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Photosynthetic pigment quantification from two green microalgal species of *Scenedesmus*

J. A. Lone[1], F. A. Lone[2], M. R. Suseela[3]

[1,2] Division of Environmental Sciences,
Sher-e-Kashmir University of Agricultural Sciences and Technology-Kashmir, Shalimar, Srinagar, Jammu and Kashmir, 190 025, India.
[1,3] Algology Section, Conservation Biology and Biodiversity Division,
National Botanical Research Institute, Lucknow, Utter Pradesh 226 001, India.

ABSTRACT

The chlorophyll extraction from two fresh water green microalgal speces of Scenedesmus was performed using Dimethyl sulfoxide as a solvent. Scenedesmus dimorphus and Scenedesmus quadricauda were isolated from the fresh waters of Dal lake Kashmir, Himalaya. The growth analysis pattern of these two robust algae in the BBM media showed that both the species are fast growing and reached a stationary phase on 14th day of incubation and are also suitable for large scale biomass production. Dimethyl sulfoide (DMSO) is regarded as a reliable solvent for extracting chlorophyll, however modification of standard methods is necessary for a particular species. The photosynthetic pigments (Chl-a, Chl-b, Total Chl, Total Carotenoids) of these two microalge were calculated using two set of equations viz., Arnon's formulas and Wellburn equations. The high amount of total pigments (mg g^{-1} fw) were calculated when Wellburn equations were applied (S. dimorphus = 19.806 and S. quadricauda = 27.099) and Arnon's equations (S. dimorphus = 7.274, S. quadricauda = 9.713) were found to be inefficient in calculating the photosynthetic pigments.

Key words: *Dal Lake, Microalgae, Scenedesmus dimorphus, Scenedesmus quadricauda, Chlorophyll.*

1. INTRODUCTION

Microalgae are microscopic organisms capable of converting solar energy into food energy via photosynthesis. They have enormous economic implications, not only as primary producers and pollution indicators [1] but also as a source of several natural products, biofertilizers and fine chemicals. They are an inseparable associate of environment and also help in the purification of the environment. The early accumulation of oxygen in the earth's atmosphere was due to photosynthesis of ancient algal forms. It is estimated that, the algal photosynthesis contributes nearly 90 per cent of oxygen release in the earth's atmosphere. Globally algae are considered to fix 50 per cent of CO_2 and are the primary producers in aquatic habitat supporting rich food chains

and oxygenate the aquatic systems [2]. The photosynthesis in algae is similar to that found in all plants, but algae are especially effective in converting carbon dioxide and other nutrients into organic compounds. Microalgae are usually cultivated in open ponds or photobioreactors. The cultivation process requires carbon dioxide, light, water and other nutrients which facilitate the photosynthetic process. This cultivation captures greenhouse gas and simultaneously produces biomass containing high-value consumer products [3].

Chlorophyll is one of the most valuable bioactive compounds that can be extracted from microalgal biomass. Several methods have been employed by the researchers to extract chlorophyll form the plant biomass. DMSO has also been widely used by many researchers to extract chlorophyll from the plants viz [4,5,6,7,8,9,10] etc. Our primary objective was also to extract photosynthetic pigments from two fresh water green microalgae *Scenedesmus dimorphus* and *Scenedesmus quadricauada* using DMSO as a solvent by employing two different extaction methods.

2. MATERIALS AND METHODS

The two green microalgae *Scenedesmus dimorphus* and *Scenedesmus quadricauda* were isolated from the fresh water Dal lake of Kashmir Himalaya, as per different Isolation techniques (Culture enrichment, Streaking, Inoculation**)** given by Kaushik (1987) [11]. The identification of microalgae was carried out by using advanced microscope (LEICA DM 500, U.K) connected with computer having digital image analyzer and software (LAS EZ 1.8.0). The identification of the microalgae was also authenticated based upon standard keys given by Tiffany [12]; Prescott [13]; Phillipose [14] etc. for morphological characteristics.

2.1 Growth measurement

Six Erlenmeyer flasks (500ml capacity) were arranged in two series, each containing 250 ml BBM media [15,16] and 12 per cent (30 ml) inoculum. These flasks were arranged in two series, each series containing three flasks. The first series was containing the inoculum of *Scenedesmus dimorphus* and the second was containing the inoculum of *Scenedesmus quadricauda.*

3 ml culture was taken from the flask in the first cuvette and 3 ml BBM media was used as blank in second cuvette. The maximum absorbance was inspected by scanning a culture sample between 400 and 1100 nm using double beam UV Vis spectrophotometer (Chemito Spectrascan UV 2700, Thermo Scientific,) loaded with Spectrum PC software. The highest absorbance peak value was then used to measure the optical density.

Growth rate of cultures was determined by measuring the optical density (OD_{680nm}) after every 24 hours at 11:00 am. For the measurement of OD, 3 ml culture was drawn from the flask and BBM media was used as a blank. OD was measured at 680 nm as per the scanning process initially using a double beam spectrophotometer. The sample cultures were diluted to an OD of less than one, to fall within the linear range of measurement. The actual OD was determined by multiplying the OD value with the dilution factor [17].

2.2 Photosynthetic pigment quantification, Wellburn [9]

Pigments were extracted from algal cells using dimethyl sulphoxide (DMSO 1 ltr=1.10 kg, M 78.13 g mol^{-1}, Purity≥99.8%, Merck). Culture samples (2 ml) were centrifuged in eppendorf tubes at 10,000 rpm for 5 minutes and the supernatant was discarded. Hot (60^{o}C) DMSO (2 ml) was added and cells were resuspended by vortexing. Samples were incubated at 60^{o} C, with occasional shaking, for 10 minutes before centrifuging. The supernatant pigment extract was removed and diluted with DMSO to an OD of less than one. The OD at 649, 665 and 480 nm was determined and the pigment content was calculated using the equations below [9].

Chlorophyll-a (Chl-a) = 12.47 (OD_{665})-3.62 (OD_{649})

Chlorophyll-b (Chl-b) = 25.06 (OD_{649})-6.5 (OD_{665})

Total chlorophyll = Chl-a + Chl-b

Total carotenoids = [1000(OD_{480})-1.29 (Chl-a)-53.78 (Chl-b)]/220

Total pigment mg L^{-1} = Sum of the above

All the measurements were carried out in triplicates. DMSO solution was used as blank and the results were converted into mg g^{-1} fw.

2.3 Photosynthetic pigments estimation, Hiscox and Israelstam [5]

A suitable amount of microalgae was taken in centrifuge tubes and was centrifuged at 10,000 rpm for 10-15 minutes. Now supernatant from the tubes was removed and the microalgae (sediment) were transferred into 25 ml beakers for Lyophilisation in lypholizer for 3 hours. 100 mg of microalgae was weighed and placed in the test tubes. 20 ml of DMSO was added in the same tubes. The tubes were covered with the aluminium foil (to avoid photo- oxidation of pigments) and kept in an oven at 65^{o}C for 5 hours or kept overnight at room temperature. The absorbance of chlorophylls contained in solution at 663, 645, and 630 were recorded. Chl-a, Chl-b, Chl-c and total Chl were calculated by using the formulas given by Arnon [18]. DMSO solution was used as blank and the results were expressed in terms of mg g^{-1} fw.

$$\text{Chorophyll-a (Chl-a)} = \frac{[(12.7\times A_{663}) - (2.69\times A_{645})] \times V}{1000 \times W}$$

$$\text{Chlorophyll-b (Chl-b)} = \frac{[(22.9\times A_{645}) - (4.68\times A_{663})] \times V}{1000 \times W}$$

$$\text{Total Chlorophyll} = \frac{[(8.02\times A_{663}) + (20.2\times A_{645})] \times V}{1000 \times W}$$

$$\textbf{Total Carotenoids} = \frac{A_{480} + [(0.114 \times A_{663}) - (0.638 \times A_{645})] \times V}{1000 \times W}$$

Where A=Absorbance at given wavelength, V= Volume of DMSO and W= Weight of microalgae in milligrams.

3. STATISTICAL ANALYSIS

The measurements of the values were done in triplicates and the mean ± standard deviation (SD), mean ± standard error (SE) were calculated using GraphPad Prism 5 statistical software.

4. RESULTS

4.1 Growth measurement

The maximum absorbance was inspected by scanning a culture sample between 400 and 1100 nm and the highest absorbance peak value obtained at 680 nm was then used to measure the optical density as shown in the Figs. 1 and 2. Therefore, growth of both the species was read in this wavelength. The specific data pertaining to the growth measurements of two tested microalgae *S. dimorphus* and *S. quadricauda* is shown in Fig. 3. During the whole experiment, the measurements of the OD values at 680 nm were done in triplicates and the mean ± standard error (±SE) was calculated and OD values in tabular form were converted into growth curve using GraphPad Prism 5 statistical software. The plot clearly shows distinct phases of a typical growth curve of two microalgae where the growth reached a stationary phase on 14^{th} day of incubation and during the investigations it was found that both the species thrive well in the BBM media. During all the phases of growth curve it has been observed that *S. quadricauda* is slightly fast growing as compared to *S. dimorphus.* Initially the cultures showed gradual growth rate and from the 4^{th} day onwards, both the species had significant increase in total number of cells. As evident by the growth curve, both the species show lag phase of 5 days and on 6^{th} day both the cultures showed signs of exponential phase. During the stationary phase, maximum growth was found in *S. quadricauda* with OD of 3.37 compared with initial reading of 0.084 and minimum growth was found in *S. dimorphus* with OD of 2.97 compared with initial reading of 0.045.

4.2 Estimation of photosynthetic pigments

The photosynthetic pigments of two algal species were determined by DMSO method using two different set of formulae provided by Arnon, [18] and Wellburn, [9]. The comparison of pigment concentration is presented in the Table 1.

By using Wellburn equations total photosynthetic pigments were maximum in *S. quadricauda* (27.099 mg g^{-1}) as compared to *S. dimorphus* (19.806 mg g^{-1}). In case of Arnon formulae, total photosynthetic pigments were also maximum in *S. quadricauda* (9.713 mg g^{-1}) as compared to *S. dimorphus* (7.274 mg g^{-1}).

The results show that by using Wellburn equations maximum chlorophyll-a (16.711 mg g^{-1}), chlorophyll-b (5.447 mg g^{-1}), total chlorophyll (22.158 mg g^{-1}) and total carotenoids (4.941mg g^{-1}) were reported in *S. quadricauda.* On the other hand minimum chlorophyll-a (11.609 mg g^{-1}), chlorophyll-b (4.447 mg g^{-1}), total chlorophyll (16.056 mgg^{-1}) and total carotenoids (3.750 mg g^{-1}) were reported in *S. dimorphus.*

Similarly, maximum chlorophyll-a (8.015 mg g^{-1}), chlorophyll-b (1.168 mg g^{-1}), total chlorophyll (8.701 mg g^{-1}) and total carotenoids (0.530 mg g^{-1}) were reported in *S. quadricauda* and minimum chlorophyll-a (6.008 mg g^{-1}), chlorophyll-b (0.796 mg g^{-1}), total chlorophyll (6.803 mg g^{-1}) and total carotenoids (0.471 mg g^{-1}) were reported in *S. dimorphus* by using Arnon formulae.

Overall in both the tested microalgal species the photosynthetic pigments were reported maximum by applying Wellburn (1994) equations, while as minimum pigments were reported by Arnon (1994) formulae as shown in Fig. 4.

5. DISCUSSION

5.1 Pigment estimation in two microalgae by using different equations

In the present research work DMSO was used as a solvent as compared to acetone because DMSO is superior to acetone for the extraction of chlorophyll from green algae. Shoaf and Lium [4] reported that DMSO is superior to acetone giving 2-60 times more chlorophyll depending on the algal species. Also, as no maceration of algal tissue is required, chances of losing chlorophyll due to filtration step (acetone method) are totally eliminated. Moreover acetone does not extract all the major pigments completely [19].

The extraction of chlorophyll depends upon many factors like solvent type [20], solvent impurity [21], tissue type and degree of maceration [22] and the equations used to calculate Chl- concentration [9,22]. The equations used to calculate Chl- concentration might also influence the results because the absorption spectra of Chl-a and Chl-b are different in different types of equations. Wellburn [9] has presented accurate extinction coefficients and relevant simultaneous equations for use with various solvents including DMSO [22]. However, in the present study two different sets of equations provided by Arnon [18] and Wellburn [9] using DMSO as a solvent were used for calculations. The data revealed that the maximum photosynthetic pigments were estimated with Wellburn equations and minimum pigments were calculated with Arnon's formulas. Our results are in conformity with the results of Wellburn [9] and Tait and Hik [10]. The much-quoted equations of Arnon [18] to determine individual levels of Chl-a and Chl-b in 80 per cent acetone in water are still used by many researchers despite the fact that they are inaccurate and that particular solvent mix has many disadvantages [9]. The Arnon's equations will give inaccurate results for DMSO [23] and several studies have modified Arnon's equations and most importantly Wellburn [9] provided equations that give accurate results. There are many reasons for this inaccuracy including the poor resolution of the spectrophotometers of the 1940's but the main problem is the

solvent itself.

Chlorophyll content decides the photosynthetic rate of a particular strain. Changes in chlorophyll level are probably controlled as algal density and climatic factors (light and temperature). Photosynthetic efficiency of microalgae is directly related to its growth and hence production also. Using the Wellburn equations in our study the Chl-a content in *S. quadricauda* was reported to be 16.711 mg g^{-1}, while as in *S. dimorphus* Chl a was found to be 11.609 mg g^{-1}. The Chl-a content was highest in both the species, as the photo-systems in tested microalgae contain Chl-a. However Chl-b also augments the overall fluorescent signal and enables green algae to perform the photosynthesis. Our results are well supported by [24] who reported the Chlorophyll content of 27.99 mg g^{-1} in *C. vulgaris* under photoautotrophic nutritional modes. Carotenoids are important components of photosynthetic apparatus of vegetative cells serving as additional pigments [25]. They protect chlorophyll molecules against photo destruction and oxidation by molecular oxygen [26]. Appreciable amounts of carotenoids were also present in both the microalgal species and the total carotenoids in *S. quadricauda* and *S. dimorphus* were reported 4.941 and 3.750 mg g^{-1} respectively. The total photosynthetic pigments in *S. quadricauda* and *S. dimorphus* were 27.099 and 19.806 mg g^{-1} respectively. Shaaban *et al.* [27] also found the total chlorophyll contents of the identified algae during summer 2007 from 9.66 to 23.0 mg L^{-1} respectively comparing with the total chlorophyll contents in other seasons using DMSO solvent. The Chl-values of Shaaban *et al.* [27] support our pigment values. In our study both the microalgal species possess appreciable amounts of photosynthetic pigments which is due to the fact and obvious due to its enormous growth as shown in its growth curve (Fig.3).

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Table-1: Chlorophyll estimation (mg g^{-1} fw) in two microalgae by using two different set of equations

Microalgae	(Chl-a)	(Chl-b)	Total Chl	Total Carotenoids	Total pigment
Chlorophyll concentration as estimated by Wellburn equations, 1994:					
S. dimorphus (±SD)	11.609 (±0.131)	4.447 (±0.042)	16.056 (±0.173)	3.750 (±0.109)	19.806 (±0.064)
S. quadricauda (±SD)	16.711 (±0.0891)	5.447 (±0.1181)	22.158 (±0.0297)	4.941 (±0.0212)	27.099 (±0.0085)
Chlorophyll concentration as estimated by Arnon's formulae, 1949**:**					
S. dimorphus (±SD)	6.008 (±0.625)	0.796 (±0.476)	6.803 (±1.100)	0.471 (±0.008)	7.274 (±1.109)
S. quadricauda (±SD)	8.015 (±0.0262)	1.168 (±0.597)	8.701 (±0.110)	0.530 (±0.009)	9.713 (±0.561)

(±SD) = Standard deviation

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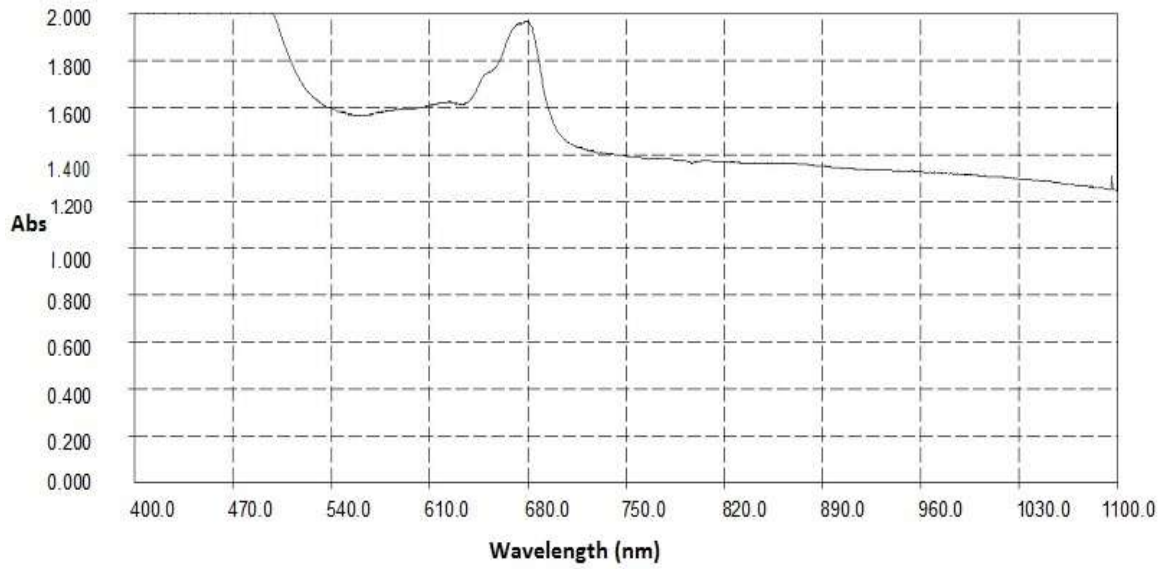

Fig. 1: Spectrascan of *S. quadricauda* with scan speed of 1800 nm/min screened between 400 and 1100 nm

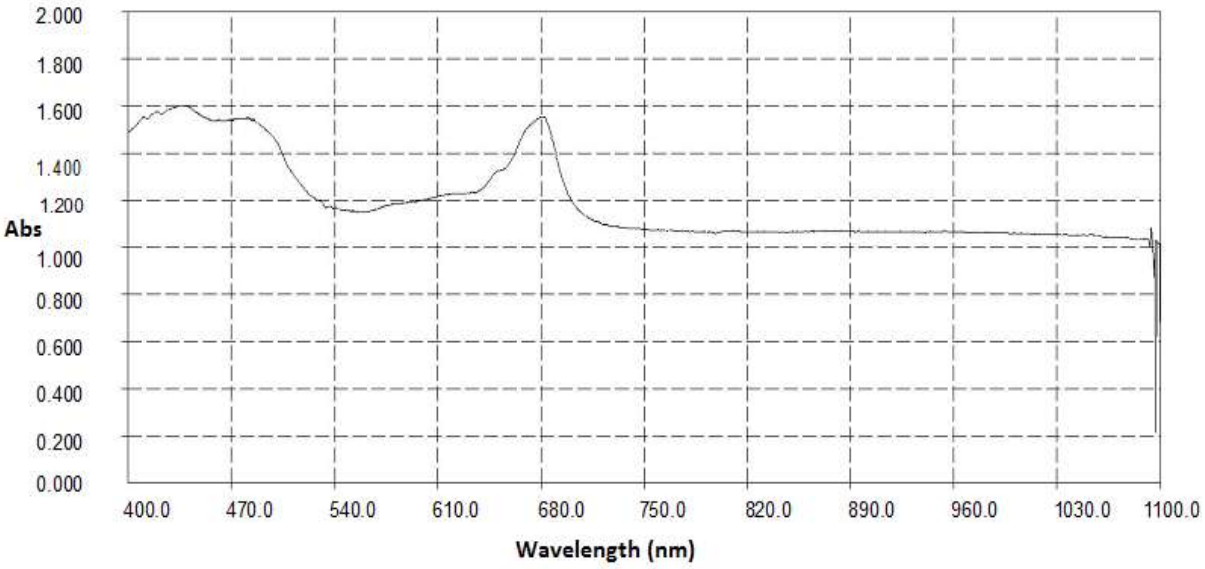

Fig. 2: Spectrascan of *S. dimorphus* with scan speed of 1800 nm/min screened between 400 and 1100 nm

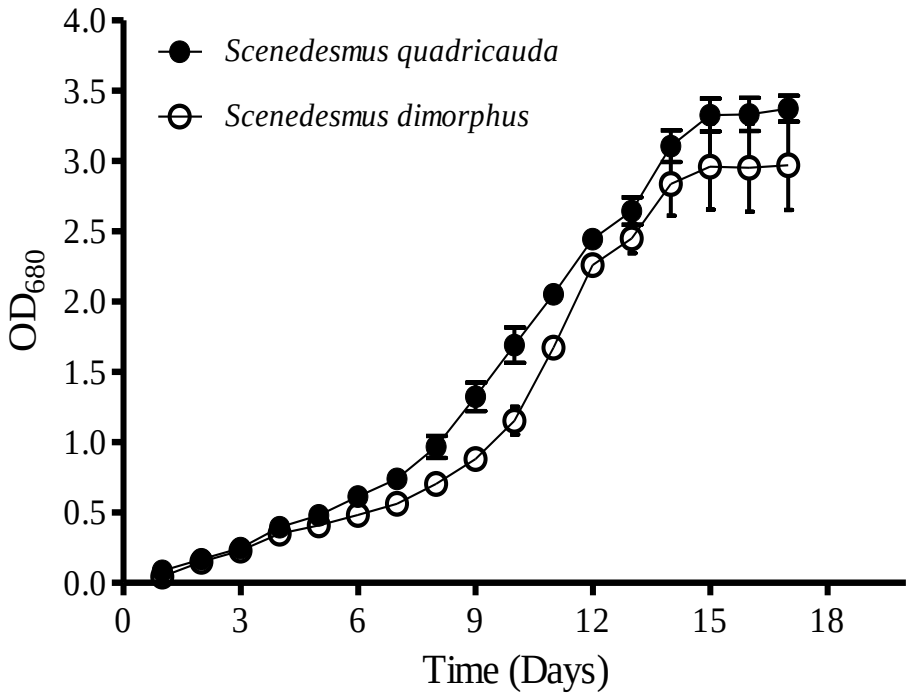

Fig. 3: Growth curve of two microalgae under batch mode

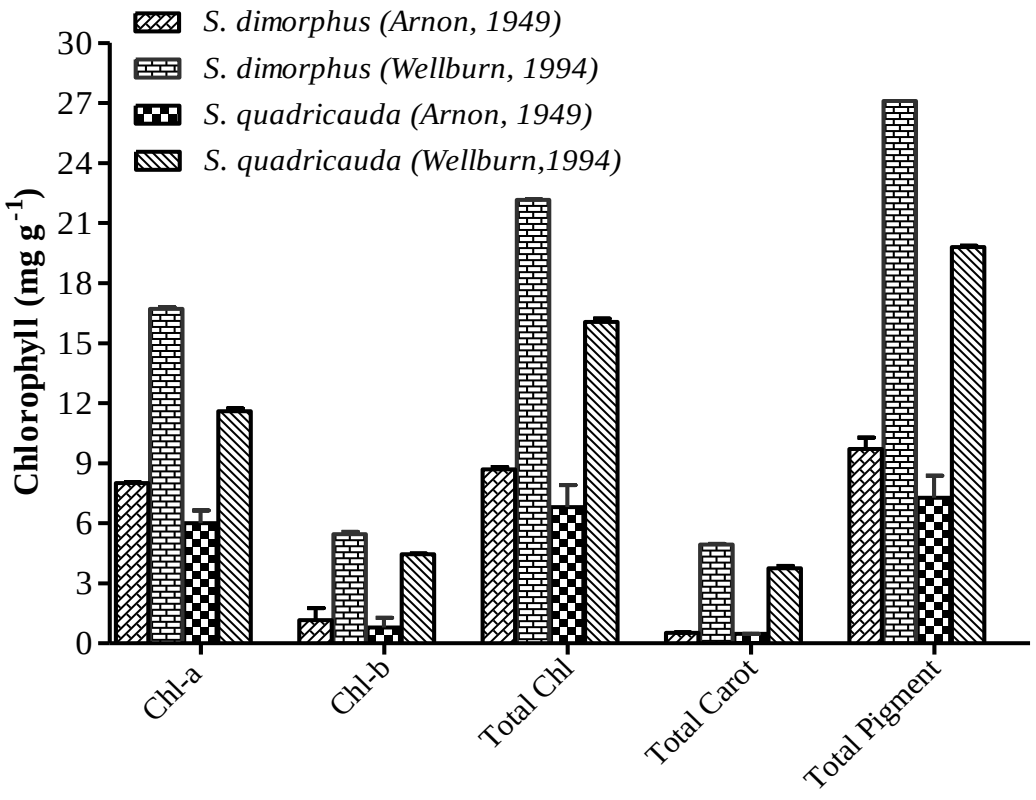

Fig. 4: Chlorophyll estimation in two microalgal species by using two different set of equations: Arnon, 1949 and Wellburn, 1994 equations.