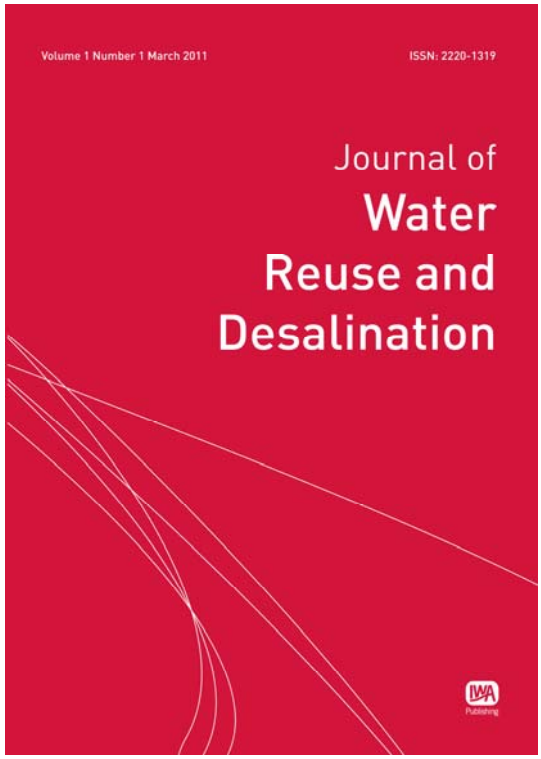

Utilization of inland saline water for *Spirulina* cultivation

K. P. Sandeep, S. P. Shukla, V. Harikrishna, A. P. Muralidhar, A. Vennila, C. S. Purushothaman and R. Ratheesh Kumar

ABSTRACT

An attempt was made to assess the suitability of the inland saline water of Rohtak (Haryana) for mass cultivation of *Spirulina platensis*, a salt loving cyanobacterium. Cultivation of *S. platensis* was performed in indoor and outdoor culture units. The investigation revealed that the yield of biomass in de-calcified inland saline water was comparable to the yield obtained in synthetic chemical-based prescribed growth medium. Further, the quality of biomass in terms of protein, chlorophyll *a*, carotenoids and phycocyanin contents was also comparable to the prescribed medium. The downstream processing of the biomass through a three-step process resulted in an appreciable quantity of a highly valuable pigment, phycocyanin (purity ratio: $A_{620}/A_{280} = 3.13–3.39$). The overall observations of the study suggest that inland saline water can be used for cost-effective production of *Spirulina* biomass and value-added chemicals. The removal of calcium and salts from inland saline water by *S. platensis* cultivation also offers an added advantage for the reuse of the spent medium for agricultural and aquacultural purposes.

Key words | inland saline water, phycocyanin, *Spirulina*

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INTRODUCTION

Due to increasing trends of global population, urbanization and rapid industrialization, a large number of land and water resources are on the verge of ecological degradation. This can lead to a considerable loss in productivity of agricultural lands. Therefore, to ensure food and nutritional security, appropriate measures should be initiated, not only to enhance the productivity of agricultural lands, but also to make judicious use of the land and water resources such as inland saline water and land that is not suitable for agriculture. Globally, about one-third of all agricultural land is either non-fertile or is becoming infertile due to salinity. Therefore, use of the salts in ground-water resources as nutrients for biomass production of valuable salt-tolerant plant species can offer a lucrative option for sustained utilization of inland saline resources.

Salt-affected land has adversely affected about 1,000 million hectares of land in more than 100 countries. In India, about 6.73 million hectares of salt-affected land (CSSRI 2011) is causing huge losses to the poor and marginal farmers, especially in states like Maharashtra and Haryana.

During the recent past, a few initiatives have been taken to utilize inland saline groundwater for the culture of species of fish and prawns like *Lates calcarifer* (Jain *et al.* 2006; Patridge & Lymbery 2008), *Penaeus latisulcatus* (Prangnell & Fotedar 2006), *Penaeus monodon* (Tantulo & Fotedar 2006) and *Pagrus auratus* (Fielder *et al.* 2001), and also for an agar-yielding seaweed *Gracilaria cliftonii* (Kumar *et al.* 2010), etc. Although such practices offer an attractive option for the farmers affected by inland salinity, there is an urgency to further explore the potential plant and animal species which can be grown in inland saline water so that wide-ranging options can be made available to the farmers of salt-affected areas.

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This paper describes the findings of an investigation on feasibility aspects of the production and downstream processing of a commercially viable species of blue-green algae (Cyanobacteria) *Spirulina* (*Arthrospira*) *platensis* grown in inland saline-water-based medium utilizing the saline groundwater of Rohtak, Haryana. *S. platensis* has high nutritional value. It has been declared a 'Superfood' for the 21st century by the World Health Organization.

An attempt was also made to extract phycocyanin, a valuable compound, by using a low-cost extraction process. Phycocyanin is a compound with a wide range of applications, such as antioxidant and anticancer agent, fluorescent tracer in molecular techniques and non-toxic food colourant (Eriksen 2008). Currently, the cost of food-grade phycocyanin (purity $A_{620}/A_{280=}0.7$) is around 0.13 US$ mg^{-1}, whereas the cost of analytical grade (purity > 4.0) is 15 US$ mg^{-1} or above (Cisneros & Rito-Palomares 2004).

This is a first report on utilization of inland saline water for biomass production of a commercially viable species of cyanobacteria. The findings of this investigation will provide baseline information for sustainable utilization of inland saline resources by adopting low-cost and high-return culture practices in inland saline areas.

MATERIALS AND METHODS

Organism and growth conditions

A unialgal culture of cyanobacterium, *S. platensis* was obtained from the Algal Biology Laboratory of Central Institute of Fisheries Education (CIFE), Mumbai. The mother culture was sub-cultured in modified Nallayam Research Centre medium (prescribed by Nallayam Research Centre, Chennai; termed NRC medium in the following text) under photoautotrophic conditions. The batch and airlift culture experiments were carried out at an illumination of 3,500 ± 100 lux using compact fluorescent lamps (Philips, 23 W). The intensity of light was measured using a lux meter (LX-103, Taiwan). The photoperiod was fixed at 12:12 hours light and dark periods. The temperature was maintained at 26 ± 2 °C.

The medium used for cultivation of *S. platensis* was NRC medium with certain modifications. In the modified medium, the urea and phosphoric acid of the medium were replaced by sodium nitrate (2.5 g L^{-1}) and di-potassium hydrogen phosphate (0.5 g L^{-1}), and also the concentration of ferrous sulphate heptahydrate was reduced from 0.5 to 0.01 g L^{-1}. The comparison of the cost of media is presented in Table 2. Indoor batch culture experiments were performed at the Rohtak Centre of CIFE using this medium, and the specific growth rate (SGR) was calculated. The cultures were grown in 250-mL Erlenmeyer flasks containing 100 mL of m-NRC medium inoculated with a known volume of cell suspension of *S. platensis*. SGR was calculated by measuring the change in optical density (OD) at 750 nm every day using a double beam spectrophotometer (UV 1 model, Thermospectronic, UK).

Cultivation of *S. platensis* in inland saline-water-based media at Rohtak

Indoor and outdoor cultivation of *S. platensis* was conducted at CIFE, Rohtak Centre, Haryana, using inland saline water. Inland saline water was collected from different bore wells of the centre. The water with 11 g L^{-1} (11,000 ppm) salinity was used for the present investigation. This concentration corresponds to the very saline water category of the United States Geological Survey classification of inland saline water (Robinove *et al.* 1958). Water quality parameters of inland saline water are given in Table 1.

Indoor culture

Indoor cultivation was conducted in 250-mL Erlenmeyer flasks. A known volume of inoculum was added to different

Table 1 | Water quality parameters of inland saline water of Rohtak, Haryana

S. No.	Water quality parameters	Values
1	pH	8.29
2	Dissolved oxygen	5.6–7.1 mg L^{-1}
3	Phosphate (PO_4-P)	0.31 mg L^{-1}
4	Nitrate (NO_3-N)	0.114 mg L^{-1}
5	Ammonia (NH_3-N)	0.148 mg L^{-1}
6	Salinity	11 g L^{-1}
7	Calcium	580–600 mg L^{-1}

inland saline-water media. The inoculum for the outdoor culture was prepared in the air lift culture assembly. Various treatments were used as follows.

1. Decalcified Inland Saline Water (DISW)
   Raw inland saline water passed through a decalcification unit consisting of a bed of negatively charged zeolite. This DISW was directly used for the *Spirulina* culture. Sodium ions in the zeolite exchange with positively charged Ca^{++} ions from the raw inland saline water up to a certain extent. The treated inland saline water can be used for various aquaculture activities. This system can be used year round without replacing the chemical. Initially, the zeolite should be charged with saline solution (400 g NaCl in 10 L fresh water). The schematic diagram of the unit is given in Figure 1.
2. ISW1 (ISW + $NaHCO_3$ + NaCl)
   Raw inland saline water was mixed with $NaHCO_3$ (8 g L^{-1}) and NaCl (2 g L^{-1}). Calcium precipitate was removed after the addition of $NaHCO_3$.
3. ISW2 (ISW + $NaHCO_3$ + NaCl + K_2SO_4)
   Raw inland saline water was mixed with $NaHCO_3$ (8 g L^{-1}), NaCl (2 g L^{-1}) and K_2SO_4 (0.5 g L^{-1}). Calcium precipitate was removed after the addition of $NaHCO_3$.
4. ISW3 (ISW + $NaHCO_3$ + HWW)
   Hatchery wastewater (HWW) was collected from a *Macrobrachium* hatchery and was mixed with inland saline water after the treatment with $NaHCO_3$ (8 g L^{-1}) in a proportion of 1:2.

Outdoor culture

The outdoor cultivation was carried out in circular FRP (fibre-glass reinforced plastic) tanks of 1,000 L capacity and the volume of the medium was 60 L. The culture was mixed using an air-injection tube and the tank was covered with a polythene sheet to avoid contamination by dust particles and bird and animal droppings and also to prevent water loss due to evaporation. A perforated glass head was attached at the end of the air-injection tube to achieve the uniform distribution of air throughout the medium.

Outdoor cultivation at Rohtak was conducted with the three best inland saline-water-based media selected after indoor cultivation. Along with this, modified NRC medium was also used for culture as a control. Parameters such as temperature, pH and light intensity were measured thrice a day at 10 a.m., 2 p.m. and 5 p.m. Growth of the organisms was monitored every day by measuring turbidity using a spectrophotometer.

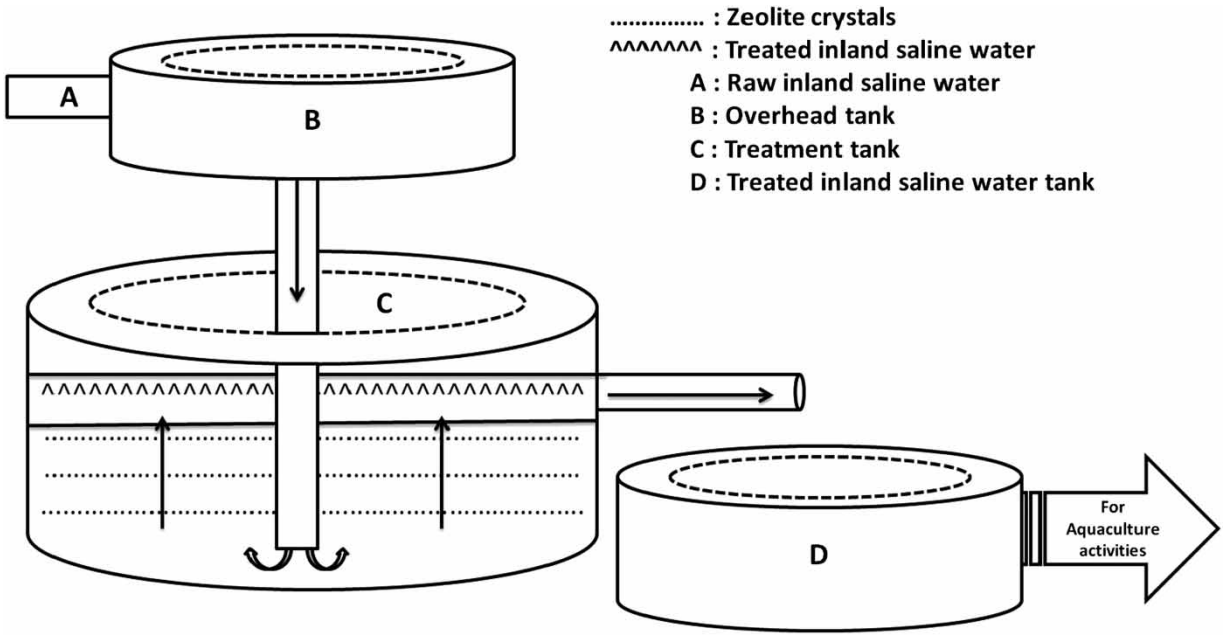

Figure 1 | Schematic diagram of decalcification unit for the removal of calcium.

Growth measurement

The samples were collected aseptically each day and the OD of the cell suspension (turbidity) was measured at 750 nm using a double-beam spectrophotometer (UV 1 model, Thermospectronic, UK). The SGR and generation time were calculated using the formula of Guillard (1973).

Specific growth rate (μ)

SGR was measured during the exponential growth phase and was calculated using the formula given below:

$$\mathrm{SGR}(\mu) = \frac{\ln N_t - \ln N_0}{t_t - t_0}$$

where, N_0 and N_t are the values of absorbance at 750 nm during the exponential phase at time t_0 and t_t, respectively.

Generation time (T_2)

The generation time or mean generation time (days) was calculated using the formula:

$$\text{Generation time} = \frac{\ln(2)}{\mu} = \frac{0.693}{\mu}$$

Pigment estimation

Major pigments in *Spirulina*, such as chlorophyll *a*, phycocyanin and total carotenoids were estimated to assess the quality of the biomass produced in different media.

Estimation of chlorophyll *a*

Chlorophyll was extracted with acetone as a solvent (APHA 2005). The procedure was carried out in subdued light to avoid degradation and amber-coloured bottles were used for storing the acetone solution. Chlorophyll *a* content was estimated using the following formula:

$$\text{Chlorophyll } a\ (\mathrm{mg/L}) = \frac{26.7 \times (A_{664b} - A_{665a}) \times V_1}{V_2 \times L}$$

Subtracted the 750 nm OD value from the readings before (OD 664 nm) and after acidification (OD 665 nm).

Where, V_1 = volume of extract (mL), V_2 = volume of sample (mL); L = path length (cm); A_{664b}, A_{665a} = OD of 90% acetone extract before and after acidification respectively; 26.7 = absorbance correction.

Estimation of phycocyanin

The phycocyanin was extracted by Repeated Freezing and Thawing (RFT) of cells in 50 mM sodium phosphate buffer at a pH of 6.8 (stage 1), and estimated by the method of Siegelman & Kycia (1978). Further, ammonium sulphate treatment (stage 2) was given and dialysed overnight at 4 °C (stage 3) using a D-Tube Dialyzer Max (MWCO 50 KDa). The procedure is given in a flow chart (Figure 2).

The amount of phycocyanin was calculated as mg of phycocyanin per mL using the following equation:

$$\text{Phycocyanin}\ (\mathrm{mg/mL}) = [A_{620} - 0.474 \times A_{652}]/5.34$$

where, A_{620} and A_{652} are the OD values at 620 and 652 nm, respectively.

The purity of phycocyanin extract was calculated in terms of the ratio of optical densities at A_{620}/A_{280} (Boussiba & Richmond 1979).

Estimation of total carotenoids

The Cyanotech (2002) method was adopted to estimate the total carotenoids in dried *Spirulina*. All procedures were performed at low temperature in subdued light conditions, as carotenoids are very sensitive to light, temperature and oxygen.

Estimation of protein

The protein content (%) of *S. platensis* biomass produced in different culture media and from different culture conditions (indoor and outdoor) was estimated by the micro-Kjeldahl method (Pelican Equipments, India). Dry biomass was used for the estimation of protein (AOAC 1984).

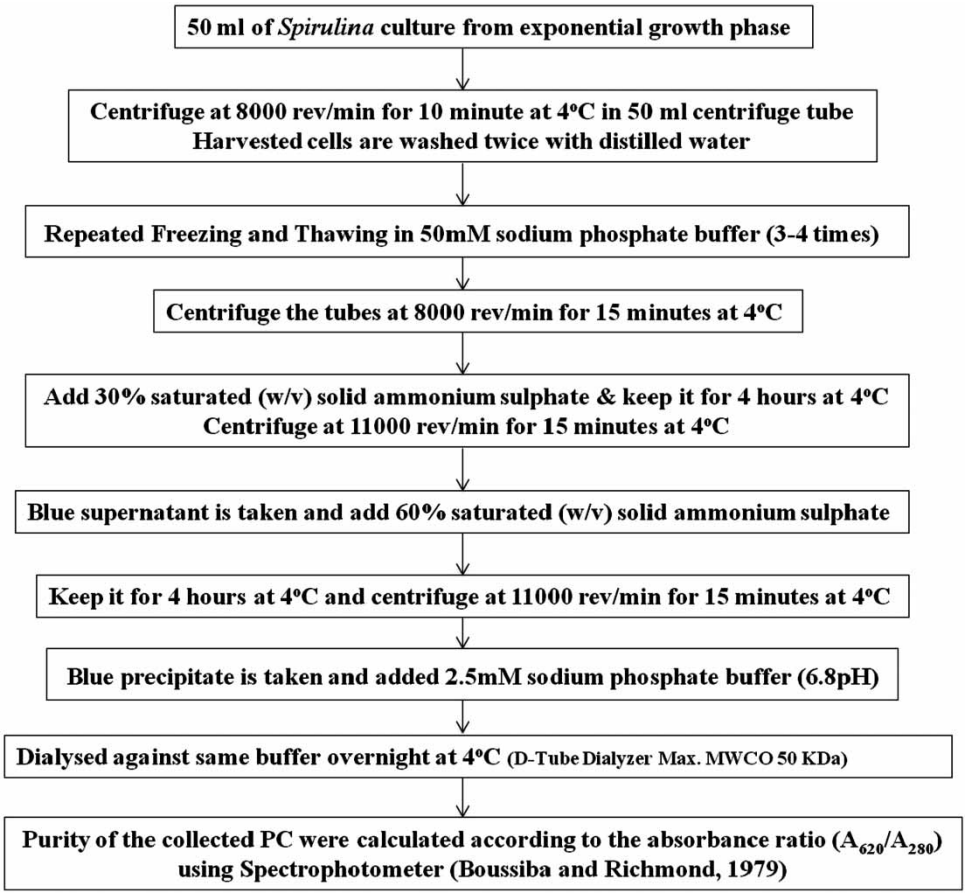

Figure 2 | Flow chart for the extraction and purification of phycocyanin.

Determination of water quality parameters

Water quality parameters, such as temperature, salinity and pH, were estimated before and after cultivation of *S. platensis*. The phosphorus (PO_4-P) content of different media was determined by the ascorbic acid method (APHA 2005). Ammonia (NH_4^+-N) nitrogen concentration was measured spectrophotometrically at 640 nm by phenate method (APHA 2005).

Reagents

Unless otherwise specified, all the reagents used for the cultivation of *S. platensis* were of general reagent grade procured from Merck, India. De-ionized water used for preparation of reagents was produced using a Milli-Q system (Millipore, France).

Statistical analysis

The data were statistically analysed using the statistical package SPSS version 16.0 in which data were subjected to one-way analysis of variance (ANOVA). Duncan's multiple range test was used as a post hoc test to determine the significant differences between the means at 5% significant level.

RESULTS AND DISCUSSION

Growth of *S. platensis* in inland saline-water-based media

Growth of *S. platensis* was measured on the basis of change in turbidity (Abs 750 nm) over 7 d in the indoor culture system using different inland saline-water-based media.

SGR of the populations grown in inland saline water were compared with that of m-NRC medium. The organism exhibited the highest growth rate in DISW, which showed 85.4% growth as compared to m-NRC medium (Figure 3). The treatment ISW1 consisting of ISW with $NaHCO_3$ (8 g L^{-1}) and NaCl (2 g L^{-1}) showed 83% of the growth as compared to m-NRC medium. A 24.4% decrease in growth was observed in the ISW2 as compared to m-NRC medium. The observations indicate that in spite of approximately 15% less growth in comparison to the prescribed growth medium (m-NRC), DISW can be successfully utilized for the cultivation of *S. platensis* without supplementing with any synthetic chemical. The only prerequisite was a treatment with zeolite to reduce the concentration of calcium ions in raw inland saline water; however, this process does not escalate the cost of biomass production as the cost of zeolite is negligible. It was interesting to note that use of inland saline-water-based medium reduced the cost of the medium by more than 90% compared to the prescribed medium which requires various commercially produced ingredients (Table 2).

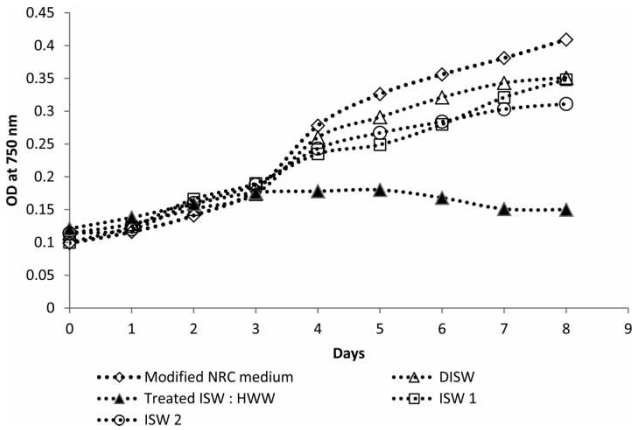

Figure 3 | Growth of *S. platensis* in indoor culture using inland saline-water-based media. DISW = Decalcified Inland Saline Water, ISWI = Inland Saline Water + 8 g L^{-1} $NaHCO_3$ + 2 g L^{-1} NaCl, ISW2 = Inland Saline Water + 8 g L^{-1} $NaHCO_3$ + 2 g L^{-1} NaCl + 0.5 g L^{-1} K_2SO_4, ISW3 = Inland Saline Water + 8 g L^{-1} $NaHCO_3$; Hatchery waste water (2:1).

In an earlier attempt, Leema *et al.* (2010) evaluated the feasibility of utilizing seawater for biomass production of *S. platensis*. They obtained the highest growth rate (0.255 d^{-1}) in a medium consisting of seawater supplemented with $NaHCO_3$ (19.5 g L^{-1}), K_2HPO_4 (0.5 g L^{-1}), $NaNO_3$ (3.0 g L^{-1}) $FeSO_4.7H_2O$ and Fe_2 EDTA (0.01 g L^{-1}). In our investigation, a considerably higher growth rate (0.377 ± 0.004 d^{-1}) was recorded for *S. platensis* grown in inland saline-water-based media. Although decalcification of inland saline water using zeolite was an essential requirement for the softening of raw inland saline water, inland saline-water-based media biomass production of *S. platensis* was more cost-effective than seawater-based media because supplementation of seawater with chemicals (mentioned above) increases the cost of production. We also attempted to increase the growth in inland saline water by amendments with chemicals, viz. $NaHCO_3$, K_2SO_4, NaCl, and HWW but

Table 2 | Comparison of the cost of media used for *Spirulina* cultivation

			Price in USD[a] per 1,000 L			
S.No.	**Ingredients**	**Quantity of chemicals in kg 1,000 L^{-1}**	**Modified NRC medium**	**Decalcified ISW**	**ISW1**	**ISW2**
1	$NaHCO_3$	8	73.3	–	73.3	73.3
2	NaCl	5	22.9	–	9.16	9.16
3	$NaNO_3$	2.5	27.3	–	–	–
4	K_2SO_4	0.5	6.7	–	–	6.65
5	K_2HPO_4	0.5	9.2	–	–	–
6	$MgSO_4.7H_2O$	0.16	1.6	–	–	–
7	$FeSO_4.7H_2O$	0.01	0.13	–	–	–
8	Na-Zeolite (1.2 US $/kg)	–	–	–	–	–
9	Media cost per 1,000 L	–	141.1	2	82.46	89.11

ISW = Inland Saline Water, ISW1 = Inland Saline Water + 8 g L^{-1} $NaHCO_3$ + 2 g L^{-1} NaCl, ISW2 = Inland Saline Water + 8 g L^{-1} $NaHCO_3$ + 2 g L^{-1} NaCl + 0.5 g L^{-1} K_2SO_4.
[a]The exchange rate of USD is Rs 55/- as on 15/09/2012.

no improvement in the growth was observed with these amendments (Figures 3 and 4). Dilution with HWW might have altered the ionic composition in ISW3, which did not support the growth of *S. platensis* to the extent observed in other treatments. In view of the lower cost of chemicals required for growth of the organism in inland saline water, and a comparatively higher growth as compared to sea-water-based media (Leema *et al.* 2010), the inland saline water can be utilized for more cost-effective production of *Spirulina* biomass.

The observations on outdoor cultivation of *S. platensis* in various compositions of inland saline-water-based media revealed that the growth rate in DISW was almost comparable to that observed in the control (m-NRC medium). Maximum growth was noted in DISW, which showed 86.7% growth as compared to m-NRC medium (Figure 4). The treatment ISW1 also exhibited a similar extent of growth (84.5%). However, less growth was recorded in the treatment ISW2, which showed 76% growth as compared to that in m-NRC medium.

In the indoor culture conducted at Rohtak using different inland saline-water media, the SGR was calculated for 7 d culture time (Table 3). Only 8% reduction in SGR from that of m-NRC medium was recorded in the DISW treatment. The SGR of *S. platensis* in DISW was not significantly different ($p > 0.05$) from that of m-NRC medium (control). The SGR in DISW and control was significantly higher ($p < 0.05$) than those of ISW1 (0.213 ± 0.002), ISW2 (0.257 ± 0.003) and ISW3 (0.153 ± 0.004). However, in the outdoor cultivation of *S. platensis* at Rohtak, the SGR in the control was significantly higher than that of the DISW treatment. The SGR in DISW was significantly higher ($p < 0.05$) than those of ISW1 and ISW2 as observed in indoor cultivation.

Table 3 | Specific growth rate of *S. platensis* grown in indoor and outdoor culture using inland saline-water-based media

Specific growth rate	Indoor	Outdoor
Control	0.434 ± 0.004^{d}	0.449 ± 0.015^{d}
DISW	0.401 ± 0.004^{d}	0.377 ± 0.021^{c}
ISW1	0.213 ± 0.002^{b}	0.278 ± 0.015^{b}
ISW2	0.257 ± 0.004^{c}	0.241 ± 0.002^{a}
ISW3	0.153 ± 0.010^{a}	–

ISW1 = Inland Saline Water + 8 g L^{-1} $NaHCO_3$ + 2 g L^{-1} NaCl, ISW2 = Inland Saline Water + 8 g L^{-1} $NaHCO_3$ + 2 g L^{-1} NaCl + 0.5 g L^{-1} K_2SO_4, ISW3 = Inland Saline Water + 8 g L^{-1} $NaHCO_3$: Hatchery wastewater (2:1).
Values with different letters in superscript in the same column differ significantly ($p < 0.05$).

Comparison of protein content in different inland saline-water-based media

The protein content (%) of *S. platensis* biomass obtained from different culture media was estimated using the micro-Kjeldahl method after 7 d of culture. Dry biomass was used in the estimation. The highest protein content was obtained in the DISW ($53.46 \pm 0.20\%$) though it was significantly lower ($p < 0.05$) than that of control

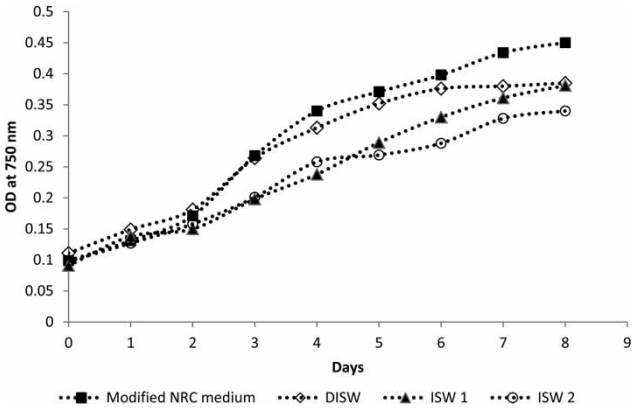

Figure 4 | Growth of *S. platensis* in outdoor culture using inland saline-water-based media. DISW = Decalcified Inland Saline Water, ISWI = Inland Saline Water + 8 g L^{-1} $NaHCO_3$ + 2 g L^{-1} NaCl, ISW2 = Inland Saline Water + 8 g L^{-1} $NaHCO_3$ + 2 g L^{-1} NaCl + 0.5 g L^{-1} K_2SO_4.

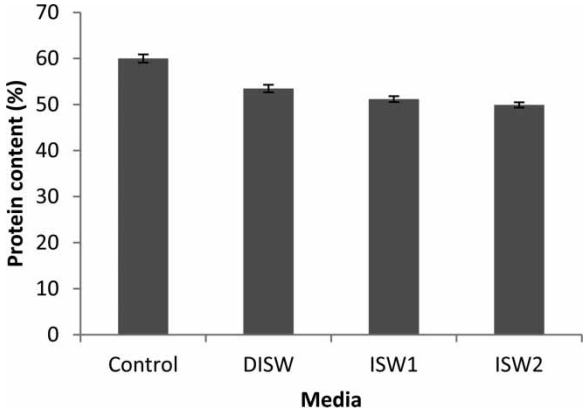

Figure 5 | Protein content (after 7 d of culture) of *S. platensis* in outdoor culture using inland saline-water-based media. ISWI = Inland Saline Water + 8 g L^{-1} $NaHCO_3$ + 2 g L^{-1} NaCl, ISW2 = Inland Saline Water + 8 g L^{-1} $NaHCO_3$ + 2 g L^{-1} NaCl + 0.5 g L^{-1} K_2SO_4.

Table 4 | Yield of pigments during the culture cycle of *S. platensis* under outdoor conditions

	Avg. temperature & light intensity		Yield (mg g^{-1} DW) and percentage (given in the parenthesis) increase of various pigments											
			Control			DISW			ISW1			ISW2		
Day	Temp (°C)	Light intensity (kilo lux)	PHY	CAR	CHL	PHY	CAR	CHL	PHY	CAR	CHL	PHY	CAR	CHL
0	31.66	73.00	14.84 (–)	0.46 (–)	1.36 (–)	14.88 (–)	0.45 (–)	1.40 (–)	12.06 (–)	0.30 (–)	1.39 (–)	14.34 (–)	0.30 (–)	1.44 (–)
1	35.00	77.50	15.67 (5.6)	0.64 (39.0)	1.60 (17.6)	19.97 (34.2)	0.72 (60.0)	2.00 (43.0)	18.28 (51.6)	0.45 (50)	1.82 (30.9)	18.59 (29.6)	0.38 (26.6)	1.87 (29.9)
2	34.33	76.30	20.45 (30.5)	0.80 (25.0)	2.10 (31.2)	24.27 (21.5)	0.87 (20.8)	2.40 (20.0)	19.87 (8.7)	0.49 (8.8)	1.98 (8.8)	23.13 (24.4)	0.48 (26.3)	2.30 (22.9)
3	35.66	79.30	32.06 (56.7)	1.24 (55.0)	3.30 (57.1)	35.39 (45.8)	1.28 (47.1)	3.41 (50.4)	26.23 (32.2)	0.65 (32.6)	2.61 (31.8)	29.42 (27.2)	0.61 (27.1)	2.96 (28.7)
4	38.00	73.00	40.67 (24.8)	1.57 (26.6)	4.20 (27.3)	41.97 (18.6)	1.52 (18.7)	4.23 (17.2)	31.53 (20.2)	0.78 (20)	3.14 (20.3)	37.44 (28.4)	0.78 (27.8)	3.80 (28.4)
5	40.66	74.30	44.38 (9.1)	1.71 (8.9)	4.56 (8.6)	47.19 (12.4)	1.71 (12.5)	4.73 (11.8)	38.39 (21.4)	0.95 (21.8)	3.82 (21.6)	39.38 (4.3)	0.82 (5.1)	3.96 (4.2)
6	39.66	61.33	47.61 (7.3)	1.84 (7.6)	4.86 (6.6)	50.41 (5)	1.83 (7.0)	5.00 (5.7)	43.72 (14.2)	1.08 (13.7)	4.36 (14.1)	42.16 (7.0)	0.88 (7.3)	4.24 (7.0)
7	38.66	60.00	51.92 (9.0)	2.01 (9.2)	5.29 (8.8)	50.95 (1.1)	1.85 (1.1)	5.14 (2.8)	47.83 (9.4)	1.18 (9.3)	4.77 (9.4)	48.01 (13.8)	1.00 (13.6)	4.83 (13.9)
8	39.00	58.66	53.83 (3.7)	2.08 (3.5)	5.49 (3.8)	51.62 (1.2)	1.87 (1.0)	5.21 (1.4)	50.48 (5.5)	1.25 (5.9)	5.03 (5.5)	49.77 (3.7)	1.04 (4.0)	5.01 (3.7)

PHY = Phycocyanin, CAR = Total carotenoids, CHL = Chlorophyll-*a*, DISW = Decalcified Inland Saline Water, ISW1 = Inland Saline Water + 8 g L^{-1} $NaHCO_3$ + 2 g L^{-1} NaCl, ISW2 = Inland Saline Water + 8 g L^{-1} $NaHCO_3$ + 2 g L^{-1} NaCl + 0.5 g L^{-1} K_2SO_4.

(60 ± 0.58%). Similar observations were reported by Tredici *et al.* (1986) and Olguin *et al.* (1997) in the case of outdoor culture of *S. maxima* in seawater supplemented with nitrate or urea. The other inland saline-water-based media, ISW1 and ISW2 also showed a comparable protein content with 51.17 ± 0.24 and 49.90 ± 0.78%, respectively (Figure 5). The value recorded for protein content (50–53% DW) of the population grown in inland saline water was lower than the values reported (59–66% DW) by Leema *et al.* (2010) for the biomass produced in seawater media, however, the protein content of the biomass produced in inland saline water meets the required value of protein content for feed and food-grade biomass of *S. platensis*. The lower protein content of ISW-grown biomass was well compensated by comparatively higher phycocyanin content (about 20% higher) than that reported by Leema *et al.* (2010). The phycocyanin content is shown in Table 4.

Comparison of pigment content in different inland saline-water-based media

Three major pigments, phycocyanin, chlorophyll *a* and carotenoids (mg g^{-1} dry weight), were estimated from the biomass of *S. platensis* harvested during the culture. The yield and daily increases in percentage were estimated. In the case of phycocyanin, 51.62 ± 0.35 mg g^{-1} yield was obtained in DISW, while the pigment content was 53.83 ± 0.42 mg g^{-1} in the control. Chlorophyll *a* content was 5.21 ± 0.04 mg g^{-1} in the DISW and 5.49 ± 0.02 mg g^{-1} in the control. Total carotenoid content estimated in the control and DISW was 2.08 ± 0.04 and 1.87 ± 0.02 mg g^{-1}, respectively (Table 4).

The phycocyanin obtained was subjected to a simple three-stage purification process to obtain high value pigment. The purity of the pigment calculated as ratio of absorbance at 620 and 280 nm (A_{620}/A_{280}) is given in the Table 5. It is clear from the data that the purity ratio increased remarkably from the first step to third step.

Phycocyanin content of the biomass produced in inland saline water was considerably higher (51.62 mg g^{-1} DW) than the values reported for seawater-based media (39.64 mg g^{-1} DW) by Leema *et al.* (2010). The purity of phycocyanin produced in seawater-based media was higher (Abs 620/280 = 4.10) than inland saline water grown cultures (Abs 620/280 = 3.32). In spite of about 20% lower purity than seawater-based media (Leema *et al.* 2010), the extraction procedure used in present investigation did not include expensive steps such as DEAE Sepharose column chromatography.

From the experiment, it is obvious that there is a remarkable reduction of cost in the preparation of culture media. Approximately 98% reduction in the cost per litre was observed in the case of DISW as compared to the prescribed medium (Table 2). A reduction in cost of 41.6 and 36.8% was obtained with ISW1 and ISW2, respectively. For 1,000 L of the m-NRC medium costs US$141.1. While in the case of DISW, ISW1 and ISW2 the cost is US$82.46 and 89.11, respectively. Thus, the production cost of DISW medium is much less than the organic fertilizer medium (US$90) proposed by Ilknur (2012).

The calcium removal in different inland saline-water media per gramme of *Spirulina* biomass is expressed in Figure 6. The maximum amount of calcium removal was observed in the DISW medium followed by raw ISW. A reduction in salinity was observed in all the media after the culture period (Figure 7). Maximum reduction was observed in ISW2 (ISW + 8 g L^{-1} $NaHCO_3$ + 2 g L^{-1} NaCl + 0.5 g L^{-1} K_2SO_4). The utilization of salts present in

Table 5 | Purity ratio (A_{620}/A_{280}) of three stage purification of phycocyanin from outdoor culture of *S. platensis* using inland saline water based media after 7 d (values are mean ± SE)

Stage	Inland saline-water-based media			
	Control	DISW	ISW1	ISW2
Stage 1	0.537 ± 0.0005^{d}	0.504 ± 0.0038^{c}	0.479 ± 0.0043^{b}	0.451 ± 0.001^{a}
Stage 2	1.484 ± 0.0145^{d}	1.234 ± 0.033^{b}	1.373 ± 0.018^{c}	1.10 ± 0.049^{a}
Stage 3	3.551 ± 0.05^{c}	3.328 ± 0.059^{b}	3.40 ± 0.045^{bc}	3.13 ± 0.060^{a}

ISW1 = Inland Saline Water + 8 g L^{-1} $NaHCO_3$ + 2 g L^{-1} NaCl, ISW2 = Inland Saline Water + 8 g L^{-1} $NaHCO_3$ + 2 g L^{-1} NaCl + 0.5 g L^{-1} K_2SO_4. Values with different letters in superscript in the same row differ significantly ($p < 0.05$).

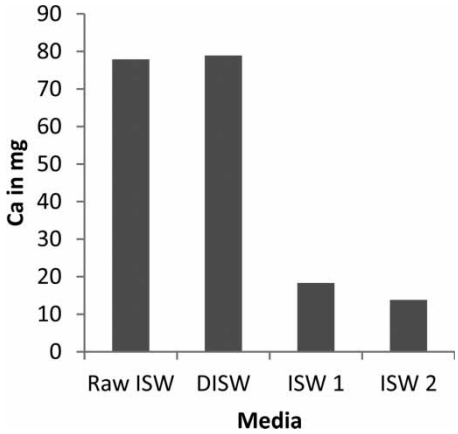

Figure 6 | Calcium removal (per gramme of *Spirulina* biomass) in different inland saline-water media. DISW = Decalcified Inland Saline Water, ISWI = Inland Saline Water + 8 g L^{-1} $NaHCO_3$ + 2 g L^{-1} NaCl, ISW2 = Inland Saline Water + 8 g L^{-1} $NaHCO_3$ + 2 g L^{-1} NaCl + 0.5 g L^{-1} K_2SO_4.

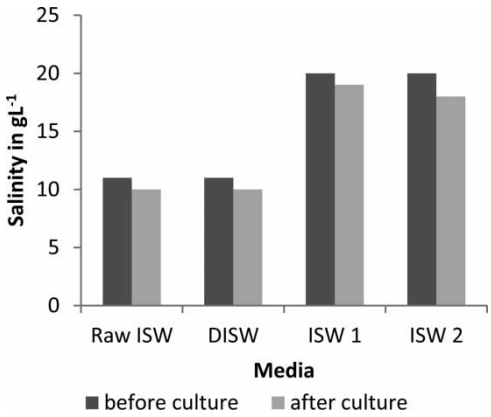

Figure 7 | Changes in salinity of different inland saline-water media before and after *S. platensis* culture. DISW = Decalcified Inland Saline Water, ISWI = Inland Saline Water + 8 g L^{-1} $NaHCO_3$ + 2 g L^{-1} NaCl, ISW2 = Inland Saline Water + 8 g L^{-1} $NaHCO_3$ + 2 g L^{-1} NaCl + 0.5 g L^{-1} K_2SO_4.

inland saline water by *S. platensis* for its growth resulted in a decrease in calcium and salt content of the water at the end of the culture cycle. Apart from the production and cost benefits, the removal of calcium and salts from inland saline water is an added advantage for the reuse of the spent medium for agricultural and aquacultural purposes.

CONCLUSIONS

On the basis of the findings of the present investigation, it is recommended that inland saline water can be utilized as a low-cost growth medium for biomass production of halophilic algae such as *Spirulina platensis*. The type and extent of amendment required may vary and will depend on the physicochemical properties of raw inland saline water of different groundwater sources, therefore, location-specific strategies for utilization of inland saline groundwater resources need to be evolved on the basis of further studies.

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