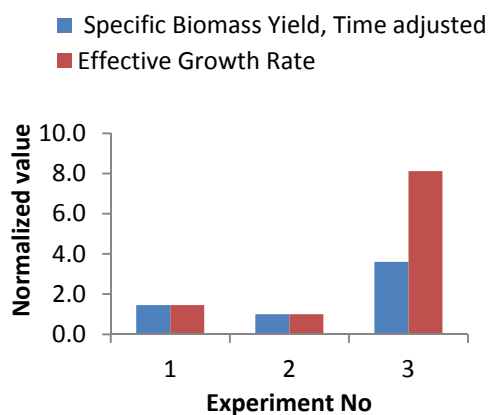


Figure 3: Effective Growth Rate and Specific Biomass Yield Normalized against the values of Experiment 2.

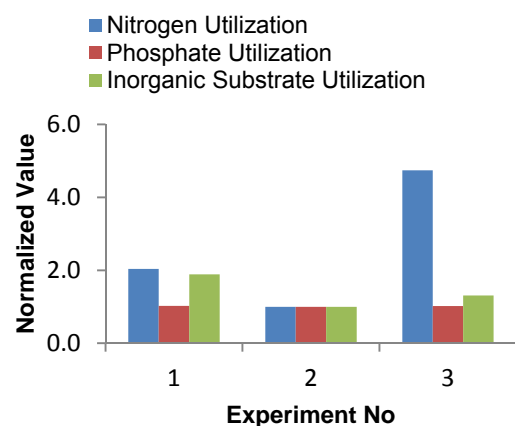


Figure 4: Phosphate, Nitrogen and Inorganic substrate utilization Normalized against the values of Experiment 2.

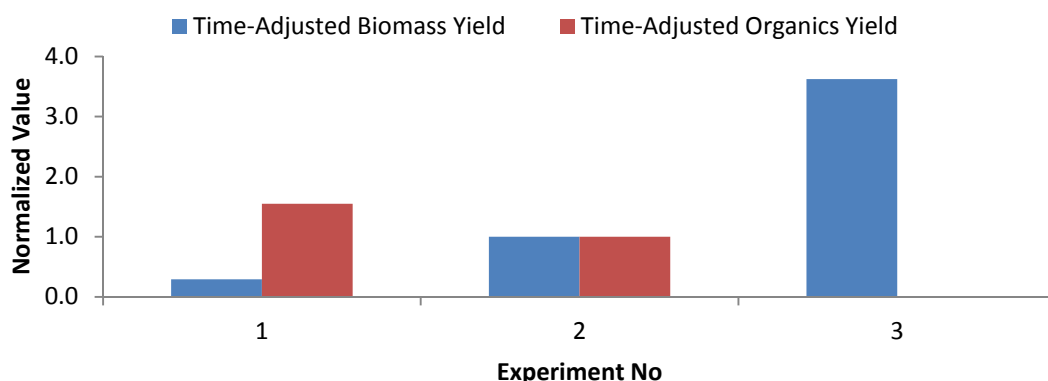


Figure 5: Biomass and Organics Yields, Normalized against Experiment 2. This figure clearly indicates that Experiment 3 had much more success in converting substrate to Biomass than Experiment 1 with CaCO_3

4. Conclusion

The results of the study clearly indicate that growing *D. multivariabilis* in Calcium Carbonate delivers suboptimal biomass productivity, due to a metabolic shift that occurs, possibly as defensive mechanism of the organism to counter the pH increase of the water. The result of this shift presents other opportunities, as the metabolites produced from Calcium Carbonate in significant quantity have unexplored potential. Further studies are needed to characterize the nature of the metabolites and explore their industrial potential.

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