Journal of Environmental Science and Technology

ISSN 1994-7887

science alert

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OPEN ACCESS Journal of Environmental Science and Technology

ISSN 1994-7887
DOI: 10.3923/jest.2019.164.176

Research Article
Studies on the Growth Rate, Oil Yield and Properties of Some Indigenous Freshwater Microalgae Species

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Abstract
Background and Objective: Freshwater bodies within Nsukka, Nigeria had been shown to be rich in indigenous strains of *Desmodesmus armatus*, *Desmodesmus subspicatus*, *Chlorella lewinii*, *Dictyosphaerium* sp. and *Cosmarium* sp. with outstanding growth and bio-oil characteristics. Objective of this study was to undertake studies on the growth rate, oil yield and properties of some indigenous fresh water microalgae species, for biodiesel applications. **Materials and Methods:** Microalgae species were isolated from freshwater pool within Nsukka environs. Two-litre bubble column photobioreactors (PBRs) were used for the cultivation. Growth rate for the autotrophic and mixotrophic were assessed. Chemical flocculation using aluminum sulfate (4 g cm^{-3}) assisted by centrifugation, at 3500 rpm for 15 min was employed for harvesting of the microalgae biomass. Oil was extracted from biomass by means of accelerated solvent extraction (ASE) using Soxhlet extractor with n-hexane. Experimental design in completely randomized design (CRD) was conducted. Data was analyzed using descriptive and inferential statistics with SPSS, version 21. **Results:** Results showed that growth and specific growth rate for the mixotrophic cultivation (i.e., with glucose) were higher than that of the autotrophic (i.e., without glucose). Extraction temperature has significant effect ($p \leq 0.05$) on most of the oil properties evaluated. *Desmodesmus armatus* gave the optimum oil yield of 72.62% at 92.53°C, whereas *Cosmarium* sp. produced the least oil yield of 45.53% at 91.72°C. **Conclusion:** These findings suggested that indigenous freshwater microalgae species from Nigeria have high growth rate, oil yield and promising oil properties that made it a suitable 3rd generation alternative feedstock for biodiesel applications.

Key words: *Desmodesmus* sp., photobioreactor, bio-oil yield, kinematic viscosity, specific growth rate

Citation: Felix Uzochukwu Asoiro, Wilfred Ifeanyi Okonkwo and Nkechinyere Onyekwere Nweze, 2019. Studies on the growth rate, oil yield and properties of some indigenous freshwater microalgae species. J. Environ. Sci. Technol., 12: 164.176.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Current global research is geared towards renewable energy, away from fossil resources. Fossil-based power-generating resources contribute to increase in global carbon footprints (a greenhouse gas) in the atmosphere, global warming, environmental degradation, greenhouse effects, climate change[1] and political instability for some oil producing regions.

Renewable energy is known to ameliorate these challenges. Carbon dioxide (CO_2) and other harmful compounds produced by industries and automobiles can be absorbed by microscopic plants like microalgae[1-3]. Microalgae is a 3rd-generation feedstock with high growth rate (GR), photosynthesis efficiency[4], efficient CO_2 sequestration[2,3] and great capability to be cultivated on marginal agricultural lands, salty water, brackish water, thereby eliminating the food versus fuel conflict.

According to Wu *et al.*[5], oil extraction represents one of the first critical step in biodiesel production from microalgae. Research showed that oil extraction from microalgae is dynamic in nature and complex. Biodiesel quality from microalgae could be positively manipulated by selecting process extraction conditions that favors extraction of oils over optimal extraction conditions thereby producing positive effects on both oil yield and properties[6]. Numerous researches have studied the effects of process conditions on oil yield and oil properties of exotic microalgae species. Liauw *et al.*[7] determined the impact of oil extraction temperatures on acid value (AV) of neem seed oil. Ejim and Kamen[8] investigated the physiochemical characterization of algae oil from Nike lake, Enugu, Nigeria in comparison with other oil seeds. Sanjay *et al.*[9] did a study on the isolation of diatom *Navicula Cryptocephald* and characterization of the extracted oil for biodiesel production. The physico-chemical properties of *cynobacteria* oils for biodiesel production was assessed by Da Ros *et al.*[10]. Song and Shi[11] had conducted a study on the exploitation of oil-bearing microalgae for oil and biodiesel production while Bordoloi *et al.*[12] characterized bio-oil from *Scenedesmus dimorphus* and its sub-fractions. Islam *et al.*[6] investigated the effect of moisture content and temperature on high pressure liquid/oil extraction form microalgae using accelerated solvent extraction (ASE) method. The feasibility of biofuel production form *Chlorella protothecoides* oil with respect to its fuel properties and blending characteristics with petroleum diesel was investigated by Chen *et al.*[13]. Using central composite design with response surface methodology (RSM), Zeb *et al.*[14] also investigated the effects of process conditions (biomass-to-Solvent (BS) ratio, time, temperature) on bio-oil properties and yield from *Saccharina japonrica*, using supercritical solvent extraction means. Rahman and Nahar[15] conducted a study on the production and characterization of algal biodiesel from Spirulina maxima. Stanley *et al.*[16] extracted and characterized microalgae oil. In a review, Kumar and Sharma[17] assessed the potential of microalgae oil for biodiesel production. Kanda *et al.*[18] conducted research on the oil yield and properties of numerous blue-green microalgae species by solvent extraction using dimethyl ether (DME). However, very little information in literature on the growth characteristics as well as the optimum oil extraction temperature, time and oil properties for some indigenous freshwater microalgae species.

Therefore, this study seeks to determine the growth and specific growth rate as well as the effects of temperature and time on oil yield and properties of indigenous fresh water microalgae species.

MATERIALS AND METHODS

Materials: In the research work which lasted 3 years, species of *Desmodesmus armatus*, *Desmodesmus subspicatus*, *Chlorella lewinii*, *Dictyosphaerium* spp. and *Cosmarium* spp. were isolated from Onuiyi, Nsukka, in Nigeria in pools of freshwater, that indicated greenish colour as evidence of microalgae presence, between April and November, 2016 and at the hours of 10 am and 5 pm. Species were identified, subcultured and cultivated at laboratory of the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka. Fifteen bubble column 2 L photobioreactors (PBR) with 1.5 L working volume were used for the cultivation. The PBR systems consist of 6 fluorescent lamps (15 W, 150 V) with continuous illumination of 129 µmol photons m^{-2} sec^{-1}, determined using conversion and calibration factors of 800 lumens and 0.0135, respectively per lamp mounted by the sides and stationed at 20 cm from each other with an aquarium air pump. Chemical flocculation using aluminum sulfate (4 g cm^{-3}) assisted by centrifugation operating at 3500 rpm for 15 min was used for harvesting of the microalgae biomass.

Determination of growth rate (GR) and specific growth rates (SGR): Cell GR is normally determined as change in cell concentration per given period of time. GR was evaluated using the expression (Eq. 1) by Ogbonna[19]:

$$\frac{dX}{dt} = \frac{X_2 - X_1}{t_2 - t_1} \tag{1}$$

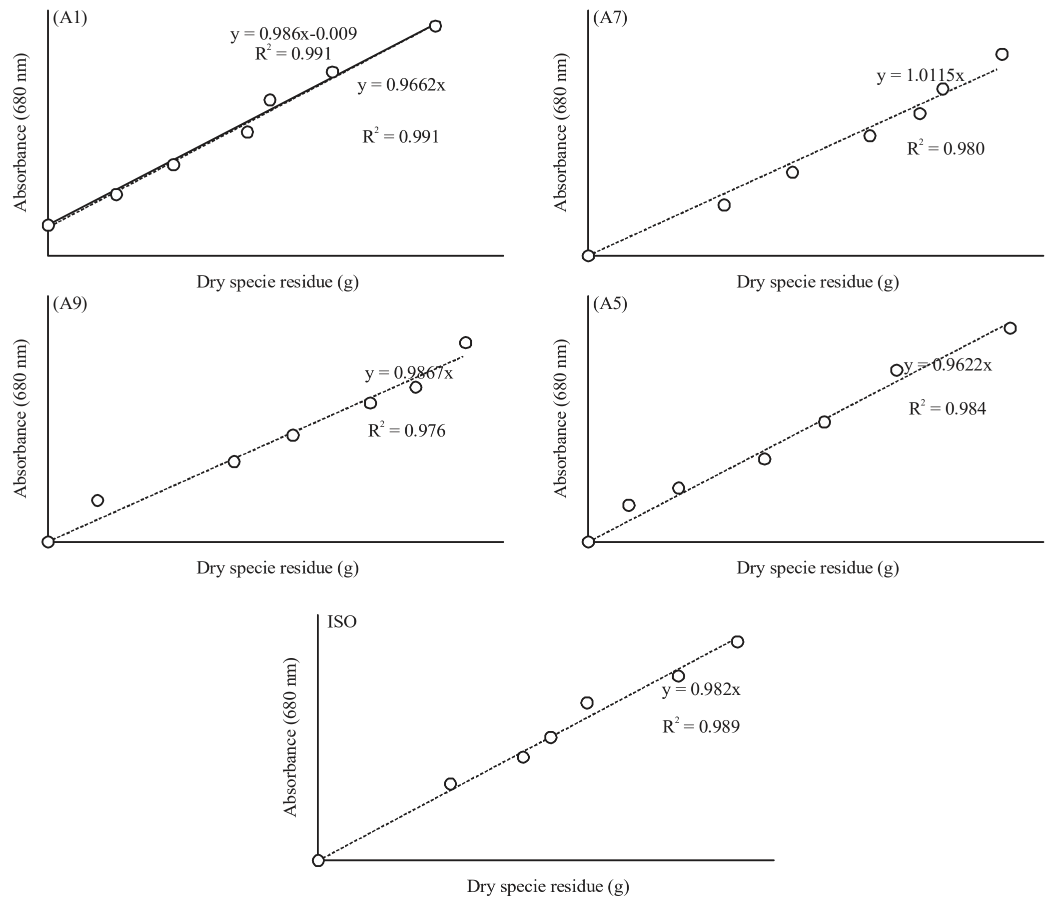

Fig. 1: Standard curve for extrapolating the microalgae cell concentrations (X, g cm^{-3}/day) of (A1) *Desmodesmus armatus*, (A7) *Desmodesmus subspicatus*, (A9) *Chlorella lewinii*, (A5) *Dictyosphaerium* sp. and (ISO) *Cosmarium* sp.

where, X_1 and X_2 are cell concentrations (g cm^{-3}) which were interpolated from the Standard Curve (SC) (Fig. 1) while t_1 and t_2 were the days of cultivation.

The SGR (/day) is the growth rate for individual unit cell. It is the ratio of the cell GR per cell concentration. The SGR (μ) was calculated using Eq. 2 developed by Ogbonna[19]:

$$\mu = \frac{X_2 - X_1}{t_2 - t_1} \times \frac{1}{x} \quad (2)$$

where, x is average cell concentration between t_1 and t_2 which was computed using Eq. 3:

$$\chi = \frac{X_1 + X_2}{2} \quad (3)$$

Determination of oil yield (OY): Oil was extracted from the harvested dried microalgae biomass at temperature ranging from 40 -120°C at 20°C intervals and time ranging from 30-210 min at 30 min intervals by accelerated solvent extraction (ASE) using Soxhlet extractor with normal hexane. OY (% by wt) was evaluated using Eq. 4:

$$OY = 100\left(\frac{M_O}{M_{MA}}\right) \quad (4)$$

where, M_o is mass of oil in grams and M_{MA} is mass of microalgae biomass in grams.

Determination of bio-oil properties: Association of Official Analytical Chemists (AOAC)[20] was employed to investigate the microalgae oil density (OD). The Kinematic viscosity (KV) (centistokes) of the oil was determined by using a kinematic viscometer (Model K21590, Koehler Instrument Company, incorporated, NY, USA)[20]. American Oil Chemists' Society (AOCS)[21] was used to access the acid value (AV) and oleic acid value (OAV) (% by wt) of the extracted oil, using potassium hydroxide and phenolphthalein as indicator. Ash content (AC), iodine values (IV) and saponification value (SV) were determined according to the method by AOAC[20] with slight modification. Hydroxyl value (HV) was determined by Wikipedia. An improvised method by Garba *et al*.[22] was used to evaluate the flash point (FP) (°C). Manual method according to El-Refaie *et al*.[23], Akbar *et al*.[24] and Dijkstra and Van Opstal[25] were used to evaluate the pour point (PP). In determining the cloud point (CP), about 3 cm^3 of the oil was placed into a test tube and kept on an ice bath with a thermometer fixed. The temperature at which the oil begins to condense was recorded as the CP. About 5 cm^3 of the oil was placed in a container with a thermometer kept inside and heated until the first bubble was observed. The temperature at this time gives the measurement of the boiling point (BP). Cetane number (CN) and peroxide values (PV) were evaluated according to Otamiri (Unpublished data) while sulphur content (SC) was analyzed using sulphur analyzer (Model: Asooma T 2000), following American Society for Testing and Material (ASTM) method 4294. The instrument was calibrated in the range of 0.001-1.00 wt % by using commercially available standards. The method by Ejim and Kamen[8] was used in the determination of the moisture content (MC) of the extracted microalgae oil.

Statistical analysis: During cultivation, an experimental design in Completely Randomized Design (CRD) with a total of 30 observations (5 levels of microalgae species ×2 levels of mode of nutrition ×3 replications) was conducted. During oil extraction a 5×7×5 factorial in CRD with a total of 175 observations (5 levels of extraction temperature ×7 levels of extraction time ×5 microalgae species) was carried out. During oil characterization a 5×18×5×3 factorial in CRD with a total of 1350 observations (5 levels of extraction temperature × 8 levels of oil properties ×5 microalgae species ×3 replications) was also conducted. Data was analyzed using descriptive and inferential statistics with SPSS, version 21, Excel package, Windows 10, Prism Graph 6 and Minitab 16. ANOVA, F-test, Duncan multiple range test and LSD.

RESULTS

Growth rate (GR) and specific growth rate (SGR): Table 1 presented the dry weight and absorbance readings of *Desmodesmus armatus*, *Desmodesmus subspicatus*, *Chlorella lewinii*, *Dictyosphaerium* spp. and *Cosmarium* spp. at various dilutions while Table 2 summarized the results of Fig. 1

Table 1: Dry weight and absorbance reading of microalgae cells at different dilutions

	Initial weight of filter paper					Weight of filter paper with microalgae residue					Weight of microalgae residue					Absorbance reading at (λ = 680 nm)				
S/N	A1	A7	A9	A5	ISO	A1	A7	A9	A5	ISO	A1	A7	A9	A5	ISO	A1	A7	A9	A5	ISO
Control	0.781	0.781	0.781	0.781	0.781	0.781	0.781	0.781	0.781	0.781	0.00	0.00	0.00	0.00	0.00	0.000	0.00	0.00	0.00	0.00
1	0.781	0.781	0.781	0.781	0.781	0.901	1.081	0.891	0.871	1.071	0.12	0.30	0.11	0.09	0.29	0.101	0.25	0.20	0.15	0.31
2	0.781	0.781	0.781	0.781	0.781	1.001	1.231	1.191	1.071	1.231	0.22	0.45	0.41	0.20	0.45	0.199	0.41	0.39	0.22	0.42
3	0.781	0.781	0.781	0.781	0.781	1.131	1.401	1.321	1.171	1.291	0.35	0.62	0.54	0.39	0.51	0.305	0.59	0.52	0.34	0.50
4	0.781	0.781	0.781	0.781	0.781	1.171	1.511	1.491	1.301	1.371	0.39	0.73	0.71	0.52	0.59	0.409	0.70	0.68	0.49	0.64
5	0.781	0.781	0.781	0.781	0.781	1.281	1.561	1.591	1.451	1.571	0.50	0.78	0.81	0.68	0.79	0.499	0.82	0.75	0.70	0.75
6	0.781	0.781	0.781	0.781	0.781	1.461	1.691	1.701	1.711	1.701	0.68	0.91	0.92	0.93	0.92	0.651	0.99	0.97	0.87	0.89

A1: *Desmodesmus armatus*, A7: *Desmodesmus subspicatus*, A9: *Chlorella lewinii*, A5: *Dictyosphaerium* sp., ISO: *Cosmarium* sp.

Table 2: Summary of cell concentration of the various microalgae species

Species	Symbol	Absorbance vs dry specie residue	R^2
Desmodesmus armatus	A1	Y = 0.9662x	0.990
Desmodesmus subspicatus	A7	Y = 1.0115x	0.980
Chlorella lewinii	A9	Y = 0.9867x	0.976
Dictyosphaerium sp.	A5	Y = 0.9622x	0.984
Cosmarium sp.	ISO	Y = 0.9822x	0.989

showing the relationship between absorbance and cell concentration of the various microalgae species with very perfect fit (R^2). The GR values (g cm^{-3}/day) and SGR (/day) of the cultivated microalgae species for autotrophic (without glucose) and mixotrophic (with glucose) cultivations are shown in Table 3 and 4, respectively. It was obvious that the GR and SGR for the mixotrophic cultivation (i.e., with glucose) were higher than the other (i.e., without glucose) for the species of microalgae cultivated.

Effects of temperature and time on oil yield (OY) of microalgae species: Table 5 presented the ANOVA for the growth rate of microalgae species. Oil yield of microalgae species at various temperatures and time are presented in Table 6 while 7 Summarized the effects of temperature and time on oil yield from microalgae. Glucose and microalgae species have significant effect ($p \leq 0.05$) on growth rate, with glucose having more effect than microalgae species (Table 5). The grand mean, OY values for *Desmodesmus armatus* (37.82%), *Desmodesmus subspicatus* (35.29%), *Chlorella lewinii* (37.43%) and *Dictyosphaerium* sp. (34.37%) are higher than the average grand mean values for all the microalgae species, except for *Cosmarium* sp. (23.17%) which had relatively lower values than the average grand mean (Table 6). Extraction temperature, time and microalgae species have significant effects on oil yield with temperature having the most effects (Table 7).

Effects of extraction temperature and microalgae species on oil properties: The mean values of the effect of extraction temperature on microalgae oil properties for (*Desmodesmus armatus*, *Desmidesmus subspicatus*, *Chlorella lewinii*, *Dictyospoerium* sp. and *Cosmarium* sp. in Duncan Multiple Range Test (DMRT) format is presented in Table 8. Temperature and microalgae species had significant effects ($p \leq 0.05$) on oil properties (Table 8). All the oil properties investigated were affected by extraction temperature.

DISCUSSION

The SGR for all the individual microalgae species were higher than the GR (Table 3 and 4). All the microalgae species had growth phases. The lag phase lasted 2-6 days. Exponential phase took 6th-8th days. Thereafter the stationary growth phase and the death phase which was due to depletion in nutrient and the presence of some harmful secondary metabolites. Similar trend had earlier been reported by Asoiro and Okonkwo[26].

Table 3: Growth rate for the five different cultivated microalgae species (g cm^{-3}/day)

Days of	*Desmodesmus armatus* (A1)		*Dictyosphaerium* sp. (A5)		*Desmodesmus subspicatus* (A7)		*Chlorella lewinii* (A9)		*Cosmarium* sp. (ISO)	
cultivation	Glucose	Non glucose	Glucose	Non glucose	Glucose	Non glucose	Glucose	Non glucose	Glucose	Non glucose
2nd-4th	0.16715	0.03570	0.03689	0.070671	0.182674	0.005175	0.087159	0.006081	0.022912	0.146130
4th-6th	0.01656	0.05174	0.14134	0.111723	0.024840	0.021217	0.006081	0.028377	0.004582	0.176171
6th-8th	0.00155	0.02432	0.03949	0.045729	0.013972	0.019665	0.048140	0.030404	0.010692	0.013747
8th-10th	0.01190	0.04605	0.04832	0.022864	0.004140	0.053819	0.000507	0.104388	0.122200	0.028513
10th-12th	0.00517	0.13247	0.00155	0.104968	0.085904	0.004140	0.010135	0.025844	0.360489	0.116599
12th-14th	0.50093	0.44038	0.109125	0.334650	0.253053	0.615815	0.050167	0.452518	0.055499	0.323829

Table 4: Specific growth rate for different cultivated microalgae species (/day)

Days of	*Desmodesmus armatus* (A1)		*Dictyosphaerium* sp. (A5)		*Desmodesmus subspicatus* (A7)		*Chlorella lewinii* (A9)		*Cosmarium* sp. (ISO)	
cultivation	Glucose	Non glucose	Glucose	Non glucose	Glucose	Non glucose	Glucose	Non glucose	Glucose	Non glucose
2nd-4th	0.219877	0.09732	0.031968	0.126866	0.17536	0.00947	0.059147	0.015152	0.031142	0.373212
4t-6th	0.017544	0.113895	0.106084	0.151089	0.019884	0.037037	0.003881	0.065116	0.006004	0.24679
6th-8th	0.001614	0.045854	0.026099	0.050985	0.010848	0.03204	0.029697	0.061475	0.013734	0.015211
8th-10th	0.012202	0.076658	0.030185	0.023681	0.00317	0.078313	0.000303	0.165862	0.134078	0.03014
10th-12th	0.005214	0.169987	0.000944	0.096008	0.061527	0.005556	0.006031	0.034023	0.258583	0.10686
12th-14th	0.334254	0.325679	0.061947	0.218305	0.14584	0.451099	0.028821	0.365534	0.030661	0.211436

Table 5: Summary of ANOVA of growth rate of the microalgae species

Parameters	Source of variation	Df	SS	MS	F_{cal}	F_{tab}
Effect of microalgae species on growth rate	Microalgae species (A)	4	0.002	0.005	2.349	2.565
Error	Error	4	0.008	0.002		
Effect of glucose on growth rate	Glucose-fed (B)	1	0.023	0.023	10.962	4.040
Error	Error	1	0.008	0.002		
Effect of the interaction microalgae species and glucose on growth rate	Microalgae species×glucose-fed	4	0.008	0.002	0.098	2.565
Error	Error	50	1.063	0.021		
Total	Total	64	1.112			

Table 6: Oil yield of microalgae species at various extraction temperatures and times

		Time (min)							
	Temperature (°C)	30	60	90	120	150	180	210	Mean
Desmodesmus armatus (A1)	40	0.92^{a}	1.2^{a}	2.06^{b}	2.16^{b}	6.3^{c}	29.6^{d}	0.73^{a}	6.14
	60	49.36^{bc}	49.59^{c}	49.59^{c}	49.118^{b}	49.768^{c}	50.196^{d}	1.38^{a}	42.72
	80	56.66^{d}	54.984^{b}	55.43^{bc}	55.89^{c}	57.152^{d}	57.824^{e}	1.466^{a}	48.49
	100	52.66^{b}	53.17^{c}	53.1^{bc}	55.252^{d}	55.39^{d}	55.27^{d}	2.48^{a}	46.76
	120	52.31^{c}	52.43^{c}	52.77^{c}	50.12^{b}	52.65^{c}	53.49^{d}	1.2586^{a}	45.00
	Mean	42.38	42.27	42.59	42.51	44.25	49.28	1.46	37.82
Desmodesmus subspicatus (A7)	40	0.62^{a}	1.50^{b}	1.76^{b}	1.86^{b}	3.06^{c}	26.42^{d}	0.81^{a}	5.15
	60	46.116^{bc}	46.346^{bc}	46.286^{bc}	45.933^{b}	46.524^{c}	46.952^{d}	1.1^{a}	39.89
	80	53.422^{e}	51.686^{b}	52.132^{c}	52.592^{d}	53.914^{f}	54.526^{g}	0.36^{a}	45.52
	100	49.348^{b}	49.918^{c}	49.848^{c}	52.00^{d}	52.078^{d}	52.018^{d}	0.316^{a}	43.65
	120	48.984^{c}	49.104^{c}	49.444^{c}	46.794^{b}	49.324^{c}	50.904^{d}	1.267^{a}	42.26
	Mean	39.70	39.710	39.890	39.84	40.98	46.16	0.77	35.29
Chlorella lewinii (A9)	40	0.96^{a}	1.744^{a}	2.198^{a}	2.36^{a}	6.320^{b}	25.98^{c}	0.74^{a}	5.76
	60	49.36^{b}	48.59^{b}	49.576^{b}	49.118^{b}	47.568^{b}	50.014^{b}	1.438^{a}	42.24
	80	56.748^{cd}	54.004^{b}	55.03^{bc}	55.89^{cd}	56.032^{cd}	57.03^{d}	1.306^{a}	48.01
	100	52.66^{b}	53.17^{bc}	53.1^{bc}	54.652^{cd}	55.59^{d}	52.67^{b}	2.52^{a}	46.34
	120	51.71^{c}	52.43^{cd}	52.77^{d}	50.12^{b}	52.45^{cd}	52.49^{cd}	1.60^{a}	44.80
	Mean	42.29	41.99	42.54	42.43	43.59	47.64	1.52	37.43
Dictyosphaerium sp. (A5)	40	0.20^{a}	0.746^{ab}	1.47^{bc}	1.69^{c}	5.614^{d}	26.1^{e}	0.127^{a}	5.14
	60	44.646^{bc}	44.876^{bc}	44.876^{bc}	44.404^{b}	45.054^{cd}	45.482^{d}	0.84^{a}	38.60
	80	51.944^{d}	50.268^{b}	50.726^{bc}	51.174^{c}	52.436^{d}	53.108^{e}	1.486^{a}	44.45
	100	47.946^{b}	48.456^{b}	48.386^{b}	50.538^{c}	50.676^{c}	50.556^{c}	1.92^{a}	42.64
	120	47.666^{c}	47.782^{c}	48.122^{c}	45.472^{b}	48.002^{c}	48.842^{d}	1.46^{a}	41.05
	Mean	38.48	38.43	38.72	38.65	40.360	44.82	1.17	34.37
Cosmarium sp. (ISO)	40	0.76^{a}	1.00^{a}	1.70^{a}	1.82^{a}	4.820^{b}	9.202^{c}	0.57^{a}	2.84
	60	28.702^{bc}	29.012^{bcd}	28.924^{bc}	28.527^{b}	29.11^{cd}	29.538^{d}	0.256^{a}	24.87
	80	36.142^{e}	34.398^{b}	34.912^{c}	35.372^{d}	36.674^{f}	37.306^{g}	0.206^{a}	30.72
	100	32.056^{b}	32.566^{c}	32.496^{c}	34.598^{d}	34.786^{d}	34.666^{d}	0.212^{a}	28.77
	120	31.734^{c}	31.854^{cd}	32.194^{d}	39.544^{b}	32.074^{cd}	32.914^{e}	0.135^{a}	28.63
	Mean	25.880	25.770	26.040	27.970	27.490	28.730	0.280	23.17

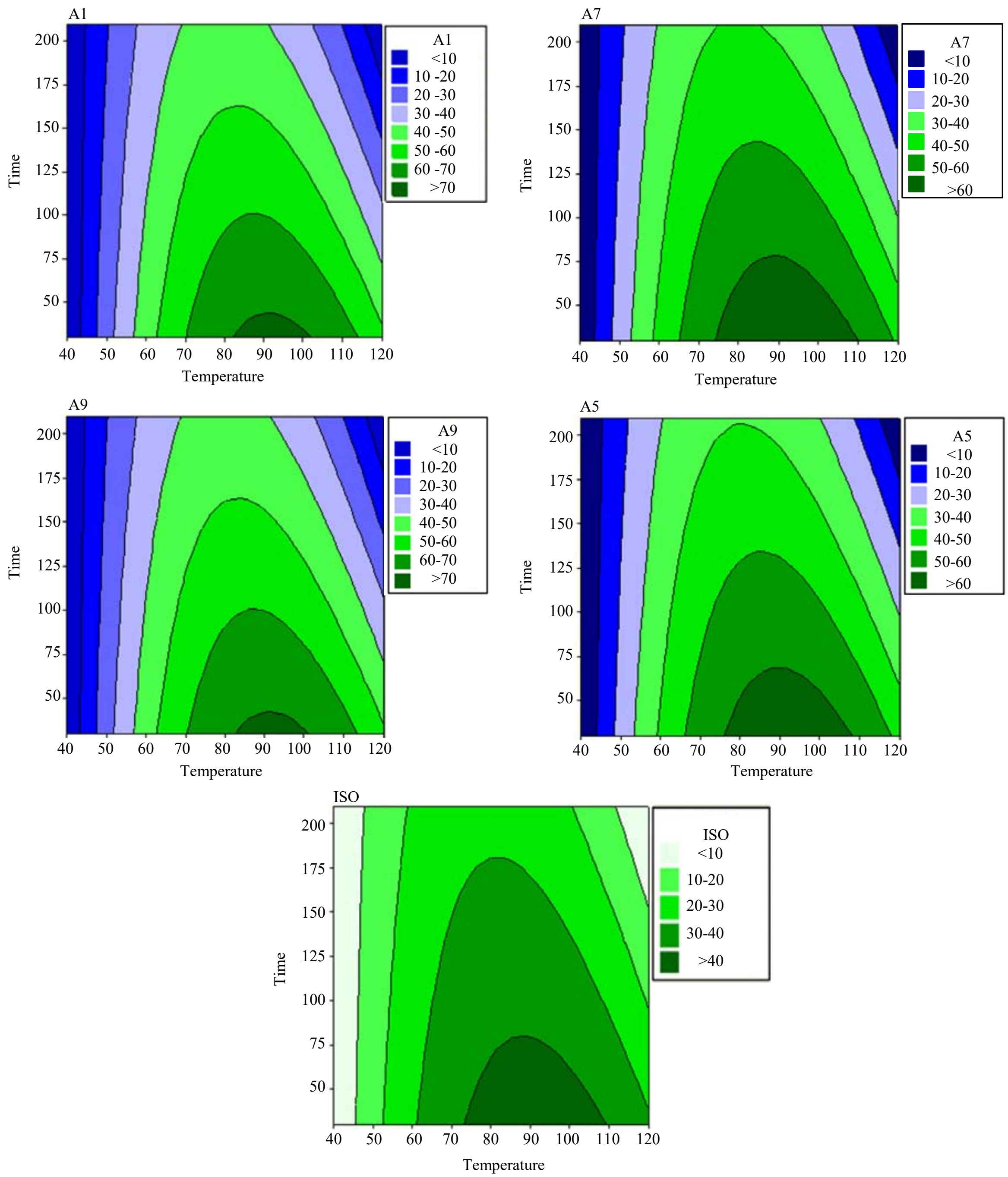

Fig. 2: Contour plots of oil yield, extraction temperature and time for *Desmodesmus armatus* (A1), *Desmodesmus subspicatus* (A7), *Chlorella lewinii* (A9), *Dictyosphaerium* sp. (A5) and *Cosmarium* sp. (ISO)

Extraction temperature and time significantly affected microalgae oil yield and oil properties. *Desmodesmus armatus* gave an optimum oil yield of 72.62% at 92.53°C, whereas *Cosmarium* sp. produced the least oil yield of 45.53% at 91.72°C, after optimization (Fig. 2). The mean oil yield values for all the species were higher than their average mean, except for *Cosmarium* sp. that had relatively lower value (Fig. 3).

The OY of *Desmodesmus armatus* was higher than that of 56.31% from *Chrysophy*, 45.6% from *Chlorella protothecoides* earlier reported by Wang *et al.*[27] and 63.78% from *Chrysophy* and *Chlorella* sp. reported by

Table 7: Summary of the effects of temperature and time on oil yield from microalgae

Parameters	Source of variation	Df	SS	MS	F_{cal}	F_{tab}
Effect of extraction time on oil yield	Time	6	157296.216	26216.036	30600.000	2.09
Effect of extraction temperature on oil yield	Temperature	4	181517.249	45379.312	52970.000	2.37
Effect of microalgae on oil yield	Microalgae	4	26398.366	6599.591	77040.000	2.37
Effect of interaction of extraction time and temperature on oil yield	Time×Temperature	24	36485.635	1520.235	17750.000	1.52
Effect of interaction of extraction time and microalgae on oil yield	Time×Microalgae	24	3985.592	166.066	193.861	1.52
Effect of interaction of extraction temperature and microalgae on oil yield	Temperature×Microalgae	16	4158.129	259.883	303.380	1.68
Effect of interaction of extraction time, temperature and microalgae on oil yield	Time×Temperature×Microalgae	96	1519.204	15.825	18.474	1.00
Error	Error	700	599.638	0.859		
Total	Total	874	411960.027			

Table 8: Mean values of the effect of extraction temperature (°C) on oil properties for *Desmodesmus armatus* (A1)

	Microalgae species/Oil extraction temperature (°C)														
	Desmodesmus armatus (A1)					*Desmodesmus subspicatus* (A7)					*Chlorella lewinii* (A9)				
	Oil extraction temperature (°C)					Oil extraction temperature (°C)					Oil extraction temperature (°C)				
Oil properties	40	60	80	100	120	40	60	80	100	120	40	60	80	100	120
Density (g cm^{-3})	0.92^{a}	0.918^{a}	0.9174^{a}	0.92^{a}	0.924^{a}	0.92^{a}	0.922^{a}	0.921^{a}	0.924^{a}	0.918^{a}	0.922^{a}	0.918^{a}	0.918^{a}	0.922^{a}	0.918$_{a}$
Kinematic viscosity (Cst)	27.56^{a}	28.254^{a}	28.306^{a}	27.421^{a}	27.96^{a}	27.56^{a}	28.654^{ab}	28.706^{ab}	27.821^{a}	30.661^{b}	27.58^{a}	28.274^{a}	28.326^{a}	27.441^{a}	30.083$_{a}$
Acid value (mg KOH g^{-1})	0.561^{a}	0.561^{a}	0.673^{b}	0.900^{c}	0.565^{a}	0.561^{a}	0.565^{a}	0.677^{b}	0.899^{c}	0.899^{c}	0.563^{a}	0.563^{a}	0.675^{b}	0.897^{c}	0.897$_{c}$
Oleic acid value (mg KOH g^{-1})	0.281^{a}	0.281^{a}	0.337^{b}	0.449^{c}	0.285^{a}	0.281^{a}	0.285^{a}	0.341^{b}	0.453^{c}	0.453^{c}	0.301^{a}	0.301^{a}	0.357^{a}	0.469^{b}	0.469$_{b}$
Ash content (%)	0.0054^{a}	0.0062^{ab}	0.0065^{b}	0.0064^{b}	0.0058^{a}	0.0054^{a}	0.0066^{ab}	0.0069^{ab}	0.0068^{ab}	0.0080^{b}	0.0056^{a}	0.0064^{a}	0.0067^{a}	0.0066^{a}	0.0078$_{a}$
Iodine value (g/100 g)	119.7^{c}	119.32^{b}	119.5^{bc}	118.68^{a}	119.74^{c}	119.7^{c}	119.36^{b}	119.54^{bc}	118.72^{a}	118.64^{a}	119.72^{bc}	119.34^{b}	119.52^{b}	118.7^{a}	118.62$_{a}$
Hydroxyl value (g/mole)	0.21^{d}	0.162^{bc}	0.18^{cd}	0.13^{ab}	0.214^{b}	0.21^{d}	0.166^{ab}	0.182^{ab}	0.13^{a}	0.15^{a}	0.212^{d}	0.164^{bc}	0.182^{cd}	0.13^{ab}	0.112$_{a}$
Saponification value (mg KOH g^{-1})	178.58^{d}	173.91^{c}	173.91^{c}	170.16^{b}	178.78^{d}	178.58^{d}	174.11^{c}	174.11^{c}	170.36^{b}	166.63^{a}	178.604^{d}	173.93^{c}	173.93^{c}	170.18^{b}	166.65$_{a}$
Flash point (°C)	221^{a}	220^{a}	220^{a}	221^{a}	221.4^{a}	221^{a}	220.6^{a}	220.4^{a}	221.4^{a}	226^{b}	221.2^{a}	220.2^{a}	220.2^{a}	221.2^{a}	225.8$_{b}$
Pour point (°C)	-2^{a}	-2^{a}	-2^{a}	-2^{a}	-2.04^{a}	-2^{a}	-2.04^{a}	-2.04^{a}	-2.04^{a}	-2.04^{a}	-2.02^{a}	-2.02^{a}	-2.02^{a}	-2.02^{a}	-2.02$_{a}$
Cloud point (°C)	-8^{a}	-9^{a}	-8^{a}	-8^{a}	-8.4^{a}	-8^{a}	-9.4^{a}	-8.4^{a}	-8.4^{a}	-8.52^{a}	-8.2^{a}	-9.2^{a}	-8.2^{a}	-8.2^{a}	-8.32$_{a}$
Fire point (°C)	241.6^{b}	239.74^{a}	241^{ab}	240^{ab}	241.64^{a}	241.6^{b}	239.78^{a}	241.4^{a}	240.4^{a}	240.4^{a}	241.8^{b}	239.94^{a}	241^{ab}	240.2^{ab}	240.2$_{ab}$
Boiling point (°C)	24^{a}	36^{b}	54^{c}	52^{c}	24.04^{a}	24^{a}	36.04^{b}	54.04^{c}	52.04^{c}	68.04^{d}	24.2^{a}	36.2^{b}	54.2^{c}	52.2^{c}	68.2$_{d}$
Heating/calorific value (33300) (kJ kg^{-1})	1.001^{a}	1.002^{d}	1.003^{e}	1.002^{b}	1.002^{a}	1.001^{a}	1.002^{d}	1.003^{e}	1.002^{b}	1.002^{c}	1.002^{a}	1.002^{d}	1.003^{e}	1.002^{b}	1.002$_{c}$
Cetane number	49.73^{a}	50.82^{b}	50.78^{b}	51.67^{c}	50.13^{a}	49.73^{a}	51.22^{ab}	51.18^{ab}	52.07^{bc}	53.06^{c}	49.93^{a}	51.02^{b}	50.98^{b}	51.87^{c}	52.66$_{d}$
Sulfur content (mg kg^{-1})	1.397^{a}	1.44^{a}	1.385^{a}	1.318^{a}	1.437^{a}	1.397^{a}	1.48^{a}	1.425^{a}	1.318^{a}	1.327^{a}	1.399^{a}	1.46^{a}	1.387^{a}	1.318^{a}	1.327$_{a}$
Peroxide value (mg kg^{-1})	2.863^{e}	2.783^{c}	2.766^{b}	2.806^{d}	2.903^{b}	2.863^{e}	2.823^{b}	2.806^{b}	2.826^{b}	2.458^{a}	2.865^{d}	2.787^{bc}	2.768^{b}	2.808^{c}	2.438$_{a}$
Moisture content (% wet basis)	0.513^{d}	0.449^{c}	0.368^{a}	0.427^{b}	0.553^{b}	0.513^{d}	0.489^{ab}	0.388^{a}	0.467^{ab}	0.457^{ab}	0.515^{d}	0.451^{c}	0.37^{a}	0.429^{b}	0.419^{b}

Table 8: Continue

	Microalgae species/Oil extraction temperature (°C)									
	Dictyosphaerium sp. (A5)					*Cosmarium* sp. (ISO)				
	Oil extraction temperature (°C)					Oil extraction temperature (°C)				
Oil properties	40	60	80	100	120	40	60	80	100	120
Density (g cm^{-3})	0.926^a	0.919^a	0.918^a	0.924^a	0.919^a	0.928^a	0.919^a	0.918^a	0.928^a	0.919^a
Kinematic viscosity (Cst)	28.16^a	28.854^a	28.906^a	28.021^a	30.663^a	28.36^a	29.054^a	29.106^a	28.221^a	30.863^a
Acid value (mg KOH g^{-1})	0.567^a	0.567^a	0.674^b	0.903^c	0.903^c	0.569^a	0.569^a	0.681^b	0.905^c	0.905^c
Oleic acid value (mg KOH g^{-1})	0.287^a	0.287^a	0.343^b	0.455^c	0.455^c	0.361^a	0.361^a	0.417^a	0.529^a	0.529^a
Ash content (%)	0.0055^a	0.0063^{ab}	0.0066^b	0.0065^b	0.0077^c	0.0055^a	0.0063^{ab}	0.0066^b	0.0065^b	0.0077^c
Iodine value (g/100 g)	119.76^a	119.52^a	119.7^a	119.28^a	119.2^a	119.78^c	119.4^b	119.58^{bc}	118.76^a	118.68^a
Hydroxyl value (g/mole)	0.216^a	0.168^a	0.186^a	0.13^a	0.17^a	0.29^a	0.242^a	0.26^a	0.13^a	0.19^a
Saponification value (mg KOH g^{-1})	178.664^d	173.97^c	173.97^c	170.22^b	166.49^a	178.66^d	173.99^c	173.33^c	170.24^b	166.51^a
Flash point (°C)	221.60^a	220.6^a	220.6^a	221.6^a	226.6^b	221.8^a	220.8^a	220.8^a	221.8^a	226.4^b
Pour point (°C)	-2.06^a	-2.06^a	-2.06^a	-2.2^a	-2.06^a	-2.08^a	-2.08^a	-2.08^a	-2.08^a	-2.08^a
Cloud point (°C)	-8.06^a	-9.06^a	-8.06^a	-8.06^a	-8.18^a	-8.8^a	-9.8^a	-8.8^a	-8.8^a	-8.92^a
Fire point (°C)	241.66^a	239.8^a	241.06^a	240.06^a	240.06^a	242.4^a	240.54^a	241.8^a	240.8^a	240.8^a
Boiling point (°C)	24.60^a	36.6^b	54.6^c	52.6^c	68.6^d	24.8^a	36.8^b	54.8^c	52.8^c	68.8^d
Heating/calorific value (33300) (kJ kg^{-1})	1.002^a	1.002^c	1.003^d	1.002^b	1.002^b	1.002^a	1.002^c	1.003^d	1.002^b	1.002^b
Cetane number	50.33^a	51.42^{bc}	51.38^{bc}	52.27^{bc}	53.06^c	49.81^a	50.9^b	50.86^b	51.75^c	52.54^c
Sulfur content (mg kg^{-1})	1.398^a	1.446^a	1.386^a	1.318^a	1.33^a	1.398^a	1.448^a	1.386^a	1.318^a	1.327^a
Peroxide value (mg kg^{-1})	2.923^b	2.843^b	2.826^b	2.866^b	2.478^a	2.943^b	2.863^b	2.846^b	2.886^b	2.498^a
Moisture content (wet basis (%))	0.513^e	0.450^d	0.369^a	0.428^c	0.418^b	0.514^a	0.450^b	0.369^c	0.428^d	0.418^e

Mean values with different letters in the same column are significant, mean values with the same letters in the same column are not significantly different

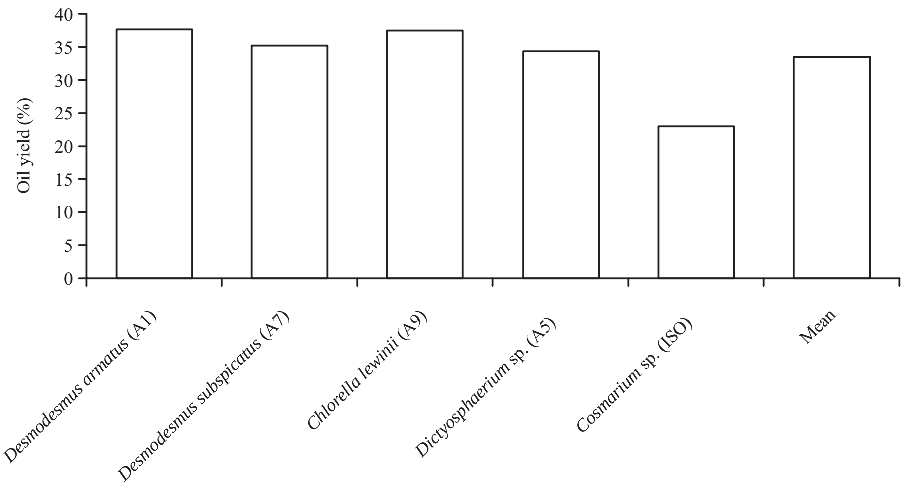

Fig. 3: Oil yield (grand mean) from the various microalgae species at different extraction temperatures and times

Zhou *et al.*[28]. Oil yield of *Desmodesmus subspicatus* (54.5%) was slightly better than that *Chlorella vulgariss* (52.5%) reported by Araujo *et al.*[29]. However, *Desmodesmus subspicatus* maximum OY was slightly lower than the value of 65.2% recorded for *Chlorella protothecoides*[14]. The maximum OY for *Chlorella lewinii* (57.03%) at 80°C after extraction time of 180 min was clearly within the range of values of total recovered oil (14-76%) for four different species of microalgae (*Isochrysis galbana*, *Nannochloropsis gaditana*, *Nannochloropsis* sp. and *Phaeodactylum tricornutum*) earlier reported by Ryckebosch *et al.* [30], depending on type of species and solvent used. Seo *et al.*[31] had earlier reported a maximum OY of 30% for microalgae enhance with florescent-painted lighting source while Drira *et al.*[32] reported a fourfold oil yield for microalgae cultivated in a PBR subjected to osmotic stress.

The maximum oil yield of 53.108% for *Dictyosphaerium* sp. at temperature of 80°C after extraction time of 180 min was very high, compared to Shankar *et al.*[33], who had earlier reported a 1.86 and 1.72 folds higher OY for *Chlorella* and *Chlorococcum* sp. respectively, using protic ionic liquid assisted cell disruption method as against the conventional (traditional) solvent extraction method. This value was also higher than the OY value of 33.9% for microalgae at 40°C and 35 Mpa reported by Tang *et al.*[34] using supercritical CO_2 extraction ($SCCO_2$).

Despite the generally low oil content of *Cosmarium* sp. compared to the four other species studied, maximum OY of 39.544% was obtained at 120°C after 120 min of extraction time. This value was clearly higher than the average oil yield of 5.8% reported by Pohndorf *et al.*[35] (2016) for *Spirulina* sp. and also higher than the value of 0.0346% reported for Nannochloropsis sp. by Pradana *et al.*[36].

The OD values from the results were in line with the value (0.92 g cm^3) earlier reported by Kumar and Sharma[17]. The OD results also showed that microalgae oil was denser than the oil of fresh water microalgae specie isolated from Nike lake Enugu, Nigeria (0.892 g cm^{-3})[8], less dense than the value of 1.305 g cm^{-3} earlier reported by Stanley *et al.*[16] for marine microalgae. The KV values at 5 temperature levels for the microalgae ranged between 27.421-30.863 cSt at temperature range of 40-120°C. This range of values was lower than the values of 59.1, 62.3 and 52.7 cSt for *M. aeruginosa*, *Trichormus* sp. and *Synechococcus* sp., respectively reported by Da Ros *et al.*[10], slightly lower than the value of 39 Centistoke (cSt) internationally accepted for biodiesel oil and less viscous (33.06 cSt) than the values reported by Kumar and Sharma[17] and Chen *et al.*[13] at an extraction temperature of 40°C. The KV values for the microalgae species were quite more than the ranges for the nonrenewable fossil fuel, which was 2-4.5 cSt at 40°C[37], higher than the values of 6.65 cSt reported for neem seed oil[38] and lower than the range of values of 41.52 to 47.83 cSt, at temperature range of 40-80°C, reported for *Jatropha curcas* L. by Asoiro and Akubuo[39]. The KV of the oil of the microalgae species were found to be lower than those of rapeseed oil (45.01 cSt) as reported by Lang *et al.*[40] and coconut oil at room temperature (43.30 cSt) as observed by Alamu *et al.*[41]. However, oil samples from some of the microalgae species were relatively greater than values reported by Kumar *et al.*[42] for coconut oil (27.23 cSt) and linseed oil (22.4 cSt), documented by Belewu *et al.*[43] and Lang *et al.*[40] respectively.

Very high KV creates injector and engine problems as a result of poor oil feeding and some deposit which could be ameliorated by transesterification and by blending. The low KV values of microalgae revealed that it is quite suitable for biodiesel purposes, even without blend with other fuel types.

The AVs at the different temperatures were generally lower than that of coconut oil (9.537 mg KOH g^{-1}) and groundnut oil (3.82 mg KOH g^{-1}) as earlier documented by Opoku-Boahen *et al.*[44], lower than palm kernel seed oil (0.834 mg KOH g^{-1}) and almond oil (0.770 mg KOH g^{-1}) as reported by Afolabi[45]. Similarly, the values were also lower than that reported by Kumar *et al.*[42] for coconut oil (2.1 mg KOH g^{-1}) but slightly higher than that of castor seed oil (0.279 mg KOH g^{-1}) *Desmodesmus armatus* recorded the least AC (0.0054%) at 40°C. *Desmodesmus subspicatus* oil had the highest AC value of 0.0080% at 120°C.

The mean IV at all the 5 temperature levels for the microalgae species was the same as the value (119.1 g I_2/100 g) earlier presented by Kumar and Sharma[17], slightly above the value of 112.2 g I_2/100 g reported for *Chlorella protothecoides* oil by Chen *et al.*[13] and value range of 57-100 g I_2/100 g for Cyanobacteria earlier reported by Da Ros *et al.*[10], However all the IVs were within the allowable international and European Standard limit, UNE-EN 14214 (100-200g/100g) for iodine in bio-oil products used for biodiesel purposes.

HV of the oil at 40 and 120°C extraction temperatures were not significant. Equally, 40 and 100°C were also not significant.

Virtually all the species of the microalgae at different temperatures had low SV (166.43-178.784 mg KOH g^{-1}) but slightly higher than the value of 152 mg KOH g^{-1} reported by Kumar and Sharma[17]. This was clearly in conformity with the value (173.56 mg KOH g^{-1}) earlier reported by Stanley *et al.*[16] for marine microalgae. However, SV of microalgae investigated were relatively low compared to the slightly high value range (203-213 mg KOH g^{-1}) earlier reported by Da Ros *et al.*[10] for Cyanobacteria.

The FP values were found to be low, compared to values reported by Kumar *et al.*[42] for coconut oil (266°C) and sunflower oil (274°C) as observed by Shereena and Thangaraj[46] but were higher than those of Babassu (150°C) as reported by Shereena and Thangaraj[46]. The CP values were low, compared to that of peanut oil (12.8°C), palm oil (31°C) and safflower oil (18.3°C) as reported by Shereena and Thangaraj[46]. PP values were much higher than those reported by Kumar *et al.*[42] for coconut (-6°C) and soya bean (12.2°C).

Kumar and Sharma[17] had earlier reported a CV of 41000 KJ kg^{-1} for microalgae oil, different from CV values for *Chlorella lewinii* oil of 33393.41 and 33363.27 at 80 and 40°C, respectively. The CV is an important property of the biodiesel that determines the suitability of the fuel as an alternative diesel fuel[47].

The mean values of CN for the microalgae species were far better than the value (51) reported by Kumar and Shamar[17]. An appropriate cetane number is required for good engine performance. The higher the CN, the better the ignition property as it ensures good cold start properties and minimizes the formation of white smoke[48]. The CN of the oil were generally higher than those of conventional diesel, animal fats and vegetable oils[49].

The PV of the five different species of microalgae oil at various temperatures and times, were generally low but slightly higher than the value of 1±0.007 Meq kg^{-1} reported by Kumar and Sharmar[17]. This low value of the oil shows that the oil is ideal for consumption, storage and other industrial purposes. Earlier, Garba *et al.*[22] had reported a higher value of 7.2 meq g^{-1} for *Jatropha curcal* L. oil.

The MC (%, wb) varied as extraction temperature increased from 40-120°C. This pattern was so for all the microalgae species studied. It was glaring from the investigation that extraction temperature and time had significant effects on both oil yield and oil properties for the indigenous microalgae species studied. It is therefore recommended for future studies that effects of other process parameters on oil yield and properties, like pressure, moisture content, particle size, type of solvent and solvent/ biomass ratio on other microalgae species of indigenous locality should be investigated.

CONCLUSION

Growth rate and specific growth rate for the mixotrophic cultivation (i.e., with glucose) were higher than that of the autotrophic (i.e., without glucose) for the microalgae species investigated. Extraction temperature and time had significant effect on oil yield and properties with largely promising attributes that makes it a suitable 3rd Generation alternative feedstock for biodiesel purposes. *Desmodesmus armatus* gave an optimum oil yield of 72.62% at 92.53°C, whereas *Cosmarium* sp. produced the least oil yield of 45.53% at 91.72°C.

SIGNIFICANCE STATEMENT

This study discovered the potential of indigenous fresh water microalgae species isolated from pool of water in Nsukka, Nigeria, with high growth rate, oil yield and promising oil properties. The oil yield and properties could be optimized by varying process parameters, which would be beneficial as 3rd generation alternative feedstock for the fast-emerging biodiesel industry. This study will help the researchers and industrialists to uncover the critical areas of some process parameters affecting microalgae growth rate, oil yield and oil properties, that many researchers were not able to explore. Thus, a new theory on greener energy may be arrived at.

ACKNOWLEDGMENT

The authors are greatly indebted to the authorities of the Department of Agricultural and Bioresources Engineering and the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka for making their laboratory and facilities available for this research.

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