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Pigment production from *Spirulina platensis* using seawater supplemented with dry poultry manure

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Abstract

Spirulina platensis is one of the most explored cyanobacteria and an attractive source of valuable protein for both human and animal consumption. The conventional nitrogen source for *Spirulina platensis* is nitrate. However, recent research has evaluated the potential of using animal waste as a low-cost nitrogen source. *Spirulina platensis* was cultivated in different liquid medium like; synthetic medium (SOT), seawater medium (SW_1, SW_2 & SW_3) supplemented with different level of poultry dry manure (PDM) as nitrogen source. Among the different media, *Spirulina platensis* biomass concentration (dry weight) of 0.623 mg ml^{-1} highest in SOT, similarly seawater based media shows (0.608 mg ml^{-1}), SW_2 (0.572 mg ml^{-1}) and the least biomass was recorded in SW_1 (0.518 mg ml^{-1}). Pigment content in cells for chlorophyll in SOT (6.04 µg ml^{-1}) & SW_3 (5.93µg ml^{-1}), phycocyanin (PC) in SOT (70.03 µg ml^{-1}) & SW_3 (63.83 µg ml^{-1}), phycoerythrin (PE) in SOT (27.85 µg ml^{-1}) & SW_3 (23.99 µg ml^{-1}) , allophycocyanin (APC) in SOT (32.82 µg ml^{-1}) & SW_3 (29.08 µg ml^{-1}) and total carotenoids in SOT (31.90 µg ml^{-1}) & SW_3 (29.08 µg ml^{-1}).

Keywords: *Spirulina plantensis*, chicken dry manure, seawater media, chlorophyll, phycobiliproteins and total carotenoids

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Introduction

The cyanobacterium *Spirulina platensis* is an attractive source of valuable proteins for both human and animal consumption. The genus *Spirulina* has gained importance and international demand for its high value phytonutrients and pigments, which have applications in health foods, feed, therapeutics and diagnostics (Becker, 1994; Richmond 1992). It has been hailed as the ‘‘Food of the future’’, besides being considered as an ideal food for astronauts by NASA (Vonshak and Tomaselli, 2000). The use of the *Spirulina* sp pigments as colorant has already been explored by the cosmetic, pharmaceutical and food industries. Phycocyanin, a blue pigment, is used as colorant for food and drinks in Japan (Callagan, 1996). Among the various pigments produced by this organism, phycocyanin has attracted maximum attention. The first synthetic medium formulated for cultivation of *Spirulina* was Zarrouk’s medium (Zarrouk, 1966) which is still used as the standard medium (SM). Subsequently, different media have been tried for cultivation of *Spirulina* such as Rao's media (Singh, S. 2006), CFTIR media (Venkataraman *et al*., 1995), OFERR media (Singh, S. 2006), Revised media (Raoof *et al.*, 2006) and Bangladesh medium (Khatum *et al*., 1994). The use of sea water as an alternative medium, after being pretreated (Faucher et al., 1979) or after being supplemented with specific nutrients under laboratory conditions (Materassi et al. 1984) or in outdoor raceways (Tredici et al., 1986; Wu et al., 1993), has been reported.

Lower costs of production by the use of a low-cost medium could be a key factor in developing a competitive process for the production of *Spirulina* as a feed and as a source of added-value products. The conventional nitrogen source for *S. platensis* is nitrate. However, recent research has evaluated the potential of using animal waste as a low-cost nitrogen source (Gantar et al., 1991; Canizares et al., 1994; Olguin et al., 2001). These studies were focused mainly on the production of *Spirulina* from swine manure and waste, and there is only limited information available on utilization of poultry manure. More recently, dry chicken manure (DCM) can supply the necessary nutrients for the culture of *S. platensis* (Ungsethaphand et al., 2007). The use of animal wastes as a source of nutrients has also been reported as feasible (Chiu et al. 1980; Yang and Duerr 1987).

The aim of this work is to study the production of *S. platensis* by the use of sea water supplemented with PDM and sodium bicarbonate. The chemical composition of the *S. platensis* biomass is also investigated.

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Materials and Methods

Culture collection and maintenance

Spirulina platensis was obtained from the Department of Microbiology, Annamalai University. The culture was routinely maintained in modified Zarrouk liquid medium and pH was adjusted to 8.8 - 9.0. All the reagents used were of analytical grade Growth and maintenance of the culture was done in an illuminated (4500 lux) growth room at 30 ± 2 °C under 12/12 hour light-dark cycles. Manual shaking of cultures was done 3 times daily.

Culture medium

The culture medium used was 20.0 g of poultry dry manure (PDM) was collected from a University Dairy farm at poultry yard. The manure was suspended in 1.0 L of sea water for 7 days before being filter through a cotton filter. Sodium metabisulfite (5 mg/L) was added to prevent microbial contamination. After 24 h, autoclaving at 121° C before the beginning of the experiment. Natural seawater was collected freshly from the coastal belt area Samiyarpettai, Cuddalore District, Tamil nadu.

Cultivation

Spirulina platensis was inoculated in four different media *viz.*, SOT (Zarrouk's media), SW_1, SW_2 and SW_3 as mention in Table. 1. Total 20 flask of 250 ml capacity containing 100 ml of each medium were inoculated with same amount of inoculums. All flasks were kept at temperature 37° C, manual shaking of cultures was done 3 times daily.

Cultivation enriched seawater

Seawater enriched with sodium bicarbonate (NaHCO3) and poultry dry manure solution (PDM) at different concentration mention in Table–1. All flask containing different level of poultry manure concentration were inoculated with same amount of inoculum *Spirulina* cell mass was filtered by filter paper and washed with buffer solution (pH-7) and resuspended in seawater by cyaclomixture for making homogenized mixture. Homogenized culture was used for inoculum. Inoculated flasks were maintained as mention above.

Table.1 Composition of culture medium for the cultivation of *Spirulina platensis*

Ingredients	**SOT (g/l)**	**SW_1 (g/l)**	**SW_2 (g/l)**	**SW_3 (g/l)**
$NaHCo_3$	8.0	1.5	1.5	1.5
$NaNo_3$	1.25	-	-	-
$CaCl_2$	0.02	-	-	-
K_2HPo_4	0.25	-	-	-
K_2So_4	0.50	-	-	-
NaCl	0.50	-	-	-
$MgSo_4$	0.10	-	-	-
FeEDTA	0.004	0.002	0.002	0.002
Sea water(ml)	-	1000	1000	1000
PDM Solution (%)	-	5.0	10.0	15.0
pH	9.0	8.53	8.58	8.60

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Harvesting & processing

Every five days interval one flask from set of 20 was harvested for dry weight determination and further processing. Cells were collected by filtration using Whatman's no 1 filter paper. Collected cells were wash with distilled water twice and dilute HCL (0.0001 N) to remove any excess salt and dust attached to cell surface.

Determination of dry weight

After filtration and washing filter paper was dried in oven at 100°C for 16 hr. Kept desiccators and cool to room temperature. Weight carefully up to 0.0001 g level by weigh balance.

Determination of Chlorophyll a content

To estimate pigments cells were harvested by centrifugation (6000 x rpm, 10 minutes), washed with distilled water. Chlorophyll a was extracted from the cell suspension with 90% (v/v) methanol at 4°C in dim light by repeated freezing and thawing. Centrifugation was carried out until total pigment recovery. The chlorophyll content in the biomass was calculated from the absorbance at 665nm of the methanolic extract (OD_{665} x 13.9 µg ml^{-1}) (Tandeu and Houmard, 1988).

Determination of Phycobiliproteins

A known volume of homogenized suspension was taken and centrifuged (6000 x rpm, 10 minutes). Phycobiliproteins were extracted completely from the pellet using equivalent volume of 0.05M phosphate buffer (0.03 g of Mono sodiumphosphate and 0.08 g of Di sodiumphosphate, pH 7.0) by repeated freezing and thawing. The absorbance of the supernatant was read at 615, 652 and 562 nm and phycobilins were estimated (Bennet and Bogorad, 1973).

Determination of total Carotenoids

A known volume of homogenized algal suspension was centrifuged at 3000 rpm for 5 minutes. The pellet was washed with distilled water 2-3 times to remove traces of adhering salts. To the pellet, added 2-3 ml of acetone (85%) which was then subjected to repeated freezing and thawing. The suspension was centrifuged and the supernatant containing pigment was collected. The extraction was repeated till the supernatant became colorless, for complete recovery of carotenoids. The pooled fractions of supernatants were made-up to a final known volume. The absorbance was taken at 450nm using 85% acetone as blank and the total amount of carotenoids was calculated in µg ml^{-1} as follows (Saleh, *et al*., 2011).

$$C = \frac{D \times V \, F}{2500 \times 100}$$

D = OD at 450nm
V = Volume of the extract, and
F = Dilution factor
(Assuming that average extinction coefficient of pigments is 2500)

Results

Cultivation

Spirulina platensis was successfully cultured in Zarrouk's medium (SOT) and Sea water medium with PDM supplementations (SW_1, $SW2_2$ & SW_3) cultivated for twenty five days, microscopic & macroscopic observation were determined on five days interval basis. *Spirulina platensis* grows well in SOT culture. Appearance of culture also shifted from light green to dark green in proportion to the increasing cell mass. While cultivation of *Spirulina platensis* in SW, both pH and appearance dose not changed as compared to cultivation in SOT medium.

Cells were collected by filtration using whatman's no 1 filter paper. Collected cells were wash with distilled water twice and dilute HCL (0.0001 N) to remove any excess salt and dust attached to cell surface.

Determination of dry weight

The Dry biomass of *Spirulina platensis* which was grown under laboratory condition on Zarrouk's medium (SOT) and Sea water medium with CDM supplementations (SW_1, $SW2_2$ & SW_3) were estimated during different period intervals and the results were showed in Table -3. After 20 days, the Dry biomass of *Spirulina platensis* was high in SOT (0.623 mg ml^{-1}) followed by SW_3 (0.608 mg ml^{-1}) and SW_2 (0.572 mg ml^{-1}). The least Dry biomass was recorded in SW_1 (0.518 mg ml^{-1}).

Determination of Chlorophyll a content

After harvesting, the chlorophyll content of *Spirulina platensis* was estimated and the results were showed in figure 1. The highest results were obtained in SOT (6.04 µg ml^{-1}) followed by SW_3 (5.93µg ml^{-1}) and SW_2 (5.21µg ml^{-1}). The least chlorophyll content was recorded in SW_1 (4.90 µg ml^{-1}).

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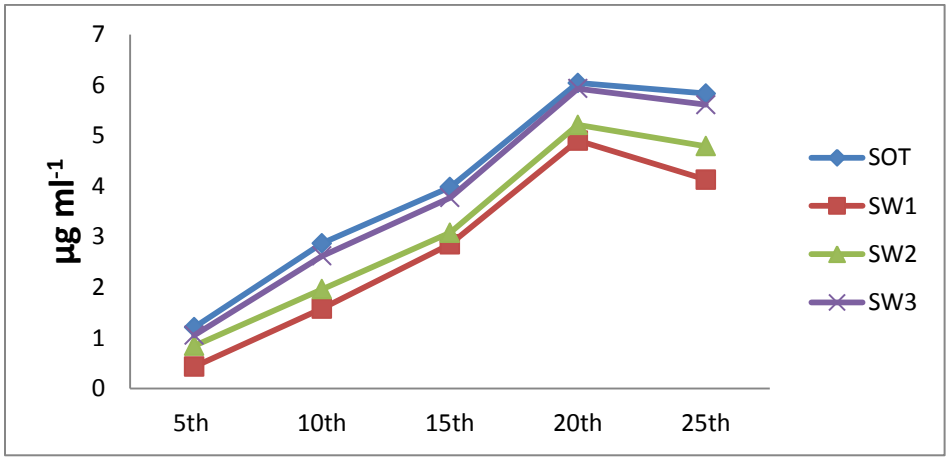
Fig. 1 Estimation of chlorophyll on different medium at periodical interval



Determination of Phycobiliproteins

The concentration of phycobiliproteins were extracted from *Spirulina platensis* (SOT, SW_1, SW_2 & SW_3) were calculated. The heights concentration of phycocyanin was recorded in SOT (70.03 µg ml^{-1}) followed by SW_3 (63.83 µg ml^{-1}) and SW_2 (58.69 µg ml^{-1}). The least phycocyanin concentration was recorded in SP_1 (39.91 µg ml^{-1}) and the results showed in Fig. 2. The high concentration of phycoerythrin was recorded in SOT (27.85 µg ml^{-1}) and the results showed in Fig. 3., followed by SW_3 (23.99 µg ml^{-1}) and SW_2 (20.06µg ml^{-1}). The least phycocyanin concentration was recorded in SP_1 (17.92 µg ml^{-1}). The concentration of allophycocyanin was high in SOT (32.82 µg ml^{-1}) followed by SW_3 (29.08 µg ml^{-1}) and SW_2 (26.08µg ml^{-1}). The least allophycocyanin concentration was recorded in SP_1 (21.38 µg ml^{-1}) and the results showed in Fig. 4.

Fig. 2 Estimation of Phycocyanin (PC) on different medium at periodical interval

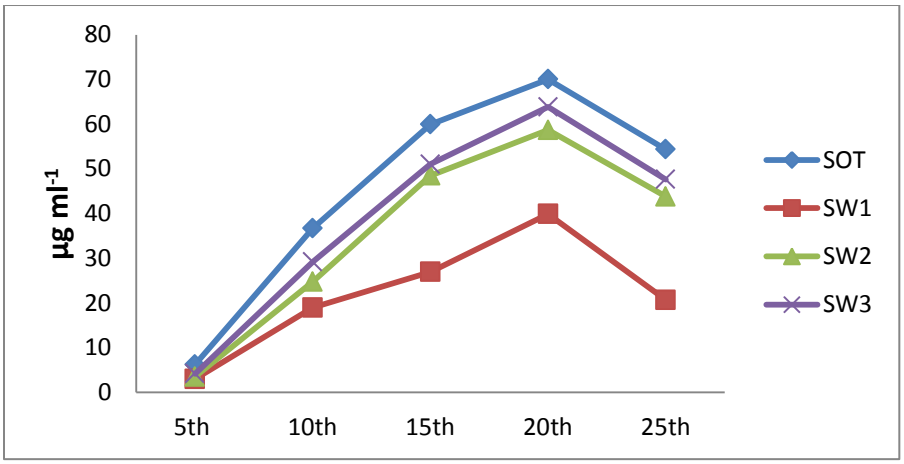


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Fig. 3 **Estimation of Allophycocyanin (APC) on different medium at periodical interval**

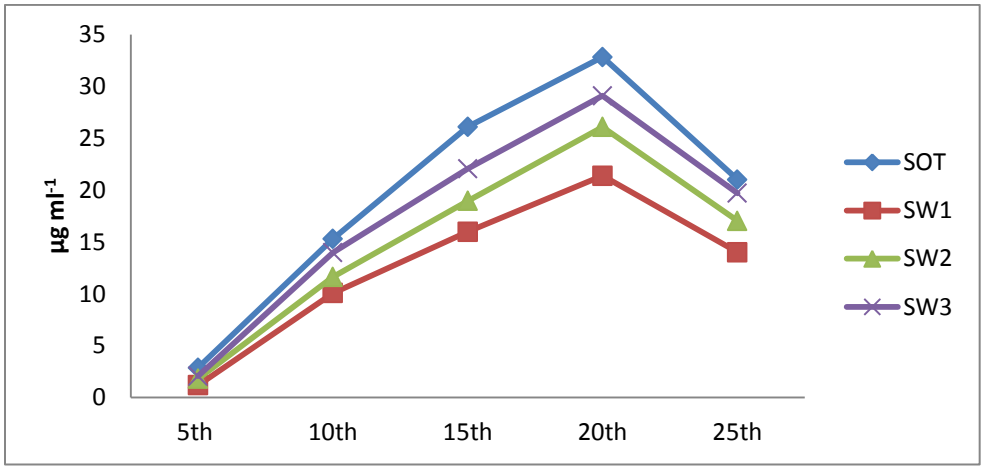
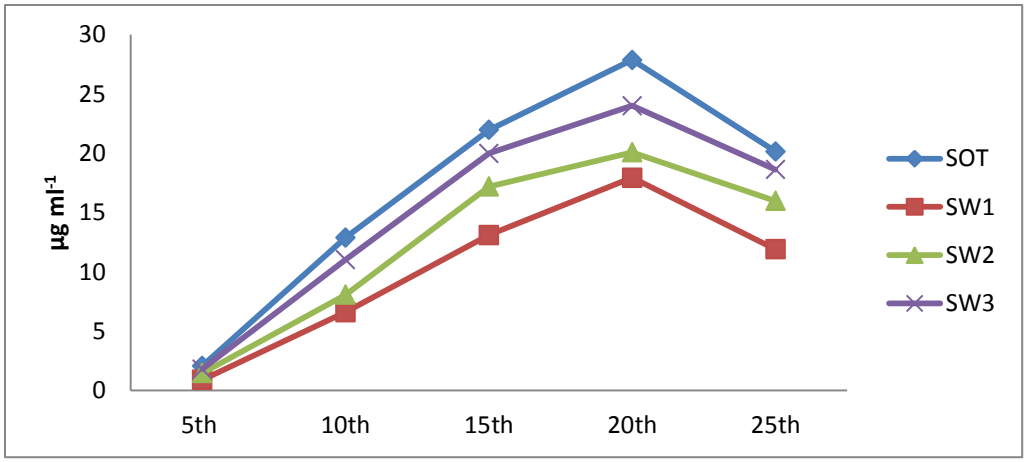


Fig.4 Estimation of Phycoerythrin (PE) on different medium at periodical interval



Determination of total Carotenoids

After harvesting, the carotenoid content of *Spirulina platensis* was estimated and the results were showed in Fig. 5. The highest results were obtained in SOT (31.90 µg ml^{-1}) followed by SW_3 (29.08 µg ml^{-1}) and SW_2 (26.78 µg ml^{-1}). The least amount of total carotenoid content was recorded in SW_1 (23.38 µg ml^{-1}).

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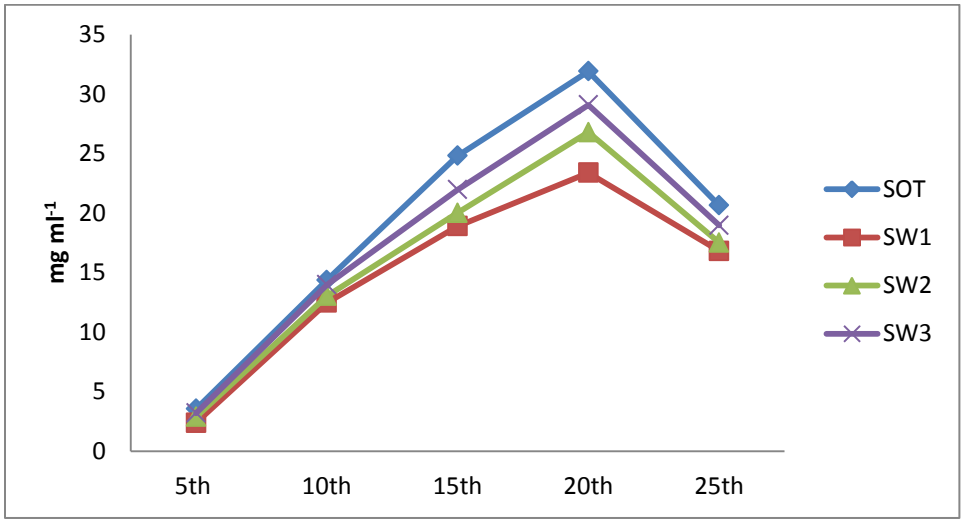
Fig.5 Estimation of total Carotenoids on different medium at periodical interval



Discussion

Spirulina platensis is an economically important filamentous cyanobacterium. The annual production of the algae is about 10, 000 tons which makes it the largest microalgal cultivation industry in the world (Zhang *et al.*, 2005). Due to its richness in protein, phycocyanin, essential amino acids, polysaccharides, carotenoids, minerals, vitamins and essential fatty acids has been regarded as an ideal bio-resource and has drawn increasing attention in recent decades (Moris *et al.*, 2001; Kawata *et al.*, 2004; Chen *et al.*, 2006). Appearance of culture colour was shifted from light green to dark green in proportion to the increasing cell mass. While cultivation of *Spirulina* in SW, both pH and appearance dose not changed as compared to cultivation in SOT medium. Microscopic & visual observation revealed culture was grown healthy and morphology of *Spirulina* filament also
maintain its colour and shape as reported by FAO (FAO, 2008).

Culturing *Spirulina* in conical flask has its limitation in providing complete information related to growth, development and production of value added chemicals, however it would give preliminary information for further demo or commercial level of cultivation (Capone, *et al.*, 1997). The maximum cell dry weight concentration, chlorophyll *a*, phycobiliprotein and total carotenoids content were significantly different among treatments. Phycobiliproteins are important accessory pigments in *Spirulina*. Theses consist of phycocyanin (PC), phycoerythrin (PE) and allophycocyanin (APC) (Saleh, et al., 2011). The highest cell dry weight (0.623 mg ml^{-1}) concentration, chlorophyll *a*(6.04 µg ml^{-1}), phycobiliprotein (PC (70.03 µg ml^{-}), PE (27.85 µg ml^{-1}), APC (32.82 µg ml^{-1})) and total carotenoids (31.90 µg ml^{-1}) content of *S. platensis* when SOT medium was used, followed by using SW_3 (seawater addition of Chicken dry manure (CDM at 15%) gave only a increase in biomass and SW_2 (seawater addition of Chicken dry manure (CDM at 10%) gave slightly increase in biomass while in SW_1 (CDM at 5%) medium, there was actually a decrease in biomass. These results agree with those of Ungsethaphand et al., (2009), found that the best cellular growth and highest protein production were observed for *S. platensis* in the biomass harvested from the culture medium containing DCM supplemented with 2.0 mg/L of urea. Olguín et al., (2003) reported that, the anaerobic effluents from digested pig waste were added in a proportion of 2% (v/v) to untreated sea-water diluted 1:4 with fresh water supplemented with 2 g L^{-1} sodium bicarbonate. A pH value 9.5 ± 0.2 was maintained. The average productivity of semi-continuous cultures during summer 1999 was 14.4 g m^{-2} $d^{-1.}$ This is the highest value reported for a *Spirulina* cultivation system utilising sea-water. The average protein

content of the semi continuous cultures was 48.9% ash-free dry weight. Danesi et al., (2004) has shown that the use of urea as nitrogen source in *S. platensis* cultivation causes an increase in the biomass.

Conclusion

The present study indicates that, natural seawater has potential to grow *Spirulina platensis* along with using dry chicken manure (20.0 g/1L). This culture medium resulted in the best cellular growth and highest pigments content. The potential of reducing production cost with the medium in a large-scale cultivation is also apparent.

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