Indian Journal of Natural Products and Resources
Vol. 14(4), December 2023, pp. 624-633
DOI: 10.56042/ijnpr.v14i4.6874

Mass culture, biomass production and antioxidant activities of two microalgae, *Arthospira platensis* and *Chlorella vulgaris*, and a fern, *Azolla pinnata*

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Received 13 November 2022; revised received 04 November 2023; accepted 07November 2023

Algae have widely been used in aquaculture as feedstuff and additives. Pharmaceutically, algae are used as nutritional supplements for treating many ailments in humans as they are considered a traditional food. The present study was carried out to optimise the culture medium for the growth and biomass production of two microalgae, *Arthospira platensis* (*Spirulina*) and *Chlorella vulgaris* (*Chlorella*), and the fern, *Azolla pinnata* (*Azolla*), under laboratory conditions. After 30 days of mass culture, the biomass production of *A. platensis* was found to be maximum in Zarrouk's medium (3.0 g/L dry wt.) than that of BG11 medium (2.02 g/L dry wt.). For *C. vulgaris*, the maximum growth was found in Conway's medium (2.86 g/L dry wt.) than that of Bold's basal medium (1.98 g/L dry wt.). In the case of *A. pinnata*, it was recorded to be 0.745 kg/L (dry wt.) in the Cow dung, followed by red soil (0.684 kg/L dry wt.) and urea (0.646 kg/L dry wt.). These algae and the fern possessed antioxidant activities, such as scavenging DPPH, ABTS and superoxide radicals, reducing power and inhibiting ascorbate autoxidation. Among these, the overall antioxidant activity was the best with *Spirulina*, followed by *Chlorella* and *Azolla.* These algae and the fern even can be grown in every household at a small scale level as and when required. Based on our previous study, it is concluded that microalgae, *Spirulina* and *Chlorella* can be consumed by humans as they are sources of protein and fibre if they are grown hygienically, and also can be used as food sources for fishes, prawns and other aquatic animals, and livestock also along with *Azolla.*

Keywords: *Azolla*, Biomass, *Chlorella*, Mass culture, *Spirulina*

IPC code; Int. cl. (2021.01)– A61K 36/00, A61K 39/00

Introduction

Algae are necessary for ecosystems in terms of functions, biomass production due to high photosynthetic efficiency and rapid growth rate, and to produce components of interest for industrial applications, including biodiesel[1]. Algae (Cyanobacteria, *Microcoleus vaginatus*) increase the water retention capacity of soil and help to reduce soil erosion[2]. Dried algae can also help improve soil aeration. In aquatic environments, algae consume carbon dioxide and produce oxygen in the carbon sequestration process, thereby stabilising aquatic food chains. Algae have widely been used in aquaculture as feedstuffs and additives, and pharmaceutically, they are used as nutritional supplements for treating many ailments in humans as they are considered traditional food[3].

The most promising representatives of microalgae are families of *Chlorella*, *Dunaliella*, *Scenedesmus*, and *Spirulina*. They constitute one of the major groups of photosynthetic eukaryotes and exist worldwide in different habitats, including fresh and marine water to terrestrial ecosystems[4]. *Spirulina* (multicellular) and *Chlorella* (unicellular) are forms of microalgae that are highly nutritious, and thus, they are incorporated into modern-day medicine for human usage. *Spirulina* (*Arthrospira*) is a spiral-shaped blue-green microalga/ cyanobacteria that thrive in saline aquatic habitats of coastal and inland areas. *Arthrospira* is rich in proteins, amino acids, vitamins (especially B12), minerals, polyunsaturated fatty acids (gamma-linolenic acid) and several pigments like carotenoids, xanthophylls, phycobiliproteins (phycocyanin), and chlorophyll-*a*, and it has therapeutic, antioxidant and anti-inflammatory properties[5]. *Chlorella* is used in medicine and cosmetics due to its unique properties, such as high proteins, polysaccharides, carotenoids, vitamins, lipids, immune-stimulator compounds, anti-oxidants and minerals[6]. *Chlorella* is well known for its antitumor, anticarcinogenic, antibacterial, antiviral, anticataract, antiulcer, and antioxidative properties[7].

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These properties of *Spirulina* and *Chlorella* are associated with many health benefits, including lowered risk factors for heart disease and improved blood sugar management. Therefore, they have been used in food, pharmaceutical, cosmetic industries and other high-value products. According to Ho *et al.*[8], *Chlorella* can be used as a high-quality and balanced supplement that provides a complete diet for humans.

Azolla, the free-floating water fern (Pteridophyta), has a good source of nutrients, such as protein with essential amino acids and minerals such as iron, calcium, magnesium, potassium, phosphorus and manganese, and appreciable quantities of vitamins A, β-carotene and vitamin B-12[9]. It is a potential feed for many animals, such as fish, insects, poultry, rabbits, pigs, and ruminants like cows, goats, and sheep due to its nutritious value, easy cultivation and high biomass yield[10]. Apart from animal feed, *Azolla* is also widely used as a bio-fertilizer[11]. *Azolla* can be used as a human food, a medicine, and an agent in the bioremediation of heavy metal pollution[12]. It may also be used for the production of hydrogen fuel and biogas, thereby helping in the absorption of large amounts of atmospheric carbon dioxide, reducing climate change[13]. It also controls the growth of weeds, acts as a bio-insecticide against mosquitoes, and reduces ammonia volatilisation accompanied by applying chemical nitrogen fertilizer[14]. Therefore, *Azolla* is a potential source of high-value products in the pharmacy, bioplastics, and nutraceutical industries. All three sources, *Spirulina* and *Chlorella* (protists), and *Azolla* (plant), have been reported to be used as feeds for prawn, *Macrobrachium rosenbergii*[15].

Several cultivation methods of microalgae, such as open ponds[16], tubular photobioreactors[17] and inclined glass panels[18], are in practice. However, there are two commonly adopted methods: (i) open cultivation in ponds, tanks and raceway ponds, and (ii) controlled closed cultivation using bioreactors. In large-scale commercial microalgae cultivation, the cost and composition of cultivation media, along with growth rate and biomass production, are challenging factors. Different cultivation media, such as Zarrouk's medium[19], Rao's medium[20], BG11 medium[21] and Conway's medium[22], are available to cultivate *Spirulina* and *Chlorella*. It has been reported that Zarrouk's medium has successfully served as the standard medium for *Arthospira* culture[23]. In the present study, the mass culture of *Spirulina* was tried with Zarrouk medium and BG11 medium under laboratory conditions. In the case of *Chlorella*, Bold's Basal medium and Conway's medium were used. Similarly, the different media used for the mass culture of *Azolla* were cow dung, red soil and urea. The biomass production of these algae and the fern in each medium was calculated. The antioxidant activities (scavenging of DPPH, ABTS and superoxide radicals), reducing power and inhibition of ascorbate autoxidation were assayed in these algae and the fern grew in their respective best medium.

Materials and Methods

Procurement of the inoculums (mother cultures) of microalgae and fern

The pure cultures of microalgae (*A. platensis* and *C. vulgaris*) were collected from M/S. Ecolive Exports, Ayalur, Gobichettipalayam, Tamil Nadu, India. The pure culture of the fern, *A. pinnata* was collected from the *Azolla* Cultivation and Research Centre, Tamil Nadu Agriculture University, Coimbatore, Tamil Nadu, India.

Morphological authentication of microalgae and a pteridophyte species

The cultured species of microalgae, *Spirulina* and *Chlorella*, and a pteridophyte, *Azolla* were morphologically identified and certified as *Arthrospira platensis* (Nordst.), Gomont, Oscilaloriaceae (BSI/SRC/5/23/2018/Tech/3440), *Chlorella vulgaris*, Chlorellaceae (BSI/SRC/5/23/2018/Tech/3442), and *Azolla pinnata* R.Br. Azollaceae (BSI/SRC/5/23/2018/Tech/3441), respectively by the Botanical Survey of India, Coimbatore, Tamil Nadu, India.

Subcultures of microalgae

Two culture media, such as Zarrouk's medium[19] and BG11 medium[21] were used for maintaining the sub culture of *A. platensis* (Table 1). The mother culture of *A. platensis* (10 mL inoculum) was inoculated in 90 mL of each of the two culture media. They were incubated for 15 days at 24±1ºC in a thermo-statically controlled room and illuminated with cool inflorescence lamps (Phillips 40 W, cool daylight 6500 K) at an intensity of 1000 lux in a 12:12 h light and dark regimes. Manual shaking of cultures was done 3 times daily. Similarly, 10 mL inoculum of *C. vulgaris* was inoculated in 90 mL of each of the two culture media, Bold's Basal medium[24] and Conway's medium[22]. The other conditions were similar to that of *A. platensis*. In the case of

Table 1 — Nutrient levels in different culture media used for the laboratory culture of *A. platensis* and *C. vulgaris*

Nutrients	Culture media			
	Zarrouk's medium	BG11 medium	Bold's Basal medium	Conway's medium
Sodium bicarbonate (g L^{-1})	16.800	--	--	--
Sodium nitrate (g L^{-1})	2.500	1.500	25.000	100.000
Sodium chloride (g L^{-1})	1.000	0.036	2.500	--
Potassium sulphate (g L^{-1})	1.000	0.001	--	--
Potassium hydrogen phosphate (g L^{-1})	0.500	0.040	7.500	--
Magnesium sulphate (g L^{-1})	0.200	0.075	7.500	--
Ferrous sulphate (g L^{-1})	0.010	0.006	4.980	--
Calcium Chloride Dihydrate (g L^{-1})	0.040	--	0.490	--
Citric acid (g L^{-1})	--	0.006	--	--
Ethylenediaminetetraacetic acid (g L^{-1})	0.080	0.020	50.000	45.000
Boric acid (g L^{-1})	--	--	11.400	33.600
Sodium phosphate, monobasic (g L^{-1})	--	--	--	20.000
Manganous chloride, 4-hydrate (g L^{-1})	--	--	--	0.360
Zinc chloride (g L^{-1})	--	--	--	2.100
Cobalt chloride,6-hydrate (g L^{-1})	--	--	--	2.100
Vitamin B_{12} (mg L^{-1})	--	--	--	10.000
Vitamin B_1 (mg/ L^{-1})	--	--	--	--
Potassium dihydrogen phosphate (g L^{-1})	--	--	17.500	--
Calcium chloride (g L^{-1})	--	--	2.500	--
Zinc sulphate (g L^{-1})	--	--	4.420	--
Manganese (II) chloride tetrahydrate (g L^{-1})	--	--	1.440	--
Molybdenum trioxide (g L^{-1})	--	--	0.710	--
Copper sulphate pentahydrate (g L^{-1})	--	--	1.570	--
Cobalt nitrate (g L^{-1})	--	--	0.010	--
Potassium hydroxide (g L^{-1})	--	--	31.000	--
Distilled water (mL)	1000.000	1000.000	1000.000	1000.000

A. pinnata, the inoculum was directly taken for mass culture.

Mass cultures of microalgae

The microalga, *Spirulina*, was mass cultured in two specified growth media (Zarrouk's and BG11) under a triplicate experimental set-up consisting of a 3 x 25 L plastic trough (total 6 x 25 L).100 mL of each sub-cultured *A. platensis* was added to 900 mL of each culture medium and added to the respective mass culture trough containing 25 L tap water and kept on in open terrace under shade (day temperature, 29-33ºC; pH, 7-8) for 30 days during February-March, 2019. The cultures were frequently stirred, the water level was maintained daily, and the growth medium (1 L) was reintroduced once every 5 days. The culture mixtures were vigorously aerated to provide adequate oxygen in order to keep the cells and media in suspension. The pH, cell count and biomass production were analysed at a 5-day interval. Similarly, the mass culture of another micro alga, *C. vulgaris* was done using two specified culture media (Bold's Basal and Conway's), each with 100 mL of sub-cultured *Chlorella*. Nylon fabric cloth with a mesh size of 30-60 microns was used to filter the cultured microalgae. After harvesting, they were carefully washed with tap water as quickly as possible and dried away from direct sunlight. Then, they were pulverised into powder forms and stored in sterilised containers for further use.

Mass culture of *Azolla*

Three culture media, such as cow dung, Red soil and urea, were used for the mass culture of *Azolla* (Table 2). The cow dung medium was prepared at a 1:10 ratio (1 kg of dried Cow dung powder was dissolved in 10 L tap water) and allowed in standby for 2 days (the water contains common macronutrients, such as calcium, chloride, fluoride, magnesium, potassium and sodium). After that, a handful of live *Azolla* (120 g/L) was added. The total volume maintained was 25 L in triplicates. The culture was maintained under shade for outdoor light (29-33ºC) exposure for 30 days with occasional stirring. The water level was maintained. The biomass production was analysed at a 5-day interval.

Table 2 — Macronutrients present in different raw materials used as culture media for the mass culture of *A. pinnata*

Nutrients (%)	Raw materials of culture media		
	Cow dung*	Red soil*	Urea
Nitrogen	0.88	0.23	46.00
Phosphorus	0.14	-	-
Potassium	1.50	0.90	-
Calcium	1.67	5.80	-
Magnesium	0.61	1.72	-
Iron	0.14	5.34	-
Carbon	-	6.80	-
Sodium	-	3.50	-

Azolla takes up nutrients from the water.
*The proximate composition was analysed by using outsourcing service available at Tamilnadu Veterinary and Animal Science University, Namakkal, India.
The common macronutrients present in water are not taken into account.

In the case of Red soil medium, 2.5 cm sediment in 25 L of water was maintained without the addition of any phosphorus. For the 10% urea medium, 2.5 kg of urea was dissolved in 25 L of water (100 g/L). The other culture conditions for red soil and urea media were similar to that of *Azolla* culture described in Cow dung medium. These three cultural media were used in order to identify the best natural culture medium for *Azolla*. The nutrient profiles of these media are presented in Table 2. The proximate composition was analysed by adopting AOAC[25] methods.

After 30 days, *A. pinnata* biomass was harvested and sun-dried for three days until they became crispy while retaining their greenish colouration. The dried leaves were pulverised into powder forms and stored in sterilised containers for further use.

Population of algal cells

Samples were taken once in five days in 10 mL vials full. The cells of *A. platensis* in each vial were preserved by adding 2-3 drops of formalin. One mL of the sample was carefully filled in a Neubauer Hemocytometer groove and covered with a glass slide. The cells were enumerated under a compound microscope[26]. A hand tally counter was used for reliable counting. Similarly, the cells of *C. vulgaris* were counted. Algal cells were calculated by the following equation.

$$\text{Algal cell population} = \frac{\text{Total number of cells counted (A + B + C + D + E)}}{5} \text{ X } 10^4 \text{ cells/mL}$$

where, A to E represent four corners and a centre squares; 10^4 is the correction factor of Neubauer chamber.

Antioxidant assays

The antioxidant activity of the algal extract deals with the kinetics of a reaction between an antioxidant and the prooxidant or radical it reduces or scavenges. In the present study, the antioxidant activities of three algae, *Spirulina*, *Chlorella* and *Azolla* were performed in methanolic and petroleum etheric extracts by following *in vitro* assays. For the stock solution, 1 mg of each extract was dissolved in 10 mL of each solvent.

DPPH radical scavenging activity

The DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical quenching experiment was performed by following the methodology of Moraes-de-Souza *et al*.[27]. The antioxidant molecules present in the algal extract can scavenge $DPPH^{\cdot+}$ free radicals by interrupting radical chain reactions, or even prevent the reactive oxidants from being formed. The stable DPPH free radical (the odd electron of nitrogen atom) is reduced in the presence of an antioxidant molecule at room temperature by receiving a hydrogen atom from antioxidants to the corresponding hydrazine. At that time, the violet-coloured DPPH solution is reduced to a yellow-coloured product, diphenylpicryl hydrazine.

The reaction mixture consists of 100 µL (1 mg) and 200 µL (2 mg) algae extract and 3.8/ 3.9 mL of 0.1 mM DPPH solution in methanol/ petroleum ether. This mixture was mixed vigorously and incubated for 20 min in the dark at room temperature. The decolorisation of the reaction mixture was measured spectrophotometrically at 517 nm (Double Beam UV-Visible spectrophotometer, LMSP-UV1900A, Labman Scientific Instruments Pvt., Ltd., India). The percentage inhibition of DPPH radicals by the algal extract was calculated using the following equation and compared with the ascorbic acid standard. Methanol/ petroleum ether was used as blank, whereas DPPH + methanol/ petroleum ether was used as an experimental control. Vitamin C was used as standard.

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \text{ X } 100$$

ABTS radical scavenging activity assay

The ABTS (2,2 azino-bis 3 ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity of algae

extract was determined according to the method of Re *et al*.[28]. The ABTS assay is based on the generation of a blue/green $ABTS^{\cdot+}$ radical cation by a strong oxidising agent (e.g., potassium permanganate or potassium persulfate) that can be reduced by antioxidants, which can be easily quantitatively detected due to the bleaching of absorption spectrum characteristic maxima at 734 nm against vitamin E standard.

The ABTS radicals ($ABTS^{\cdot+}$) were produced by allowing an ABTS stock solution with potassium persulphate to stand in the dark for 12 h. Before use, the $ABTS^{\cdot+}$ solution was diluted with distilled water to an adjusted absorbance of 0.7 at 734 nm. The reaction mixture consisted of 100 µL (1 mg), and 200 µL (2 mg) algae extract and 3 mL of the $ABTS^{\cdot+}$ radical. After incubation for 6 min under dark at room temperature the absorbance was read at 734 nm (Double Beam UV-Visible spectrophotometer, LMSP-UV1900A, Labman Scientific Instruments Pvt., Ltd., India). Methanol/ petroleum ether was used as blank. The reaction mixture devoid of algal extract was served as control.

$$\text{ABTS radical scavenging activity (\%)} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \text{ X } 100$$

Superoxide radical scavenging activity assay

The superoxide radical scavenging activity assay was done according to the method of Fontana *et al*.[29]. The non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals ($O_2^{\cdot-}$), which reduce the nitro blue tetrazolium (NBT) to purple formazan. The power of algal extract in inhibition of $O_2^{\cdot-}$ generation was measured at 560 nm.

To the 100 µL (1 mg) and 200 µL (2 mg) algal extract, 1mL of reaction mixture consisting of 20 mM sodium phosphate buffer, 75 µM nicotinamide adenine dinucleotide (NADH), 50 µM NBT, and 15 µM phenazine methosulfate (PMS) was added, mixed well and incubated for 5 min at 37°C. The absorbance was measured at 560 nm (Double Beam UV-visible spectrophotometer, LMSP-UV1900A, Labman Scientific Instruments Pvt., Ltd., India). Methanol/ petroleum ether was used as blank. The reaction mixture, devoid of algal extract, served as control. Vitamin C was used as standard.

$$\text{Superoxide anion radical scavenging activity (\%)} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \text{ X } 100$$

Activity of reducing power

The method of Hue *et al*.[30] was followed in this assay. The antioxidant substances (the ability to donate hydrogen atoms) in the algal extract have reduction potential, convert potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric–ferrous complex, at that time the colour of the solution changed from green to Perl's Prussian blue depending on the reducing power of the compounds, that has an absorption maximum at 700 nm.

To the 100 µL (1 mg) and 200 µL (2 mg) algae extract prepared in methanol/ petroleum ether, 0.2 mL of 0.2 M phosphate buffer (pH 6.6) and 0.2 mL of 1% potassium ferricyanide were added. The mixture was incubated in the water bath for 20 min at 50°C. Then 0.2 mL of 10% (w/v) trichloroacetic acid was added to stop the reaction. It was centrifuged at 10,000 rpm at 4°C. The supernatant (0.5 mL) was mixed with 0.1 mL of 0.1% ferric chloride and 0.4 mL of distilled water. After 10 min incubation, the absorbance was read at 700 nm (Double Beam UV-Visible spectrophotometer, LMSP-UV1900A, Labman Scientific Instruments Pvt., Ltd., India), which was compared with L-Cysteine. Methanol/ petroleum ether was used as blank. The reaction mixture devoid of algal extract served as a control.

$$\text{Activity of reducing power (\%)} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \text{ X } 100$$

Inhibition of ascorbate autoxidation

The inhibition of ascorbate autoxidation was done following the method of Mitsuda *et al*.[31]. Autoxidation is the spontaneous oxidation of a compound (becoming brown) in air. The common mechanism is a free radical chain reaction, where adding oxygen gives rise to hydroperoxides and their associated peroxy radicals (ROO•), which replacing the proton from the 3-hydroxyl group of ascorbic acid with a sodium ion yields sodium ascorbate, an organic sodium salt. It acts as a reducing agent or reductant, loses electrons (electron donor) and is oxidised in the redox reaction to form ascorbate radical ($Asc^{\bullet-}$) and dehydroascorbic acid (DHA), and lowers the acidity level of ascorbic acid. The prevention of oxidation of ascorbate by the antioxidants of algal extract was measured at 265 nm.

The algae extract (100 µL (1 mg) and 200 µL (2 mg)) was mixed with 0.1 mL of 5 mM sodium ascorbate solution and 9.8 mL of 200 mM

sodium phosphate buffer. After 10 min incubation, the absorbance was measured at 265 nm (Double Beam UV-Visible spectrophotometer, LMSP-UV1900A, Labman Scientific Instruments Pvt. Ltd., India). Methanol/ petroleum ether was used as blank. The reaction mixture devoid of the algal extract served as a control. Vitamin C was used as standard.

$$\text{Ascorbate autoxidation inhibition rate (\%)} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \text{ X } 100$$

Results and Discussion

Cell populations and biomasses of microalgae

The cell population and biomass of laboratory-cultured *A. platensis* and *C. Vulgaris* are provided in Table 3; Figs. 1 and 2. The statistically significant (P<0.05) gradual increase in the growth of *Spirulina* and *Chlorella*, respectively, was recorded between each interval up to the 30th day in all the media. Among the two media used for *Spirulina*, the Zarrouk medium (75.00 g L^{-25} dry wt.) was the best, followed by the BG11 medium (50.00 g L ^{25} dry wt.). Similarly, among the two media used for *Chlorella*, Conway's medium (71.50 g L^{-25} dry wt.) was the best, followed by the Bold's Basal medium (49.00 g L^{-25} dry wt.).

Growth rate and biomass of *A. pinnata*

The biomass of laboratory cultured *A. pinnata* is provided in Table 4; Fig. 3. The statistically significant (P<0.05) gradual increase in the growth rate of *Azolla* was recorded between each interval up to 30th day when compared with the initial day, in all the media. Among the three culture media, cow dung (25.366 Kg L^{-25} dry wt.) was found to be the best

Table 3 — Cell populations and biomasses of *Spirulina* and *Chlorella*, each in two different culture media during 30 days mass culture using 100 mL of subcultures

Duration	Population of micro algal cells (x 10^4 cells mL^{-1})			
	Spirulina		*Chlorella*	
	Zarrouk's medium	BG11 medium	Bold's Basal medium	Conway medium
5th day (cells mL^{-1})	111.66±2.08	84.66±5.13	82.66±6.02	100.66±3.78
10th day (cells mL^{-1})	134.66±6.02	93.66±5.03	91.66±8.08	128.66±9.29
15th day (cells mL^{-1})	153.33±3.51	102.66±4.04	102.66±4.04	152.33±9.45
20th day (cells mL^{-1})	166.66±5.03	129.33±6.02	128.33±7.02	162.33±5.03
25thday (cells mL^{-1})	186.33±7.05	163.33±5.50	163.33±4.04	182.66±5.50
30th day (cells mL^{-1})	205.33±4.50	182.33±6.65	182.33±6.65	203.66±7.09
Biomass (cells L^{-1})	2053.00	1823.00	1823.00	2036.00
Biomass (g L^{-1} dry wt.)	3.00	2.02	1.98	2.86
Biomass (g L^{-25} dry wt.)	75.00	50.00	49.00	71.50

Each value is a mean±SD of three replicate analysis, within each column means with different superscripts letters are statistically significant P <0.05 (one way ANOVA and subsequently *post hoc* multiple comparison with DMRT)

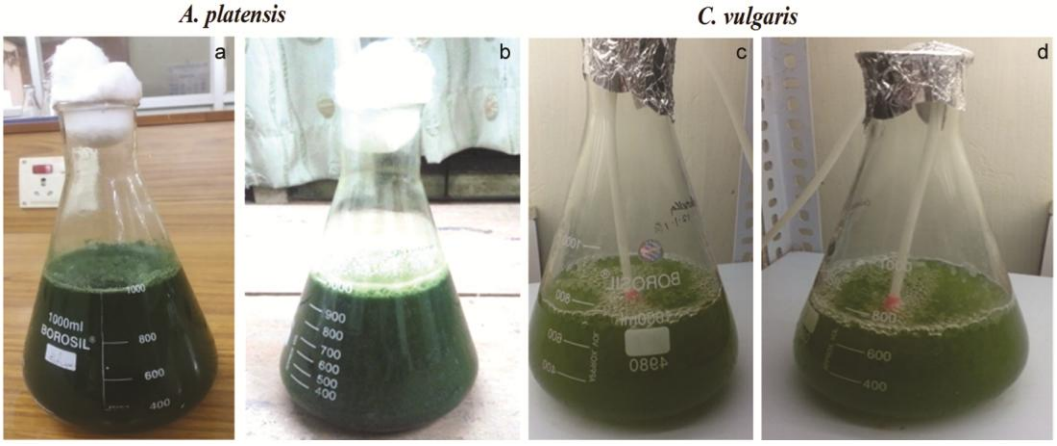

Fig. 1 — Sub-cultures of *A. platensis* and *C. vulgaris*, each using two different growth media. a) Zarrouk's medium (93.66 x 10^4 cells/mL^{-1}; b) BG11 medium (70.33 x 10^4 cells/mL^{-1}; c) Bold's Basal medium (69.66 x 10^4 cells/mL^{-1}); and d) Conway's medium (83.66 x 10^4 cells/mL^{-1}).

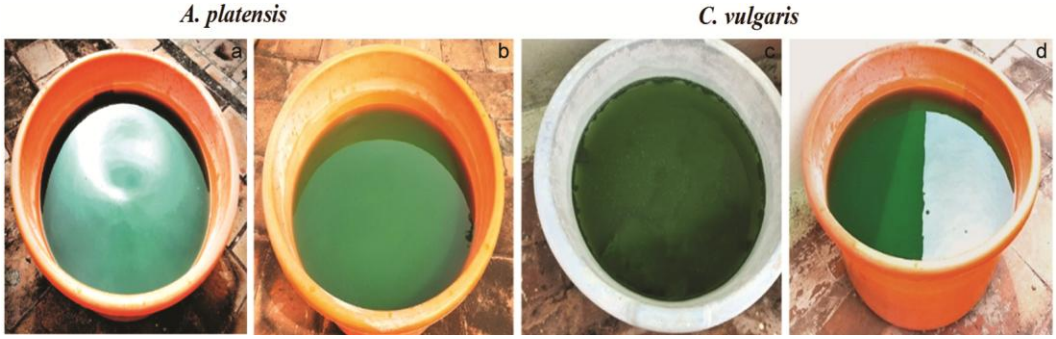

Fig. 2 — Mass cultures of *A. platensis* and *C. vulgaris*, each using two different growth media. a) Zarrouk's medium; b) BG11 medium; c) Bold's Basal medium; and d) Conway's medium.

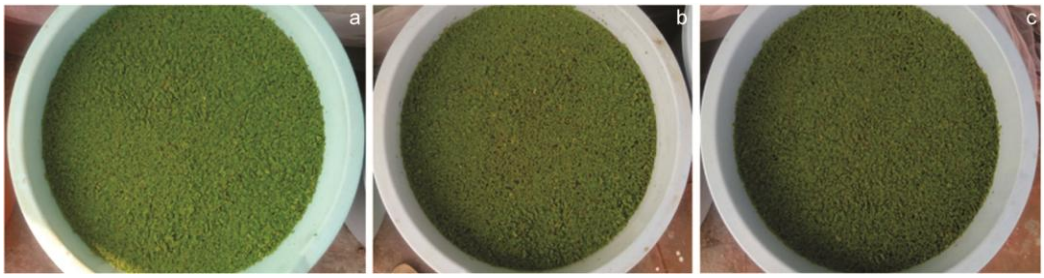

Fig. 3 — Mass cultures of *A. pinnata* using three different growth media. a) Cow dung medium; b) Red soil medium; and c) Urea medium.

Table 4 — Biomass production of *Azolla* in three different culture media during 30 days mass culture (initial introduction was 120 g L^{-1} wet wt.)

Duration	Biomass of *Azolla*		
	Cow dung medium	Red soil medium	Urea medium
5th Day (g L^{-1} wet wt.)	267.66±19.00	265.33±8.08	194.66±13.45
10th Day (g L^{-1} wet wt.)	504.33±19.85	486.66±13.50	325.66±29.30
15th Day (g L^{-1} wet wt.)	654.33±19.85	635.33±20.30	508.33±23.45
20th Day (g L^{-1} wet wt.)	743.33±25.16	726.33±24.00	653.66±34.59
25th Day (g L^{-1} wet wt.)	853.33±24.63	838.66±38.37	756.66±37.72
30th Day (g L^{-1} wet wt.)	1014.66±26.27	997.66±12.05	946.66±46.13
Biomass (Kg L^{-1} wet wt.)	1.014±0.026	0.997±0.012	946.66±0.046
Biomass (Kg L^{-1} dry wt.)	0.745±0.020	0.684±0.016	0.646±0.030
Biomass (Kg L^{-25} wet wt.)	25.366	24.925	23.650
Biomass (Kg L^{-25} dry wt.)	18.635	17.106	16.171

Each value is a mean±SD of three replicate analysis, within each column means with different superscripts letters are statistically significant P <0.05 (one way ANOVA and subsequently *post hoc* multiple comparison with DMRT)

followed by red soil medium (24.925 Kg L^{-25} dry wt.) and urea (23.650 Kg L^{-25} dry wt.).

In this study, the 30 days mass culture of *A. platensis* has yielded the maximum biomass production in Zarrouk's medium (75.00 g L^{-25} dry wt.). For *C. vulgaris*, the maximum growth was found to be in Conway's medium (71.50 g L^{-25} dry wt.). In the case of *A. pinnata*, it was recorded to be the best in the cow dung (25.366 Kg L^{-25}wet wt./ 18.635 Kg L^{-25} dry wt.).Based on our previous study, the proximate compositions of primary phytonutrients present in mass-cultured *Spirulina*, *Chlorella* and *azolla* (with Zarrouk's medium, Conway's medium and cow dung medium, respectively) consisted mainly of proteins, followed by carbohydrate and lipids[32]. *Spirulina* contains a rich amount of crude protein (58.94%), followed by *Chlorella* (47.08%) and *Azolla* (21.82%). The content of crude fat was higher in *Chlorella* (5.68%), followed by *Azolla* (4.00%) and *Spirulina* (1.54%). The crude fibre level was found to be higher in *Azolla* (26.21%), followed by *Chlorella* (2.87%) and *Spirulina* (1.00%). The content of ash was higher in *Azolla* (22.81%), followed by *Spirulina* (12.22%) and *Chlorella* (7.82%). The total

carbohydrate level was found to be higher in *Chlorella* (26.64%), followed by *Spirulina* (16.68%) and *Azolla* (15.79%). The gross energy was higher in *Chlorella* (4,377 k.cal/kg), followed by *Spirulina* (4,183 k.cal/kg) and *Azolla* (3,295 k.cal/kg). However, the biochemical composition of algae varies among species under different conditions[33,34]. An optimal medium formulation is also critical to ensure sufficient and stable supply of nutrients to attain maximal growth rate[35,36]. The chemical composition and concentrations of culture media affects the growth of the microalgae, biomass production, and fatty acid profile[37]. The conventional culture media which greatly impact on the growth are not specific to a particular micro alga. However, NSIII medium, Chu No.10 medium, BG11 medium, and Bold's basal medium (BBM) are used for microalgae cultivation[38,39]. Zarrouk's medium has a potential one among the other media in terms of biomass production of *A. Platensis*[25,38,40].

Antioxidant activity

The methanolic and petroleum etheric extracts of algae, *Spirulina*, *Chlorella* and *Azolla* possessed antioxidant activities, such as scavenging of DPPH, ABTS and superoxide radicals, reducing power and inhibition of ascorbate autoxidation (Tables 5 and 6). Among the two solvents used to obtain extracts of these algae, the methanolic extract showed more antioxidant activities than that of the petroleum etheric extract. Among the three algae, the overall antioxidant activity was found to be the best with *Spirulina*, followed by *Chlorella* and *Azolla*.

The polarity of a solvent plays a major role in exhibiting antioxidant activities. Moreover, it depends upon the content of secondary phytochemicals such as phenols, flavonoids etc.[41]. The antioxidant activities can be correlated with the biomass produced in the best medium for microalgae and fern. This study suggests that for the best growth of *Spirulina*, nutrients such as sodium bicarbonate, sodium nitrate, sodium chloride, potassium sulphate, potassium hydrogen phosphate, magnesium sulphate, ferrous sulphate, calcium chloride dihydrate and ethylenediaminetetraacetic acid are much required, and these nutrients were present in Zarrouk's medium (Table 1). Similarly, for the best growth of *Chlorella*, the nutrients such as ethylenediaminetetraacetic acid, boric acid, sodium phosphate monobasic, manganous chloride-4-hydrate, zinc chloride, cobalt chloride-6-hydrate and vitamin B_{12} are much required, and these nutrients were present in Conway's medium (Table 1).

In the case of *Azolla*, phosphorus was much required, which was found to be naturally present in cow dung (Table 2). It has been reported that

Table 5 — Antioxidant activities of condensed methanolic extract of *Spirulina*, *Chlorella* and *Azolla*

Assay Parameter	Standard	Antioxidant activity (%) in different concentrations of condensed methanolic extracts					
		Spirulina		*Chlorella*		*Azolla*	
		1.0 mg	2.0 mg	1.0 mg	2.0 mg	1.0 mg	2.0 mg
DPPH	Vitamin C	33.25±3.62	73.18±6.75	29.24±4.48	56.77±4.65	26.31±2.87	52.99±4.14
ABTS	Vitamin E	32.18±4.36	51.55±3.06	29.24±2.29	36.46±2.42	24.72±1.30	34.93±2.38
SCA	Vitamin E	33.12±1.90	51.75±2.48	28.82±2.68	45.89±3.95	28.20±1.32	43.72±2.72
RP	L-Cysteine	27.77±2.46	50.54±3.47	27.35±1.19	37.54±2.55	23.89±1.74	33.80±2.30
IAA	Vitamin C	26.59±2.69	49.06±3.54	25.72±1.66	42.11±3.47	23.86±1.35	36.22±3.12

DPPH, Diphenyl-1-picrylhydrazyl; ABTS, Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; SCA, Superoxide radical scavenging activity; RP, Reducing power; IAA, Inhibition of ascorbate autoxidation.

Table 6 — Antioxidant activities of condensed petroleum etheric extracts of *Spirulina*, *Chlorella* and *Azolla*

Assay Parameter	Standard	Antioxidant activity (%) in different concentrations of condensed petroleum etheric extracts					
		Spirulina		*Chlorella*		*Azolla*	
		1.0 mg	2.0 mg	1.0 mg	2.0 mg	1.0 mg	2.0 mg
DPPH	Vitamin C	32.38±2.28	56.43±5.49	22.89±1.63	50.60±5.94	21.53±1.66	46.80±3.26
ABTS	Vitamin E	16.35±1.89	32.24±2.84	11.78±1.73	30.60±2.52	19.19±0.84	28.17±1.04
SCA	Vitamin E	25.16±1.61	46.13±2.80	20.95±1.04	42.32±2.85	23.40±1.48	41.58±2.83
RP	L-Cysteine	22.78±1.80	45.03±2.21	25.59±1.70	36.23±3.13	17.13±1.75	33.19±1.80
IAA	Vitamin C	24.42±2.34	41.36±2.57	25.64±2.75	35.08±2.25	22.29±1.11	32.16±2.69

DPPH, Diphenyl-1-picrylhydrazyl; ABTS, Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; SCA, Superoxide radical scavenging activity; RP, Reducing power; IAA, Inhibition of ascorbate autoxidation.

methanolic and ethanolic extracts of *Spirulina platensis*, *C. vulgaris*, *A. pinnata*, *A. rubra*, *A. microphylla*, *Euglena cantabrica*, *Isochrysis galbana*, *Tetraselmis chuii*, *Dunaliella salina*, *Nannochloropsis oculata*, *Gracilaria gracilis* and species of *Ulava* (*U. compressa*, *U. linza*, *U. tubulosa*, *U. clathrata*, *U. linza*, *U. flexuosa* and *U. intestinalis*) showed good antioxidant activities[42-45].

Conclusion

In this study, the mass culture of *A. platensis*, *C. vulgaris* and *A. pinnata* were successfully done under laboratory conditions. These algae even can be grown in every household at a small scale level as and when required. Since microalgae, *Spirulina* and *Chlorella* are good nutritional sources of protein and fibre they can be consumed by humans, if they are grown hygienically. The *Azolla* can be a food source for fishes, prawns, and livestock, along with *Spirulina* and *Chlorella*, and a biofertilizer in agriculture. Moreover, these sustainable food sources, *Spirulina*, *Chlorella* and *Azolla* possess antioxidant activities that can also be taken to the pharmaceutical industry. Furthermore, to characterise the primary and secondary phytochemicals of these algae and *Azolla*, some specific studies need to be conducted.

Acknowledgement

The authors gratefully acknowledge Bharathiar University, Coimbatore, India for providing financial assistance under RUSA (BU/RUSA2.0/BCTRC/2020/ BCTRC-CT24, Dt. 14.12.2020).

Conflicts of interest

The authors report no financial or any other conflicts of interest in this work.

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