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Effect of Phosphate and Nitrate Supplement on Growth and Agar Quality in *Gracilaria dura* (C. Agardh, 1842) - *In Vitro* Culture

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ABSTRACT

The present study on growth performance of Gracilaria dura were conducted at Veraval from December 2017 to March 2018. The different concentration of nitrate and phosphate content were given to check the effect on growth, agar quality and Daily Growth Rate (DGR). Initially 200 g of G. Dura ware planted in culture tank of 5 litre capacity in 4 replication Growth and Physico chemical parameters were measured at every 30 days interval, final harvest was done at the end of 120 days. Highest mean growth was observed in treatment T-8 (2 ppm Phosphate and 2 ppm Nitrate) 709.25 ± 9.70 g FW; while, lowest (538.75 ± 7.13) in control condition. Weights of Gracilaria were found significant with increase in time period from 0 to 120 days at interval of 30 days. DGR % has been observed highest (0.651 %) in treatment - 8 at 60 days, while lowest (0.207 %) in control. Estimation of agar agar during that culture period (120 days) at an interval of 30 days show the range of 23.33 ± 1.38 to 34.52 ± 1.28.

Key words: *Gracilaria dura, Seaweed culture, Growth rate, Agar, Gel strength.*

INTRODUCTION

The word seaweed is the popular term that is loosely applied to the sea - weed is the more complex marine algae also called macroalgae. Seaweeds are found in all coastal areas of the world, in all climate zones from the warm tropics to the icy polar regions[9]. Seaweeds play very important ecological roles in many marine communities.

The species of genus *Gracilaria* comprises some of the most economically useful seaweeds in the world. The annual global harvest of *Gracilaria* has been in excess of 37,000 dry tons of which can one-third accounts for aquaculture[10]. Red seaweed production worldwide through artificial farming has gone up from 2 million wet tonnes in 2000 to above 9 million wet tonnes in 2010, *Gracilaria* used for agar production. The global agar production is about 9600 t which cost about US$173 million of which 80 % comes from *Gracilaria* spp.[13]. The red algae *Gracilaria* is world's most cultivated seaweeds with over 3.8 million tons of annual production and worth annually about US $1 billion[1].

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Gracilaria have been mostly cultivated in two Asian countries (China 70% and Indonesia 28% of global production). In the Americas, Chile is the most productive country, producing more than 12.8 tons per year with an annual value of US $29 million[1].

Gracilaria include warm temperate to subtropical eurythermal species. *Gracilaria* are also euryhaline species, which can tolerate a wide range of salinities, from about 10-40 ppt, though they grow best in ranges of 25-33 ppt and They can survive temperature ranges from 0-35°C but have an optimal range of 20-28°C[8,3].

Gracilaria is used as a source of food for both humans and shellfish (abalone). It is also used as a raw material for the extraction of the phycocolloid agar. The value of *Gracilaria* has increased with demand in the Carribbean for human consumption and the production of thickened milk drinks and in Hawaii as a luxury "vegetable/salad". The most important use of agar is in bacteriological and fungal culture work, because after nutrient materials have been added, even a dilute solution sets to a firm jelly upon which the bacteria or fungi grow. Agar is used in a number of countries by embedding preserved cooked fish in the firm jelly, which protects it from breakage. It also prevents the spoiling of foods, especially in hot countries, where cooking with some agar provides a temporary method of preservation[7].

Agar is also used in bakery products for its gelling properties. The agar that is of poorer quality is used as a coating in the manufacture of paper, and also in the manufacture of waterproof cloth. It is used as glue, and as a cleaning medium for liquids. Leather manufacturers consider agar to be very important as it helps with the finishing processes, by adding the gloss and stiffness to the final product. Agar is used as a lubricant in the hot drawing of tungsten wire for electrical lamps, as well as in the photographic industry, in the manufacture of plates and films. The present study aimed to check the effect of phosphate and nitrate nutrients on growth as well as explore the quality of agar with relation to different concentration of phosphate and nitrate of *Gracilaria dura.*

MATERIAL AND METHODS

The present study was conducted at the college of Fisheries, Veraval. The cultivation was attempted during from December 2017 to March 2018. *Gracilaria dura* plants were cultured in seawater for 120 days in aerated tank. *Gracilaria dura* plants were collected from Veraval coast. Young active uniformly growing fronds was collected, and kept in plastic tank (5 litre) and added sodium phosphate as a source of phosphate and sodium nitrate as source of nitrate. The thallus was batch-cultured in plastic tank and 200 gm of fresh weight seaweed was placed in each tank. The seawater was replaced at alternate days and aeration was provided throughout the study period. Treatment was decided according to concentration of phosphate and nitrate. (T0 Control, T1 1 ppm Phosphate, T2 2 ppm Phosphate, T3 3 ppm Phosphate, T4 1 ppm Nitrate, T5 2 ppm Nitrate, T6 3 ppm Nitrate, T7 1 ppm phosphate and 1 ppm Nitrate, T8 2 ppm phosphate and 2 ppm Nitrate, T9 3 ppm Phosphate and 3 ppm Nitrate).

Gracilaria dura were washed in tap water to remove excess salt, dried at room temperature and cut in small pieces. 10g of the samples were incubated in 1 liter of 5% NaOH solution at 70 and 80°C in a water bath for 2 h and washed in tap water for 30 min. The algae were then stirred lightly in 1 liter of 1.5% Sulphuric acid solution at room temperature for 2 h and further washed in tap water overnight to completely eliminate the acid. Samples were boiled for 90 min in 1 liter of distilled water in 2 liter Erlenmeyer flasks fitted with a reflux condenser. The agar extract was filtered through muslin cloth. The filtrate was gelled at room temperature, kept at 20°C for at 15 h.[6]. After the formation of gel at room temperature, the gel was kept at 10 °C overnight in a refrigerator. Gel strength was measured at 20 °C using a gel tester.

For measurement of gelling temperature, 10 ml of the solution containing

agar was allowed to cool gradually and a thermometer was inserted in the solution containing agar. The temperature at which the thermometer was fixed to the gel was noted. For measuring the melting temperature, an iron ball was placed on the surface of the agar gel in a test tube followed by heating of the gel on a water bath until it was melted and the temperature at which the ball touched the bottom of the tube was noted as the melting temperature of the gel[14]. After the formation of gel at room temperature, the gel was kept at 10 °C overnight in a refrigerator. Gel strength was measured at 20 °C using a gel tester.

Daily growth rate (DGR; % day−1) was calculated using the formula of Dawes *et al.*[2] as follows: DGR (% day−1) = ln (Wf / Wo) t−1×100, where Wf is the final fresh weight after t days of culture period and Wo is the initial fresh weight.

Surface seawater temperature, pH, salinity were recorded by using digital meters. Dissolve oxygen was determined by using winkler method. Nitrite nitrogen, phosphate and ammonium were measured by using standard protocol described by Grasshoff *et al.*[5]. Significance of variations in growth was tested by using two way analysis of variance (ANOVA) test as per the statistical methods[12].

RESULTS AND DISCUSSION

Monthly variation in mean growth rate

Mean growth of *Gracilaria dura* in term of weight was of observed highest in T8 treatment (2 ppm Phosphate and 2 ppm Nitrate) 709.25 ± 9.70 at end of 120 days culture period.

At the end of 30 days of culture there was no significant difference among the treatment T7, T8, and T9. However, all these three treatment exhibited higher growth with compared to control and treatment T1 to T6.

At the end of 60 days of culture, the growth of treatment - 8 showed the significant rise while treatment - 1 showed the least growth. The growth may increased because of fronds growing to young stage. After 90 days of culture treatment - 8 showed the maximum growth (609.00 ± 10.23) while lowest in (420.25 ± 9.46) in control.

At the last phase of life cycle (120 days) the highest (709.25 ± 9.70 g FW) growth were observed in T8, compared to the all other treatments.

Previous researchers have studied the effect of nitrate and phosphate supplements on growth rate, photosynthetic capacity and biochemical constitution in different season using Rhodophyceae and Chlorophyceae species. They observed that during the winter season, both nitrate and phosphate enhanced the growth over that of ambient seawater. However phosphate rather than nitrate accounted for more growth as compared to summer season. In current study the effect of phosphate and nitrate has exhibited higher growth as compare to control.

Table 1: Mean growth of *Gracilaria dura* at 30 days interval (g FW)

Treatment	0 days	30 days	60 days	90 days	120 days
T0 (c)	200 ± 0.00	230.70 ± 8.50	317.50 ± 13.8	420.25 ± 9.46	538.75 ± 7.13
T1	200 ± 0.00	247.00 ± 10.6	339.00 ± 16.3	436.25 ± 15.8	558.50 ± 9.29
T2	200 ± 0.00	251.25 ± 11.0	352.50 ± 16.3	443.25 ± 8.46	560.75 ± 11.4
T3	200 ± 0.00	258.50 ± 6.19	360.50 ± 14.7	460.50 ± 13.6	566.50 ± 7.41
T4	200 ± 0.00	255.00 ± 7.25	352.25 ± 5.05	459.75 ± 3.86	576.00 ± 4.32
T5	200 ± 0.00	256.75 ± 4.03	349.25 ± 3.77	457.75 ± 1.70	571.00 ± 3.74
T6	200 ± 0.00	263.75 ± 4.85	367.50 ± 7.72	482.25 ± 16.2	594.50 ± 15.1
T7	200 ± 0.00	281.25 ± 2.98	383.75 ± 6.65	515.00 ± 6.87	638.75 ± 13.0
T8	200 ± 0.00	286.75 ± 7.13	492.25 ± 19.5	609.00 ± 10.2	709.25 ± 9.70
T9	200 ± 0.00	285.00 ± 9.27	414.75 ± 37.7	548.00 ± 23.0	685.00 ± 8.60

T0= Control, T1= 1ppm Phosphate, T2= 2 ppm Phosphate, T3= 3 ppm Phosphate, T4= 1 ppm Nitrate, T5= 2 ppm Nitrate, T6=3 ppm Nitrate, T7= 1 ppm phosphate and 1 ppm Nitrate, T8= 2 ppm phosphate and 2 ppm Nitrate, T9= 3 ppm Phosphate and 3 ppm Nitrate

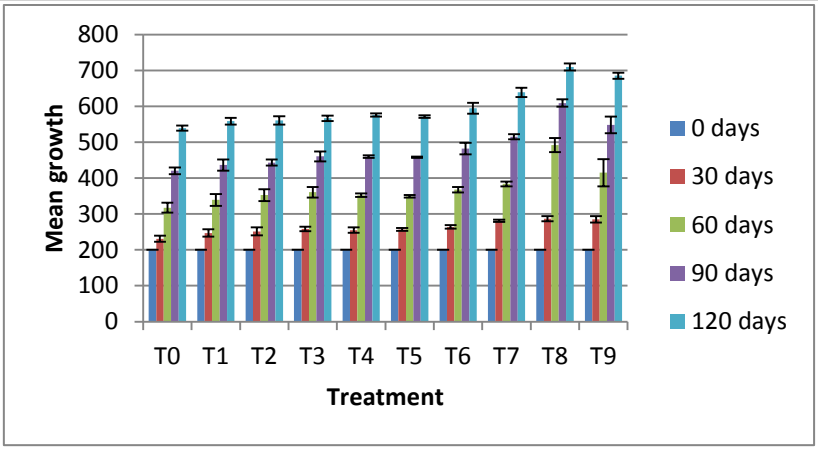

Fig. 1: Mean growth *Gracilaria dura* at 30 days interval (g FW)

Interaction of time and treatment on growth of *G. dura*

The different treatment of phosphate and nitrate significantly influences on the growth (in term of weight). The weight was significantly higher obtained with the mean value of 709.25 ± 9.70 in treatment – 8 (T8) (2ppm phosphate and 2 ppm nitrate), while poor weight 538.75 ± 7.13 were observed in control. Weights of *Gracilari*a were found significant with increased in time period from 0 to 120 days at interval of 30 days. However it may decrease after the growth of 120 days as the fronds become completely matured.

Table 2: Interaction of time and treatment on growth of *Gracilaria dura*

Treatment	P1 (0 days	P2 (30 days)	P3 (60 days)	P4 (90 days)	P5 (120 days)
T0 (c)	200 ± 0.00	230.70 ± 8.50	317.50 ± 13.8	420.25 ± 9.46	538.75 ± 7.13
T1	200 ± 0.00	247.00 ± 10.6	339.00 ± 16.3	436.25 ± 15.8	558.50 ± 9.29
T2	200 ± 0.00	251.25 ± 11.0	352.50 ± 16.3	443.25 ± 8.46	560.75 ± 11.4
T3	200 ± 0.00	258.50 ± 6.19	360.50 ± 14.7	460.50 ± 13.6	566.50 ± 7.41
T4	200 ± 0.00	255.00 ± 7.25	352.25 ± 5.05	459.75 ± 3.86	576.00 ± 4.32
T5	200 ± 0.00	256.75 ± 4.03	349.25 ± 3.77	457.75 ± 1.70	571.00 ± 3.74
T6	200 ± 0.00	263.75 ± 4.85	367.50 ± 7.72	482.25 ± 16.2	594.50 ± 15.1
T7	200 ± 0.00	281.25 ± 2.98	383.75 ± 6.65	515.00 ± 6.87	638.75 ± 13.0
T8	200 ± 0.00	286.75 ± 7.13	492.25 ± 19.5	609.00 ± 10.2	709.25 ± 9.70
T9	200 ± 0.00	285 ± 9.27	414.75 ± 37.7	548.00 ± 23.0	685.00 ± 8.60
Mean	200	261.60	362.92	483.20	599.90
Treatment		**S.Em ±**	**C.D. @ 5%**	**C.V%**	
T		1.86	5.22		
P		2.94	8.25		
T X P		5.59	16.50	2.70	

Daily growth rate (DGR %)

Daily growth rate was observed that the highest (0.651 %) DGR (%) was observed in treatment-8 at 60 days, while lowest (0.207 %) in control. DGR % has been reduced during 90 to 120 days of culture period, which show, that plant attain maturity at this stage; that is why the growth has reduced. Daily growth rate was found to be very low in comparison of work of previous researcher. Gerung *et al.*4 has shown the range of DGR % in *Gracilaria edulis* and *Gracilaria salicornia* ranging from 2.4 to 4.90 % while during study rate of DGR found less than 1 %.

For any commercial cultivation DGR % should be more than 1.5 %, it is economically not viable if the DGR % is less than 1. From this study it can be said that *in vitro* culture of *Gracilari dura* (in confined condition) is economically not viable.

Table 3: Daily growth rate (%)

Treatment	30 days	60 days	90 days	120 days
T0	0.207	0.334	0.358	0.358
T1	0.306	0.382	0.376	0.372
T2	0.330	0.410	0.384	0.373
T3	0.371	0.426	0.402	0.377
T4	0.352	0.841	0.402	0.383
T5	0.362	0.404	0.400	0.380
T6	0.401	0.440	0.425	0.394
T7	0.493	0.471	0.456	0.420
T8	0.521	0.651	0.537	0.458
T9	0.512	0.527	0.486	0.445

Estimation of agar agar

The raw extraction method suggested by Istini *et al.,* 1994 were used for agar estimation. Agar yield (%) ranged from 23.33 ± 1.38 to 34.52 ± 1.28. It was observed that higher agar content (%) was exhibited by treatment - 3 during all the culture time. Highest agar yields (34.52 ± 1.28) were exhibited in T3 at 90 days interval. Estimation of agar agar during that culture period (120 days) at an interval of 30 days show the range of 23.33 ± 1.38 to 34.52 ± 1.28. Higher phosphate content helps to formulate cell wall polysaccharides; this leads to increase in agar content; similar result where observed by Rebello *et al.,* 1996. on the contrary the combination of phosphate with nitrate content at 3 ppm level does not exhibited higher agar yield.

Table 4: Agar yield (%)

Treatment	0 days	30 days	60 days	90 days	120 days
T0 (c)	26.4 ± 1.25	24.2 ± 0.72	25.5 ± 1.28	27.2 ± 1.85	26.4 ± 0.87
T1	26.96 ± 1.12	24.76 ± 1.86	26.06 ± 0.78	27.76 ± 1.25	26.96 ± 1.45
T2	27.25 ± 1.12	25.34 ± 0.88	25.45 ± 0.56	26.45 ± 0.49	27.15 ± 1.23
T3	26.68 ± 1.23	31.25 ± 1.78	33.49 ± 1.66	34.52 ± 1.28	34.12 ± 1.21
T4	25.42 ± 0.88	24.22 ± 1.24	25.48 ± 1.11	26.12 ± 0.78	24.34 ± 1.22
T5	26.54 ± 1.06	25.18 ± 1.24	26.6 ± 1.29	27.24 ± 0.96	25.46 ± 1.4
T6	26.12 ± 0.46	25.18 ± 1.24	23.33 ± 1.38	24.15 ± 1.33	23.87 ± 0.56
T7	27.12 ± 1.32	25.23 ± 0.88	26.44 ± 0.75	27.11 ± 1.25	26.65 ± 1.98
T8	25.45 ± 1.33	26.75 ± 1.62	26.18 ± 0.89	27.11 ± 1.25	26.65 ± 1.98
T9	26.48 ± 1.23	26.08 ± 0.87	25.54 ± 1.62	26.9 ± 1.45	27.18 ± 1.11
ANOVA	*	*	**	*	*

** Significant at 5 %, * Significant at 1%

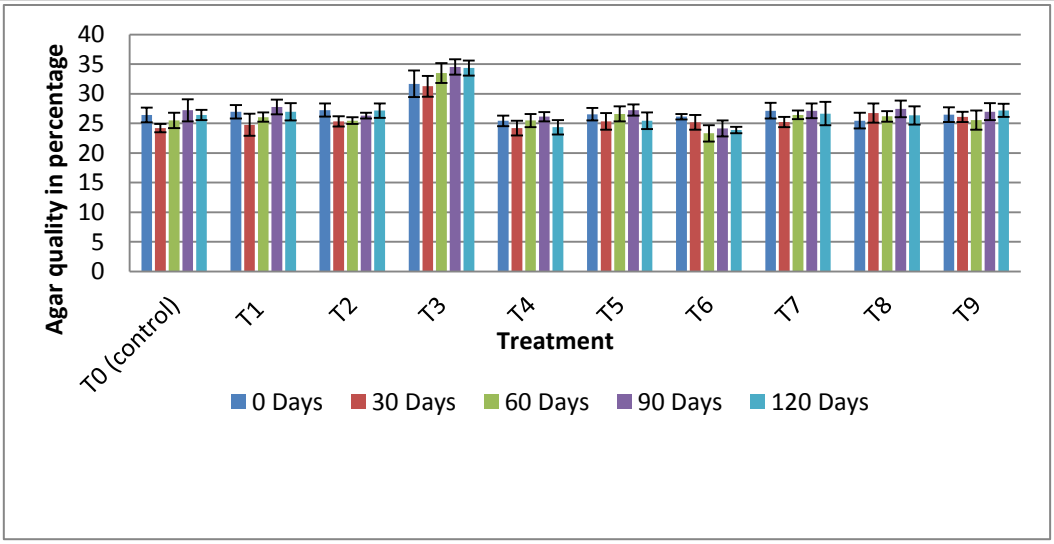

Fig. 2: Agar quality at 30 days interval

Characteristics of agar agar

The properties of Agar agar where characterized at the end of culture period (120 days) the fully mature plants are selected for estimation of agar agar estimation. The basic properties of phycocolloids like gel strength (gcm^{-1}) melting temperature and gelling temperature where estimated in all the treated *Gracilaria dura*.

Table 5: Agar properties in treated *Gracilaria dura*

Treatment	% Agar (at 120 Days)	Gel Strength (g cm^{-1})	Melting Temp (^oC)	Gelling Temp (^oC)
T0	26.4 ± 0.87	475	62.0	32
T1	26.96 ± 1.45	448	58.5	35
T2	27.15 ± 1.23	465	56.5	40
T3	34.12 ± 1.21	435	60.5	35
T4	24.34 ± 1.22	460	55.5	35
T5	25.46 ± 1.4	458	60.5	35
T6	23.87 ± 0.56	465	60.5	38
T7	26.65 ± 1.98	455	55.5	35
T8	26.65 ± 1.98	475	60.5	38
T9	27.18 ± 1.11	455	55.5	40
ANOVA	**	NS	*	*

** Significant at 5 %, * Significant at 1%

The highest (475 g cm^{-1}) gel strength where observed in T8 and control while lowest (435 g cm^{1}) gel strength where observed in treatment 3. It can be said from result that as the agar content increase gel strength may decrease. There were no significant changes among other treatment. Present investigation there was no positive effect for treated *Gracilaria dura* with respect to melting temperature. Melting temperature of agar agar ranged from 55°C to 62.2°C, highest melting temperature observed in control condition, while all the other treatments showed low melting temperature which exhibited the lower quality. Gelling temperature of all treated seaweed range from 32°C to 40°C maximum gelling temperature observed in treatment - 2 and treatment - 9, while lowest gelling temp (32 °C) where observed in control.

CONCLUSION

India has 8219 km of coastline (Lat 8o 4' and 37o 06' N; Long 68o 07' and 97o25' E), a tropical South Asian country has a stretch of

about 7500 km coastline, excluding its island territories with 2 million km2 Exclusive Economic Zone (EEZ) and nine maritime states. The seaweed flora of India is highly diversified and comprises mostly of tropical species, but boreal, temperate and subtropical elements have also been reported.

Results of two factor ANOVA conclude that different treatments were significantly influenced on the growth of *Gracilaria dura* during culture period of 120 days. Significant higher growth was obtained in Treatment-8 (2 ppm Phosphate and 2 ppm Nitrate), Whereas, it's corresponding lowest growth was obtained in control condition.

Different culture period (Monthly) were significantly influence on the growth of *Gracilaria dura* during cultivation period. Significantly the highest growth was obtained at 120 days of culture. which is also reflected in DGR(%). From these results it can be concluded that Treatment-8 (2 ppm Phosphate and 2 ppm Nitrate), is the best suitable nutrient supplement for higher growth of *G. dura*. However, the DGR (%) is not above 1, so it could be said that, the *in vitro* culture is not economically viable.

On the other hand if agar yield has to be considered, then the Treatment 3 (3 ppm Phosphate) should be taken for consideration. However there is not much significant change in chemical properties of agar, compared to the control.

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