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Transforming South Africa's Biodiversity into Diesel

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Abstract

The continuous use of fossil fuels is environmentally harmful and unsustainable. Concerns about energy supply, environmental pollution and global warming, have triggered the worldwide search for sources of environmentally friendly renewable energy. Global biodiesel production and research are focused on crop based feedstock's, which could become unsustainable in the long term, due to arable land and water requirements leading to competition with food crops. Algae have been identified as a second generation biodiesel feedstock and have the potential to supplement fossil fuels with negligible impact on food security. Microalgae have an oil yield at least 10 times better than oil seed crops and are capable of using environmental waste substrates such as CO_2 and nitrate rich waste water, for growth and lipid production. South Africa is amongst the 17 mega-biodiverse countries in the world. This coupled with the moderate climate, availability of sunlight make SA an ideal place to bioprospect for high lipid producing algal isolates and subsequent production of algal biodiesel. The algal team at Biosciences has been actively sampling SA's biodiversity for the past 3 years and has successfully obtained over 200 isolates. Various different methodologies related to isolation of these valuable algae from environmental samples have been used to develop a comprehensive screening and isolation protocol. This protocol included the inclusion of flow cytometry as a tool for isolation. The protocol was validated and the isolation of ~ 115 micro algal isolates conducted. The isolated samples were further screened for lipid production using a qualitative microscopic assay that rapidly identified high lipid producing isolates. Four of the best isolates were selected to be assessed at the secondary level. The secondary evaluation of lipid production required a rapid, semi-quantitative lipid assay, which was developed to equate lipid related fluorescence to actual lipid content. The lipid production profiles of these isolates ranged from a minimum of 0.4 pg/cell to a maximum of 90 pg/cell and the growth rates ranging from 0.52 to 0.13 day^{-1}. Preliminary results demonstrated that there is significant potential to increase lipid production through nutrient stress and further physiological stresses need to be investigated. The research on developing a process to optimize lipid productivity at larger scale is still ongoing.

Introduction

Energy supply and demand, environmental pollution and global warming, combined with the projected depletion of fossil fuels in 2050 have made it imperative to develop alternative energy sources that are renewable and environmentally friendly (Goldie, et al., 2005). Fossil transportation fuels are one of the environmentally harmful energy sources that are not sustainable and at risk of depletion, which has triggered research into renewable biofuels. Global biodiesel production and research are focused on oil seed crop based feedstock's, which could become unsustainable in the long term, due to arable land and water requirements leading to competition with food crop (Chisti, 2007)

Microalgae have been cited by many researchers (Aaronson, et al., 1982); (Benemann, et al., 1996); (Chisti, 2006); (Danielo, 2005) as the second generation biodiesel feedstock to provide a more sustainable approach to renewable fuels. This is supported by paleobotanical evidence that has attributed a large

portion of fossil oil reserves to prehistoric microalgae (Borrego, et al., 1996); (Modovan, et al., 1980). Microalgae have an oil yield at least 10 times better than oil seed crops such as rapeseed or palm and are capable of using environmental waste substrates such as CO_2 and nitrogen rich waste water, for growth and lipid production (Sheehan, et al., 1998);(Aaronson, et al., 1982); (Benemann, et al., 1996); (Chisti, 2006); (Danielo, 2005) Pulz, (2001) reported that for successful algal biotechnology, the right isolate for production is imperative.

For successful mass culture of algae, an isolate must fulfill certain key criteria. Research has indicated that for successful mass culture, the selected algal strain should be a local strain with a rapid growth rate, high photosynthetic efficiency and light harvesting capabilities, have a high lipid content, demonstrate reliable auto flocculation, have a high specific gravity compared to the growth medium, a large cell size (but non filamentous) as well as a wide tolerance to fluctuating environmental conditions, such as temperature, salinity, light intensity and metabolite and oxygen accumulation (Borrowitzka, 1996); (Chisti, 2007); (Griffiths, et al., 2009); (Grobbelaar, 2000); (Lee, 2001); (Benemann, et al., 1996); (Sheehan, et al., 1998).

South Africa is amongst the 17 mega-biodiverse countries in the world (www.megadiverse.org). This coupled with the moderate climate and availability of sunlight make SA an ideal place to bioprospect for high lipid producing algal isolates for subsequent production of algal biodiesel. Isolation of algae from mixed environmental samples and purification to monoculture is traditionally conducted by standard techniques such as serial streak plating, serial dilutions, enrichment culture and micromanipulation (Anderson, et al., 2005). Newer automated isolation techniques are recently emerging and include flow cytometric cell sorting (Balfoort, et al., 1992) (Cunningham, 1993). Microalgae that are able to grow on solid media as mono-algal isolates can be obtained without any further treatment after 3-5 serial streaks, unfortunately, not all microalgae are able to grow on solid medium and the growth period on solid medium can vary from a few days to six months for some micro algae (Anderson, et al., 2005) (Gerloff, et al., 1950) (Madigan, et al., 2006). Selective culturing is another method used to isolate microalgae from mixed natural samples and entails the use of specific physical and/or nutritional components and sub-culturing (Gerloff, et al., 1950) that support the growth of specific types of microalgae (Anderson, et al., 2005) (Madigan, et al., 2006). Micro-manipulation is the use of microscopy and robotics to isolate a single cell from liquid culture. Micromanipulation requires skill and patience and isolated cells are transferred to flasks containing liquid medium or onto agar plates (Anderson, et al., 2005). In serial dilutions, samples are serially diluted to achieve only one cell in an appropriate vessel and either spread on an agar plate or grown in a tube to obtain a single algal isolate (Anderson, et al., 2005) (Madigan, et al., 2006) while automated techniques which include flow cytometry, isolate individual species from mixed samples, automatically and with high purity and viability (Shapiro, 1988).

Flow cytometry (FCM) is thus an automated, efficient technique which can be used for the isolation of individual strains from mixed samples. The flow cytometer (FCM) records and measures the unique fluorescence and light scatter signal of individual cells as they pass through the light path of laser beams, in a single file, within a fluid stream. Cells of a particular algal species are very similar in terms of size, shape, granularity and pigmentation and thus exhibit similar light scatter and fluorescent signals when subjected to interrogation by the lasers. The similar fluorescence and scatter signals of each species are presented as individual discrete clusters, which can be selected for sorting individual species into separate tubes with high purity and viability (Melamed, et al., 1994) (Reckermann, 2000) (Davey, et al., 1996) (Davey, et al., 1999). One of the newer flow cytometer models has recently been acquired at the CSIR (BD FACS Aria II™) and exploratory research in separating 3 strains from a synthetic mix of isolates proved to be very effective.

Generally microalgae are isolated by the standard techniques discussed earlier and the potential of flow cytometric isolation for biodiesel algae is only now being realised and applied (Cellamare, et al., 2009) (de la Jara, et al., 2003) (Sieracki, et al., 2005). Both types have their advantages and disadvantages. Flow cytometry is purported to be faster, but is more expensive and complicated to use compared to standard isolation techniques, and the liquid samples must be pre-processed to remove filaments and debris. Standard isolation techniques are more labor intensive and time consuming, but they are simpler, cheaper and have proven themselves over time. A combination of standard and automated isolation techniques could provide an ideal high throughput algal isolation and screening protocol which is necessary for bioprospecting the majority of South Africa's aquatic bodies for lipid producing microalgae.

Materials and Methods

Sample collection and enrichment and isolation

Maps of South Africa that highlight eutrophic zones were used to guide the sampling site selection. High tropic zones were used to select the starting point for sampling and each trip started at a new trophic zone. Trophic zones formed the epicentre of a sampling region and samples were taken within a 50km radius from the centre. The samples reported herein are from the Western Cape region (coastal areas from Cape Town to George, at the intertidal zones, including harbours and jetties, rock pools and inland from dams and rivers close to the coast). Intertidal samples were collected as scrapings from rocks or liquid while only liquid freshwater samples were collected. The GPS coordinates, and site specific information, elevation as well as any other pertinent information relating to the sample sites was recorded. Samples (~20mℓ) were taken into McCartney bottles preloaded with 10 mℓ of either Artificial Fresh water (AF6) or Artificial Sea water (ASW) (Sheehan, et al., 1998), depending on the sample environment. Samples were stored in a polystyrene cooler bag for ~3 days during the sampling trip before being brought back to the laboratory. Samples were agitated and filtered with a 40µm filter to remove larger organic debris, zooplankton and filamentous algae. A volume of 10mℓ of the filtrate was dispensed into sterile tissue culture flasks (Cellstar® ; 25cm^3 Frickenhausen, Germany) and incubated at 25 ± 2°C under constant illumination (Osram® Cool White (L58W/965) fluorescent Lamps) for ~ 5 -10 days or until growth was observed. Samples were evaluated microscopically on an epifluorescence Olympus BX40, (Tokyo,Japan) microscope equipped with an ©Olympus Soft Imaging System (Analy SIS® Five, Life Science), to determine the different types of potential species of algae present in the sample.
Enriched samples (10ml) were transferred to sterile tissue culture flasks containing 30ml fresh medium though a 40µm filter, and allowed to incubate for 24 hours under constant illumination (Osram® Cool White (L58W/965) fluorescent Lamps). Samples were then streaked in duplicate onto either ASW or AF6 agar plates. Aliquots of 20 ml of each sample was transferred to two 50 ml glass round bottomed, one of which was placed on an Inova orbital shaker (New Brunswick, Edison, NJ, USA) at 70 rpm at 25 ± 2°C and under constant illumination (Osram® Cool White (L58W/965) fluorescent Lamps). The other flask was maintained without shaking under the same temperature and lighting conditions. Flasks and plates were incubated for 7 days and then examined both macroscopically and microscopically.
Agar plates which demonstrated robust growth of any unicellular green algae were subjected to a minimum of 3 serial passages on agar plates prior to being purified into single colonies. Cultures in round bottom flasks were isolates that congregated together and automatically isolated from other isolates in the sample. Samples that contained a mixture of unicellular algae were subjected to flow cytometer cell sorting.

Validation of flow cytometer cell sorting

Three unidentified axenic algal strains previously isolated from the Western Cape region of South Africa, were designated A41; A26 and A4. These were cultured in suspension in 50 ml tissue culture flasks containing 10 ml growth medium. Isolate A41 was cultured in 50% ASW (Sheehan et al. 1998) while isolates A4 and A26 were cultured in 50% AF6 (Sheehan et al. 1998). Flasks were incubated without agitation at 25 ± 2°C for 5 days under constant illumination (Osram® Cool White (L58W/965) fluorescent Lamps). A 1 ml aliquot of each isolate was then filtered through a 40 µm pore sized filter before diluting with 4 mℓ of either ASW or AF6 respectively (conducted in duplicate). One tube of each isolate was stained with 50µℓ of a 50µg/mℓ Nile Red: acetone solution (Fluka 72485) and incubated at ambient temperature for 15 minutes, while the other was left unstained. Each tube was analyzed using the BD FACSAriaII flow cytometer (FCM) (BD Biosciences, San Jose, CA, USA) and the results recorded. A solution of 0.1% NaCl in deionised water was used as sheath fluid. Aliquots of 0.5ml of each of the above isolates were mixed together and made up to 5ml with 0.1% saline solution, to create a mixed sample

(conducted in duplicate). One tube of the mixed sample was stained with NR as described above, while the other was left unstained. Both tubes were analyzed on the FCM and the results recorded. The individual populations were identified from the data and gated. The gates were used to isolate the individual populations from the mix using FCM sorting. The re-isolated algae were incubated as described above in the test tubes containing their respective mediums for 1 week before being transferred into 250ml round bottom flasks containing 100ml of the respective medium. A 10% inoculum inclusion was used for all experiments. All cultures were grown in triplicate. Samples were removed and analyzed daily for absorbance at 682nm using a Beckman Coulter DU800 spectrophotometer

Ensuring purity and viability of isolates

All pure isolates obtained were streaked onto agar plates and examined microscopically to confirm viability and purity. Once purity and viability were established, they were transferred to modified agar slants and tissue cultures for database maintenance. Filamentous algae were excluded from maintenance due to their characteristics of not satisfying the pre-defined criteria for "biodiesel strains". Original samples in tissue culture flasks were maintained for the length of the study as a reserve.

Selection

Two key criteria were used for the primary selection of isolates for biodiesel production; these were the organism specific growth rate and the qualitative lipid content.

Specific growth rate

Pure isolates were grown in 96 MicroWell^{TM} plates (NUNC, Kamstrupvej, Denmark). A single colony from maintenance slants were transferred to 10 ml of each respective media in a sterile 15 ml test tube, and incubated for 7 days at 25 ± 2°C under constant illumination (Osram® Cool White (L58W/965) fluorescent Lamps) while shaking at 100 RPM on an Inova orbital shaker (New Brunswick, Edison, NJ, USA). Each culture was then transferred at 50 µl aliquots to a micro well containing 150 µl of the respective media; each isolate was evaluated in triplicate. Plates were sealed with sterile NUNC sealing tapes before being incubated for 14 days at 25 ± 2°C under constant illumination (Osram® Cool White (L58W/965) fluorescent Lamps) while shaking at 100 RPM on an Inova orbital shaker (New Brunswick, Edison, NJ, USA). The plate was read twice a day for absorbance at 682nm using a BioTek micro well plate reader. The specific growth rate (µ) was determined from OD 682 nm measurements for data points conforming to high linearity ($r^2 > 0.85$) of a plot of ln (OD682 nm) against time (Nori, et al.)

Lipid content

Qualitative lipid assessment of isolates were conducted by fluorescent microscopy using an epoflourescent Olympus B40 microscope (Tokyo,Japan) equipped with an ©Olympus Soft Imaging System (Analy SIS® Five, Life Science. The area of fluorescent lipid (stained with Nile red) was expressed as a percentage of the total cell area. Semi-quantitative lipid analysis was conducted by transferring 2 ml of each sample to 2 ml micro centrifuge tubes and centrifuged at 14 000 rpm for 10 minutes. The supernatant was discarded and the pellet washed twice and re-suspended in clean media. Samples were then dispensed into black 96 micro well plates in 250ul aliquots. Each sample was dispensed in triplicate and the plate read for fluorescence intensity at an excitation of 485/40 nm and an emission of 600/40 nm (Cooksey, et al., 1987) (Elsey, 2006) (Lee, 1998) (Princu, 1990). Nile red (50 µg/ml in acetone) was automatically dispensed to each well (5µl), mixed and read for flourescent intensity, imediately and after 5 minutes. The flourescent intensity was calculated by subtracting the flourescence of the sample before staining and the flourescence intensity of stained media from the flourescent intensity of the stained sample. The intensity was used against a precalibarated curve of triolein standards to indicate the lipid content in ug/ml (Cooksey, et al., 1987) (Elsey, 2006) (Lee, 1998) (Princu, 1990).

Growth and Lipid production profiles

The best five isolates were inoculated into five 1L round bottom flasks containing 500 ml of its respective media, at 20% inoculum and incubated in an Inova 40 incubator shaker (New Brunswick, Edison, NJ, USA) at 25^{o}C under constant illumination (Osram® Cool White (L58W/965) for 14 days. Samples (5 ml) were taken daily and analyzed for dry cell weights (Maharajh. D), microscopic cell counts using a Thoma counting slide (Hawksley and Sons, London, UK) according to (Monteiro SM, 2005), lipid content using nile fluorescence intensity and absorbance at 683nm as described earlier.

Results

Validation of Validation of flow cytometer cell sorting

Pure populations were identified on the basis of fluorescence and scatter measurements (Figure 1). Chlorophyll auto-fluorescence is detected on the PerCP channel and FSC gives an indication of size. Cells with higher chlorophyll content demonstrate higher PerCP fluorescence. PE measures the fluorescence of NR stained lipids which fluoresce in the yellow spectral region (580 – 620 nm) ((Cooksey, et al., 1987). This fluorescence range is detected on the PE channel. Cells with higher lipid content demonstrate a higher PE fluorescence. Isolate 26 was identified on a dot plot of FSC and PercCP-Cy-5-A (PerCP) as depicted by gate P1 on figure 1A. Here it is clearly demonstrated that the majority of the population in the sample is concentrated within gate P1. As the unstained isolate 26 micrographs showed bright green cells (Fig 2A) and micrographs of the NR stained cells suggested a moderate amount of lipid content (FIG. 4B), a positive identification of isolate 26 could be made on the basis of the FCM data generated on the PerCP/PE dot plots (Figures 1D & G). All surrounding particles were considered to be debris due to their random distribution. Staining the sample with NR resulted in an increase in PE fluorescent intensity (P1 in Figure 1G) - an indication of the presence of lipids within the cell. This was confirmed by fluorescent microscopy (Figure 2B). In Fig. 1G, the appearance of intensely fluorescent PE clusters also became apparent. These clusters were identified as debris which fluoresces intensely when stained with NR. Fluorescent microscopic analysis (data not shown) confirmed this.

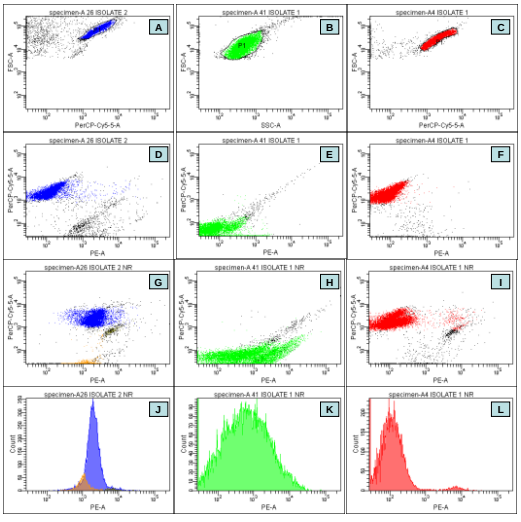

Figure 1: Flow cytometry plots for pure algal isolates.

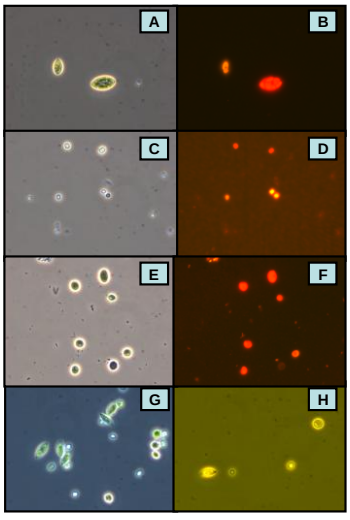

Figure 2: Fluorescent micrographs of mixed and sorted isolates. A & B isolate A26; C & D isolate A 41; E & F isolate A 4; G & H mixed isolates ; A, C, E, G (unstained) B, D, F, G (stained with NR)

Isolate 41 was identified from a plot of FSC and SSC (Figure 1B) as the population was most apparent in this plot. Chlorophyll auto-fluorescence on the PerCP channel was not intense and thus, was not used as a discriminatory factor in gating this population. A micrograph of the unstained isolate (Figure 2C) illustrated algal cells that were less green. This confirmed the lower chlorophyll content demonstrated by the FCM. The fluorescent micrograph of a NR stained sample however, showed intense lipid fluorescence (Fig. 2D). Figure 3H also shows increased fluorescence on the PE channel of the FCM, after staining. Data from both these modalities confirmed the high lipid content of isolate 41. The PE fluorescence after staining shows a wide range of intensities (Figure 1K). This can be explained by the facts that: not all of the cells take up NR at the same rate; each cell has a different lipid content; cells fall in the same range as the debris which also fluoresce intensely when stained with NR. A plot of PerCP and FSC clearly highlighted isolate A4 (Figure 1C). The unstained sample showed negative fluorescence for PE as indicated on figure 1F. After staining however, no increase was observed in PE fluorescence (Figure 1I). This indicated that isolate A4 was not a high lipid producing isolate. The fluorescent micrograph presented in figure 4F substantiates this assumption. Figure 3I shows that the majority of the population in sample A4 is negative for PE fluorescence; however upon staining a small population emerges that is highly positive for PE. The absence of this population prior to staining suggests that this is fluorescent debris.

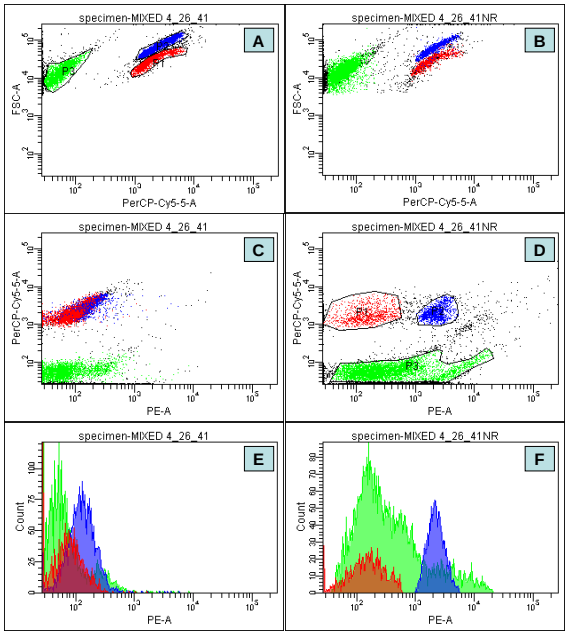

Figure 3: Flow cytometry plots of A26, A41 and A4 mixed sample

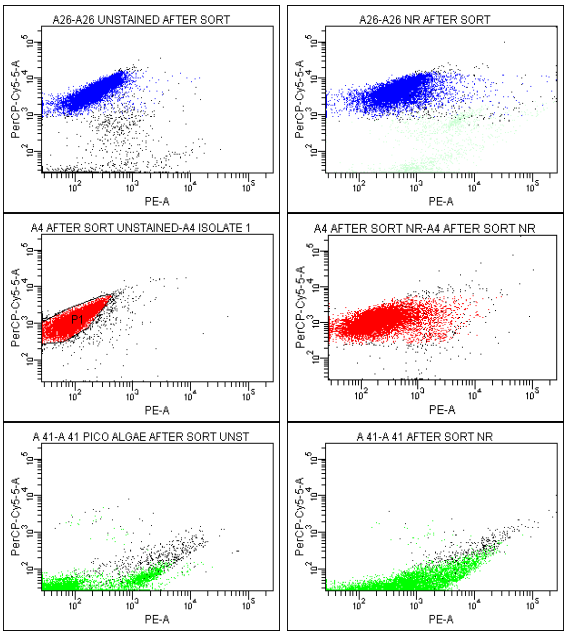

Figure 4: Flow cytometry plots of individual isolates after sorting with FCM

Figure 3 demonstrates FCM data of all isolates after being mixed together. FCM data obtained from the pure isolates (Figure 1) was used to identify relevant populations within the mixed sample. Figure 3A shows populations gated from a plot of FSC and PerCP in an unstained sample. It is apparent from this sample that, although distinguishable, isolates 26 and 4 are extremely close together, making gating and subsequent sorting of the individual populations difficult. On a plot of PerCP and PE, isolates 26 and 4 (Figure 3C) were impossible to separate. After staining with NR however, the three separate populations became apparent and were easily gated and sorted. (Figure 3D). Figure 4 shows the data for each isolate after sorting from the mixed sample.

The A26 target population was successfully sorted into pure culture as observed in figures 4A and B - as well as in figures 2a and B. The PE intensity of the sorted unstained sample has shifted marginally to the right (Fig 4A). This is probably due to the carry over of some NR stained debris and medium from the unsorted sample resulting in false positive lipid fluorescence.

Figures 2C and D show PerCP / PE dot plots for stained and unstained A4 samples respectively. When these plots are compared to Figures 3F and I, it is clear that the populations are the same. It is also apparent that the sorted sample is not contaminated by either A26 or A41 (Figure 3D). This was further confirmed by microscopic examination (Figures 2E & F)
Isolate A41 was also successfully sorted into pure culture from the mixed sample using FCM sorting Figures 4E and F show PerCP / PE plots of stained and unstained samples, respectively, after sorting. The position of the major population is similar when compared to FCM data from pure A41 (Figures 1E and H), however, the PerCP (chlorophyll) intensity has dropped in comparison. This is explained by the possible dilution that occurred during sorting as well as by life cycle changes of the culture. Pure isolates were in culture for ~ 7days (before being mixed and sorted on the FCM) and could have reached limitation of certain nutrients. The transfer to new media with excess nutrients (after sorting) could have triggered a change in growth stage from late exponential to lag and thus explain the decrease in chlorophyll content.

Sample collection and enrichment

The data obtained from the flow cytometry cell sorting experiment combined with extensive literature on isolation of algae was used to develop a detailed isolation protocol (Data not presented). The protocol demonstrated a recovery of ~94% of isolates initially viewed microscopically in environmental samples, which proved the efficiency of the developed method. A total of 46 samples were collected and subjected to the developed isolation protocol. Figure 5 demonstrates the locations of each sample. The most utilized method for isolating microalgae is serial streak plating. Our study revealed that not all isolates are capable of growth on solid media and some grow synergistically in liquid medium and cannot be separated by traditional means (Gerloff, et al., 1950) demonstrated that certain types of algae have specific growth characteristics which allow them to congregate into pure populations within a mixed culture. These congregations are easily removed using mechanical separation. The isolation method developed by Biosciences included various methods, such as streak plating, selective culturing and flow cytometry cell sorting.

Figure 5: Map of sampling sites.

Microscopic evaluation revealed the presence of 123 potential isolates, 52 of which were unicellular (42%), 26 were diatoms (21%) and 43 were filamentous (35%). Figure 6 demonstrates the typical composition of an enriched environmental sample; sample A13 indicates the presence of potentially 4 isolates. Filamentous algae have been identified as algae that are unsuitable for biodiesel production due

to its generally low lipid production capability; however the sample contains potentially 3 isolates of value to the project.

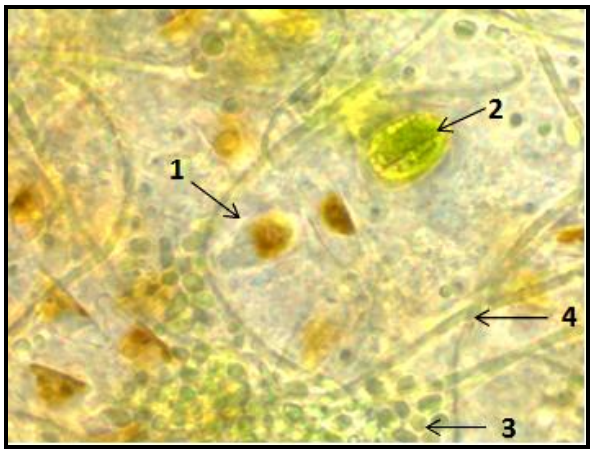

Figure 6 Micrograph of a typical sample (A13) containing 3 potential isolates.

The 46 samples obtained from aquatic habitats of the Western Cape region subjected to the isolation protocol developed and yielded a total of 115 isolates. The number of isolates obtained from each samples was not standard, with some samples yielding as many as 5 isolates and other samples results in no isolates. Majority of the isolates were successfully isolated through selective culturing (62%), which substantiates previous findings by (Gerloff, et al., 1950) while FCM cell sorting and streak plating yielded 17 and 18% respectively. Three isolates proved extremely difficult to isolate and required a combination of all 3 isolation methods to be isolated.

Isolate Selection

The resultant 115 isolates were evaluated for specific growth rate and qualitative lipid content, which was used to select the 4 most suitable organisms for lipid production. Table 1 below provides a summary of the best 4 isolates from this sample set.

Table 1: Summary of primary screen results of best five isolates

Isolate No.	Growth Rate	Doubling Time	Semi-Qualitative Lipid Analysis
#	(d^{-1})	days	%
A15.2	0.017	1.70	33.1
A11.1	0.008	3.61	8.6
A11.4	0.007	3.90	43.1
A10.3	0.018	1.60	40.2

These five isolates were cultured in large scale to assess the lipid production profiles relative to the growth of the organisms over a period of time. The growth profiles of the organisms, based on microscopic cell counts are presented in figure 7 below.

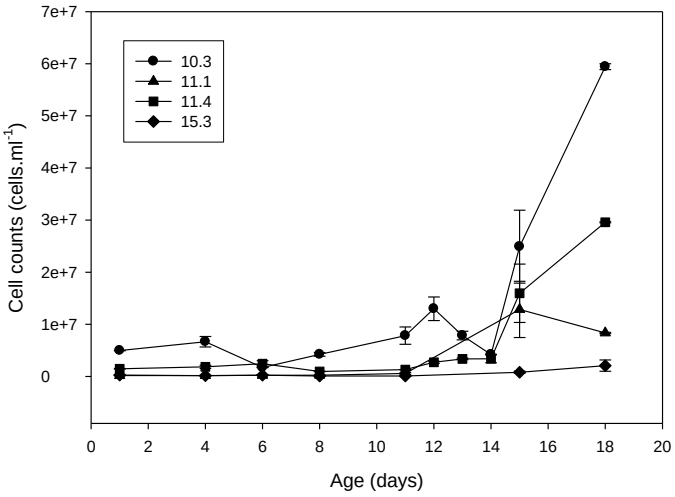

Figure 7: Growth profiles of 4 organisms

Isolates 10.3 and 11.4 grew exceptionally well reached a maximum cell count of ~ 6.0 E07 and 3.0E07 cell/ml respectively. Isolates 11.1 and 15.3 grew sufficiently but did not manage to reach cell concentrations similar to 10.3 and 11.4. Isolate 10.3 demonstrated a notable dip in growth from day 12 and started too increased again after day 14. The drop in cell number could possibly be explained by the fact that nutrient limitation was reach around 14 days due to the faster growth and substantiated by the lack of this phenomenon on other isolates. The later increase in cell number could be attributed to organism releasing some of the growth nutrients as part of a survival mechanism. The increase in lipid concentration coincides with the drop in cell counts for isolate 10.3; this further substantiates the hypothesis of nutrient limitation according (Anderson, et al., 2005).

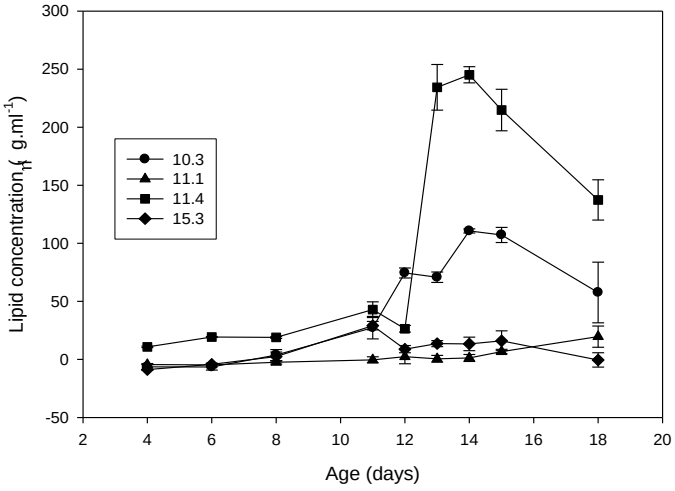

Figure 8: Total lipid production profile of 4 isolates

Figure 8 demonstrates the lipid production of the test isolates, it is apparent that isolate 11.4 and 10.3 have reached the highest respective content. Table 1 presents a summary of the performance of the 4

organism. It is clear here that 11.4 demonstrates excellent potential and requires further investigation. Although isolate 15.2 demonstrated a poor growth rate the lipid content per cell was moderate and requires further investigation to elucidate the production machinery of this organism.

Table 1: Summary of growth and lipid evaluation study.

Organism	Growth rate #	Max lipid concentration (ug/ml)	Lipid productivity (pg/cell)
A10.3	0.2919	111	5.00
A11.1	0.1668	19	0.40
A11.4	0.5223	245	90.00
A15.2	0.1333	16	9.00

Conclusion

The data presented in this paper provides a brief overview of the development of an isolation and screening protocol for microalgae from environmental samples. The developed protocol was successfully demonstrated to be efficient in recovering majority of the potential isolates in a sample. The screening process successfully selected 1 isolate for further investigation. A brief study of the evaluation of 4 isolates shows promise and further studies are currently ongoing to elucidate the lipid production capability and triggers for increased production.

References

Aaronson and Dubinsky Mass production of Microalgae // Experientia 43 (suppl): 44 -105 . - 1982.

Anderson R A and Kawachi M Traditional microalgae isolation techniques: pp 83 - 100 // In: Algal culturing techniques. Editor: Anderson R.A.. - London : Elsevier Academic Press: ISBN: 0-12-088426-7, 2005.

Balfoort H W [et al.] Automatic Identification of Algae: Neural Network Analysis of Flow Cytometric Data // J. Plankton Research. - 1992. - Vols. 14, pp 575-589.

Benemann and Oswald Systems and economic analysis of microalgae ponds for conversion of CO2 to biomass // Final report to the department of energy. - 1996.

Borrego A G [et al.] Comparative petrographic and geochemical study of the Puertollano oil-shale kerogens. // Org. Geochem. 24: 309–321.. - 1996.

Borrowitzka M.A. Closed Algal Photobioreactors: Design Considerations for Large Scale Systems // J Mar. Biotechnol.. - 1996.

Cellamare M, Rolland A and Jacquet S Flow cytometry sorting of freshwater phytoplankton // J Appl Phycol DOI 10. 1007/s 10811 - 009 - 9439 - 4. - [s.l.] : Springer, 2009.

Chisti Y. Biodiesel From Microalgae // Biotechnology Advances. - 2007.

Chisti Y. Microalgae as sustainable cell factories // Environmental Engineering and Management Journal. - 2006. - Vols. Vol.5, No.3. pp 261-274. - http://omicron.ch.tuiasi.ro/EEMJ/.

Cooksey K E [et al.] Fluorometric determination of the neutral lipid content of microalgal cells using nile red. // Journal of Microbiological Methods. 6: pp 333 - 345. - [s.l.] : Elsevier, 1987.

Cunningham A Analysis of Microalgae and Cyanobacteria by Flow Cytometry pp.131 - 142 // In: Flow Cytometry in Mirobiology, ed. D. Lloyd.. - London : Springer Verlag. ISBN: 3-540-19796-6, 1993. - Vols. pp 131-142.

Danielo Olivier An Algae Based Fuel // Biofutur. - Vol:255 : [s.n.], May 2005.

Davey Hazel M. and Kell Douglas B. Flow Cytometry and Cell Sorting of Heterogenous Microbial Populations: The Importance of Single Cell Analyses // Microbiological Reviews. - 1996. - Vols. 60 (4) pp 641 -696.

Davey Hazel M., Jones, Alun., Shaw, Adrian D. and Kell Douglas B. Variable Selection and Multivariate Methods for the Identification of Microorganisms by Flow Cytometry // Cytometry. - 1999. - Vols. 35: pp 162-168.

de la Jara A [et al.] Flow cytometric determination of lipid content in a marine dinoflagellate, Crypthecodinium cohnii // J Appl Phycol 15: pp 433-438. - [s.l.] : Kluwer Academic Publishers, 2003.

Elsey D., Jameson, D., Raleigh, B. & Cooney, J.M. Fluorescent Measurement of Microalgal Neutral Lipids. pp 6, 333-345 // Journal of Microbiological Methods.. - [s.l.] : doi:10.1016/j., 2006 .

Gerloff G C, Fitzgerald G P and Skoog F The Isolation, Purification, and Culture of Blue-Green Algae // American Journal of Botany. - [s.l.] : Botanical Society of America, 1950. - Vols. Vol. 37 , .

Goldie Jenny, Douglas Bob and Furnass Bryan (editors) In Search of Sustainability [Book]. - Collingwood, Australia : CSIRO, 2005. - ISBN: 0 643 09062 2.

Griffiths Melinda J and Harrison Susan T.L. Lipid Productivity as a Key Characteristic for Choosing Algal Species for Biodiesel Production. // J Appl Phycol. - 2009.

Grobbelaar J.U. Physiological and Technological considerations for optimising mass algal cultures // J. Appl. Phycol.. - 2000.

Lee J. S., Yoon, B-D. and Oh, H-M Rapid Method of Determination of Lipid from Green Alga Botryococcus braunii. // Biotechnol techniques.. - 1998.

Lee Y-K. Microalgal Mass Culture Systems and Methods: Their Limitations and Potential. // J Appl. Phycol.. - 2001.

Madigan M T and Martinko J M Methods in microbial ecology. pp 593 - 612 // In: Brock Biology of Microorganisms. - New Jersey, U.S.A : Pearson Prentice Hall: ISBN: 0-13-196893-9, 2006.

Maharajh. D Lalloo. R and Gorgens. J Effect of an exponential feeding regime on the production of Rhodotorula araucariae epoxide hydrolase in Yarrowia lipolytica [Journal] // Letters in Applied Microbiology. - 2008 : [s.n.]. - Vol. 47. - pp. 520-525.

Melamed M.R., Lindmo T and Mendelsohn M.L. (eds) Flow Cytometry and Cell Sorting. - New York : Wiley - Liss, 1994. - Vol. 2 nd edition.

Modovan J.M. and Seifert W.K. First Discovery of Botryococcane in Petroleum // J. S. C. Chem. Comm.. - 1980.

Monteiro SM Clemente JJ, Henriques AO, Gomes RJ, Carrondo MJ,Cunha AE A procedure for high-yield spore production by Bacillus subtilis. [Journal] // Biotechnol Progr . - 2005. - Vol. 21. - pp. 1026–1031.

Princu C.J., Priscu, R.L., Palmisano, C.A. and Sullivan, W.C. Estimation of neutral lipid levels in Antartic sea ice microalgae by Nile red florescence. // Princu, C.J., Priscu, R.L., Palmisano, C.A. and Sullivan, W.C.: Estimation of neutral lipiAntarctic Science. . - 1990.

Pulz O Photobioreactors: Production Systems for phototrophic microorganisms. // Appl. Microbiol. Biotechnol.. - 2001.

Reckermann M Flow Sorting in Aquatic Ecology. In: Aquatic Flow Cytometry: Achievements and Prospects. M. Reckermann and F. Colijn (eds) // Scientia Marina. - 2000. - Vols. 64 (2) PP 235-246.

Shapiro H.M. Practical Flow Cytometry. - New York : Alan R. Liss Inc, 1988 .

Sheehan J [et al.] A Look Back at the U.S. Department of Energy's Aquatic Species Program - Biodiesel From Algae. - 1998.

Sieracki M, Poulton N and Crosbie N Automated Isolation Techniques For Microalgae : pp 101 - 116 // In: Algal Culturing Techniques: Anderson, R.A. ed.. - New York, USA : Elsevier Academic Press. , 2005.