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Selection and development of superior strains through functional trait-based approach in agarophytic red alga *Gracilaria dura* (Rhodophyta)

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Abstract

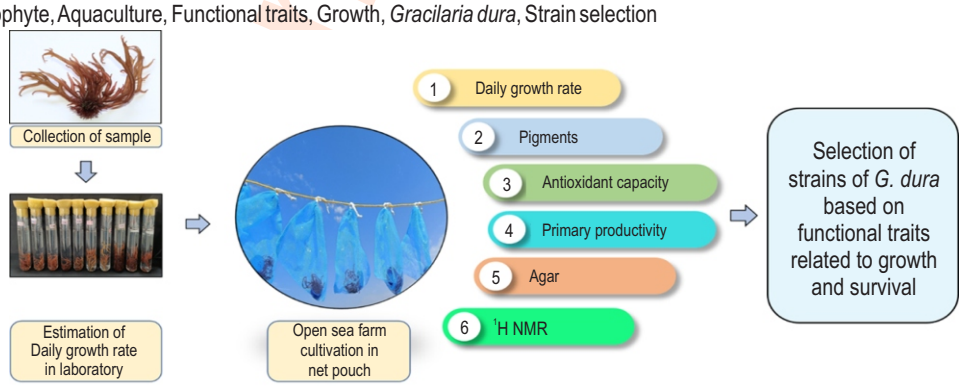
Aim: *Gracilaria dura,* a red agarophyte, is known for its high-quality agarose content and holds significant potential for commercial applications in aquaculture-related industries. The successful commercial utilization of seaweeds relies heavily on obtaining high-quality seed material with commercially valuable traits.

Methodology: This study focuses on employing a functional trait-based approach to develop superior strains of *G. dura* for commercial aquaculture in India. Cultivation through an open sea farm, variations in growth, agar yield, pigments, antioxidant capacity, and primary productivity among the strains were observed.

Results: The daily growth rate ranged from 0.5 to 3.5% per day, while pigment content exhibited variations in the Chlorophyll-a, R-Phycocyanin, and R-Phycoerythrin contents. Antioxidant capacity and gross primary productivity also displayed diverse ranges. From a total of 38 strains of *G. dura*, three were selected based on their growth and other functional traits, namely ADI0221201, VER0220090, and ADI0221202. Positive correlations were identified between growth and regeneration, as well as growth and Chl-a. Agar content showed a positive association with antioxidant capacity and productivity. 1H NMR analysis identified 12 metabolites as potential biomarkers for *G. dura* growth.

Interpretation: All three selected strains hold promise for future commercial cultivation of *Gracilaria dura*. Furthermore, six additional strains were selected based on their higher growth and agar yield for targeted breeding and hybridization to enhance desirable traits.

Key words: Agarophyte, Aquaculture, Functional traits, Growth, *Gracilaria dura*, Strain selection



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Introduction

Algae, encompassing both seaweeds and microalgae, contribute over 30% of global aquaculture production based on fresh weight, with a significant portion attributed to seaweeds (FAO, 2021). Seaweeds are valuable marine bio-resource, contain dietary fibers, unsaturated fatty acids, polyphenols, and many other nutrients (Rioux *et al.,* 2017). They are the main contributors to global aquaculture production of 34.7 million tonnes accounting for 97 per cent of world seaweed production in 2019 valuing USD 14.7 billion that comes from cultivation (FAO, 2021). Seaweeds also source important pigments such as phycoerythrin, phycocyanin, carotenoids and fucoxanthins. The genus *Gracilaria*, is found in intertidal and subtidal zones of Indo-Pacific and Western Atlantic parts of earth. It is the largest genus in Gracilariales, and belongs to the Gracilariaceae family (Torres *et al.,* 2019). The genus *Gracilaria* has been investigated for its nutritional, and biochemical potentials and valuable polysaccharide agar with a fast growth rate, ease of vegetative reproduction and other attributes favouring its cultivation (Porse and Rudolph, 2017). In 2019, a total of 3.6 million tonnes of wet biomass from farmed *Gracilaria* species was produced globally, contributed by 11 countries (FAO, 2021). India's share of this production is around 5300 tonnes (FW) of biomass, valued between 300 to 500 crore rupees (Ranjan, 2021).

The quality of seedlings for cultivation has become crucial under worse farming environments, such as increased seawater temperatures and more frequent and severe disease outbreaks. Improper management of seedling production, such as use of inbred stocks or repeated vegetative propagation, can lead to a decrease in quality and subsequently loss of agronomic value. For the improvement of seedling quality and production efficiency, genetic improvement technologies such as strain selection (Hwang and Park, 2017), tissue culture (Reddy *et al.,* 2017), breeding (Hwang *et al.,* 2019), hybridization (Zhao *et al*., 2016), as well as life history stage based genetic markers (Sambhwani *et al.,* 2022; Yong *et al.,* 2016) would be helpful. In mariculture, to promote seaweed production along with other methods, strain selection stands out as an appropriate mechanism (Santelices, 1992). Procedures related to strain selection could answer specific problems in the cultivation. For many species utilised in commercial cultivation, strain selection has been reported including *Laminaria lamouroux*, *Porphyra* C. Agardh, *Eucheuma* J. Agardh and *Kappaphycusdoty* (Santelices, 2001).

Some authors have emphasised the importance of colour strains, as in *Eucheuma* and *Kappaphycus* (Dawes, 1992; Paula et al., 2002). *Gracilaria* has been the subject of studies reporting coloration and characterizing morphological variants (Plastino *et al.,* 1999; Ferreira *et al.,* 2006). However, there has been no intensive strain selection effort with *Gracilaria* species (Levy and Friedlander, 1990). Repetitive use of same stock for vegetative propagation is exclusively used for *Gracilaria dura* in large-scale cultivation resulting in a decrease in productivity. Thus, the present work aimed to evaluate the potential strain selection using fronds of *G. dura* cultured in the laboratory over the period and then transfer to an open sea farm. The present study also describes the development of growth-based biomarkers by ^{1}H NMR spectroscopy which may be helpful in the selection of strains with a higher growth rate.

Materials and Methods

Biological material: Sample strains of red agarophyte *Gracilaria dura* were collected from the wild along the Veraval and Adri, north-western coast of Gujarat during low tide from July 2019 to February, 2022. A total of 144 strains of *G. dura* were collected in separate polyethene sealable bags containing seawater and immediately transported back to the laboratory within 8 hr. Sample strains were brought to the laboratory in zip lock plastic bags. Filtered seawater was used to clean the samples to eliminate the epiphyte contamination and calcareous and extraneous adhering materials on the thallus surface. Strains were given alphanumeric accession numbers and cultured in controlled laboratory conditions (12:12 hr photoperiod, 25°C and 50 µmole photons m^{-2} sec^{-1} light).

Growth and regeneration studies: For primary screening, the growth rate of 144 strains was estimated in laboratory. Total 30 fragments of 2 cm length were excised from each strain. Fragments were cultured for 15 days in Erdschreiber seawater media under controlled laboratory conditions (12:12 h photoperiod, 25°C and 50 µmole photons m^{-2} sec^{-1} light). Daily growth rate (DGR% Day^{-1}) was calculated according to the formula given by Dawes *et al.* (1993). All 144 strains varied DGR ranging from 0.5 to 4.6 % per day. In 38 out of 144 strains showed a DGR of more than 3 %. These 38 strains were selected for the open sea farm experiment. The fragments (5 of each strain)~3 cm raised in laboratory conditions were transferred to the open sea farm experimental site at the Coast of Simar, Gujarat. Each fragment was cultured in the net pouch (10 X 10 cm) for a period of 30 days (n=5). After 30 days, all the fragments were weighed individually and the daily growth rate (% per day) was estimated using the formula given by Dawes *et al.* (1993). Survival percent, pigment content, antioxidant capacity, and primary productivity were analyzed. To calculate regeneration, a number of new shoots developed over each fragment was manually noted and the number of shoots per fragment for each strain was estimated.

Estimation of pigment content: A 100 mg algal material was ground to a powder with liquid nitrogen. Phosphate buffer 0.1M (pH 6.8) was added, mixed and the homogenate was incubated overnight at 20°C. After incubation, the homogenate was vortexed and centrifuged at 12000 rpm for 15 min. The supernatant was collected in a separate tube, 0.2 ml buffer was added to the pellet, vortexed, and centrifuged at 12000 rpm for 15 min and both the supernatants were pooled. For the extraction of chlorophyll-a, a similar process was performed using 90 % acetone as a solvent. Chlorophyll-a content was calculated from the equation given by Jeffrey and Humphrey (1975). Phycoerythrin (R-PE) and phycocyanin (R-PC) contents were determined by UV–VIS

spectrophotometry from the equations given by Kursar and Alberte (1983).

Preparation of extract: The extract for of antioxidant activity was prepared by crushing 100 mg fresh algal sample of *G. dura* with liquid nitrogen in a mortar and pestle. Sequential extraction was done by adding 1ml distilled water to the crushed sample, incubated overnight at 4°C and centrifuged at 11, 000 rpm for 15 minutes. The supernatant was collected and to the pellet 70% methanol was added and incubated overnight at 4°C, followed by centrifugation at 11,000 rpm for 15 min. Both the supernatants were pooled and the extract was stored at 4°C for further analysis.

Estimation of total antioxidant activity: Total antioxidant activity assay was conducted following the method of Prieto *et al.* (1999). 100 µl of the above extract was used for the assay. 1 ml mix reagent consisting of 28 mM Sodium phosphate, 4 mM ammonium molybdate and 0.6 M Sulphuric acid was added to the extract. This reaction mixture was incubated for 1 hr at 100°C. After cooling at room temperature, the absorbance was recorded at 695 nm on a UV- Vis spectrophotometer (EPOCH/2 Biotek) and the total antioxidant activity was calculated as equivalent to ascorbic acid as standard.

Estimation of cupric reducing antioxidant capacity (CUPRAC): The cupric reducing antioxidant capacity (CUPRAC) spectrophotometric method was used for determining the antioxidant activity (Apak *et al.,* 2004). To 100 µl extract, 50 µl 10 mM copper chloride, 50 µl ammonium acetate buffer (pH 7) and 50 ml 7.5 mM Neocuproine were added, followed by incubation period of 1 hr at room temperature. The absorbance was recorded at 450 nm wavelength using UV- Vis spectrophotometer (EPOCH/2 Biotek) and cupric reducing antioxidant capacity was calculated equivalent to ascorbic acid as standard.

Estimation of primary productivity: Net primary productivity (NPP) and Respiration (R) were measured by light and dark bottle method (Guillemin *et al*., 2014). 0.3 g fresh vegetative fragments of *G. dura* were placed in 100 mL glass bottles filled with autoclaved filtered seawater (35 PSU). Bottles were placed in controlled laboratory conditions at 25°C, 40 µmole photons m^{-2} sec^{-1} for 12 hrs. Dark bottles were covered with black polythene sheets to stop light. Initial and final dissolved oxygen (O_2) concentration was recorded with a HACH HQ30D DO probe, USA. All oxygen values (mg O_2 L^{-1} hr^{-1}) were converted into carbon values (mg C L^{-1} hr^{-1}) by using a factor of 0.375/PQ (photosynthetic quotient). PQ value was taken as 1 and expressed on dry weight basis.

Extraction of agar: Five gram of fresh chopped algal biomass was dipped in distilled water and incubated overnight at room temperature. Thereafter, the material was incubated in an autoclave at 121 °C for 1 h. The cooked solution was filtered through a 0.45-micron filter membrane. To the filtrate, 2 volume of pre-chilled isopropanol was added to precipitate agar. The agar was collected in a pre-weighed petri plate and dried in a hot air oven at 60°C for one day. Dried agar was crushed to a fine powder using liquid nitrogen (Sambhwani *et al*., 2022).

Extraction of metabolites and developing growth-based marker by ^{1}H NMR: ^{1}H NMR analysis was performed to identify the metabolites that might be related to growth. The analysis was carried out to find and develop growth-based biomarkers in *G. dura.* These biomarkers can be directly helpful to select and identify the strains with higher growth. Two strains VER0220090 and VER0220104 with the highest growth rates were selected. The selected strains showed daily growth rates above 3% in the laboratory as well as in open sea farm conditions. The metabolites were extracted from the two strains VER0220090 and VER0220104 by following the extraction procedure. Fresh biomass (200 g) was powdered using liquid nitrogen and to it 50mM phosphate buffer (pH 6.0) was added and mixed followed by vortexing for 2 min and sonication for 30 min at 55°C. The aqueous extract was centrifuged at 10,000 rpm for 2 min. The supernatant was collected and re-centrifuged for 2 min to obtain a clear solution. This clear solution was transferred to a 5-mm nuclear magnetic resonance (NMR) tube. The NMR tube was cleaned with nitric acid and dried before use. A few drops of D_2O containing a reference standard sodium 3-(trimethylsilyl) propionate-2,2,4,4-d4 (TMSP) was added. 1H NMR spectra was recorded on a Jeol ECZR 600 MHz spectrometer. Spectra for both strains were processed and bucket integration was performed. Spectra were arranged to match their chemical shift buckets. Metabolite was identified by employing NMR databases of HMDB and BMRB (Jaiswar *et al.,* 2021).

Statistical analyses: Laboratory growth rate experiment was conducted in 3 replicates. All other experiments were conducted in 5 replicates (n=5) and the values were expressed as mean ± standard deviation. All the variables were analysed by One-way analysis of variance (ANOVA) and Fisher Least Significant Difference (LSD) tests by Infostat software (Di Renzo *et al.,* 2018). The relationships among the three selected strains and all variables were analyzed by Spearman's correlation coefficient by OrginPro (Learner edition) software.

Results and Discussion

The findings of this study demonstrated the possibility of selecting *G. dura* strains, initially cultured under controlled laboratory conditions and subsequently evaluating their performance in an open sea farm. Variations were observed among the strains, when grown under identical conditions. Heterogeneity was observed in regeneration, growth rates, pigments, antioxidant capacity, primary productivity, and yield of native agar. These differences in functional traits may be attributed to intraspecific genotypic variations. Consequently, we hypothesize that *G. dura* populations possess inherent genetic diversity at the genotypic level or exhibit heterosis. Therefore, *G. dura* populations could serve as a resource for selecting individuals with desirable traits or as matrices for crossbreeding to achieve higher biomass production and/or agar yield. The

growth rates of the strains cultured in open sea farm conditions confirmed significant differences. The daily growth rate (DGR) ranged from 0.5±0.3 to 3.5±1.3 % (Table 1). Some strains grew with DGR above 3% similar to controlled culture conditions. The highest growth rate with 3.5±1.5% was observed in strain VER0220090 (F= 3.0, p<0.0001).The average growth of *G. dura* strains tested in an open sea farm was 33 % lesser than laboratory conditions. The average growth observed was 2 % per day, which was 2-fold higher than the previous study, where the growth was found to be 0.9 % per day in *G. dura* using the net pouch method (Veeragurunathan *et al.,* 2015). Similar patterns of growth rate were observed in certain strains of *Gracilaria birdiae*, where the performance of these strains differed between the laboratory and open sea (Ursi and Plastino, 2001). Previous research has already identified discrepancies in the growth patterns of *G. dura* collected at different time periods. Furthermore, disparities in growth are also noticeable across the various life stages of *G. dura* (Mantri *et al.,* 2021). Furthermore, all the strains of *G. dura* exhibited uneven growth, and similar discrepancies in growth were observed in identical seaweed clones cultured under similar conditions (Santelices, 1992). In clonal organisms, significant variations in phenotype and genotype are expected (Bonga and Durzan, 1985). Physiological, developmental, and genetic differences among different strains are essential factors that may contribute to intra-species variations (Sosa *et al.,* 1998). New shoots emerged in all strains and the regeneration ranged from 1.2 ± 0.5 to 8.6±2.07 shoots per fragment (Table 1). The highest (8.6 ± 2.0) number of shoots per segment was found in strain VER0221206 (F= 2.55, p<0.0001).

The chlorophyll-a content significantly varied among the strains. The Chl-a content ranged from 24.21±1.34 to 62.86± 8.56 µg g^{-1} (Table 1). The highest Chl-a (62.86 ± 8.56 µg g^{-1}) was observed in strain ADI0221205 (F= 29.03, p<0.0001). Significant differences in red pigments content were observed. The R-Phycocyanin (R-PC) content ranged from 23.34±3.86 to 115.26 ± 2.13 µg g^{-1} (Table 1). The highest R-PC (115.26±2.13 µg g^{-1}) was observed in strain VER0121200 (F= 27.23, p<0.0001). The R-Phycoerythrin (R-PE) content ranged from 46.94 ± 1.66 to 267.61 ± 5.55 µg g^{-1} (Table 1). The highest (267.6 ± 5.56 µg g^{-1}) R-PE content was observed in strain ADI0121194 (F= 37.34, p<0.0001). Inorganic carbon, nitrogen and phosphorous with other environmental factors are essential for the growth of seaweeds (Roleda and Hurd, 2019). In this study, strains were cultured in a nutrient-enriched medium in the laboratory while in the open sea withouta medium. Hence, the availability of nutrients was higher in the laboratory than in the open sea, resulting in differences in the growth. The availability of higher nitrogen may increase nitrogen-based compounds. Higher amounts of N-based compounds, such as pigments (Phycobiliproteins and Chlorophyll), increase productivity and growth (Roleda and Hurd, 2019). Here, positive correlations between pigments (Chl-a, R-PE and R-PC) and productivity (NPP and GPP) are observed. The pigments play a crucial role in net productivity by enhancing their ability to capture light and convert it into energy through photosynthesis. The antioxidant capacity of the strains varied significantly in both, total antioxidant capacity (TAC) and CUPric reducing antioxidant capacity (CUPRAC) assay. The CUPRAC antioxidant capacity ranged from 0.294±0.01 to 1.053±0.03 mg g^{-1} (Table 2). The highest (1.053 ± 0.03 mg g^{-1}) CUPRAC antioxidant capacity was observed in strain VER0121197 (F= 27.38, p<0.0001). The TAC ranged from 0.316 ± 0.01 to 0.745 ± 0.02 mg g^{-1} (Table 2). The highest (0.745 ± 0.02 mg g^{-1}) TAC was observed in strain VER0719026 (F= 30.15, p<0.0001). Primary productivity significantly varied in all the strains. The respiration rate ranged from 0.095 ± 0.04 to 0.52± 0.18 mg C2 L^{-1} hr^{-1} (Table 2). The highest respiration rate (0.52 ± 0.18 mg C2 L^{-1} hr^{-1}) was observed in strain VER0221210 (F= 7.86, p<0.0001). The NPP ranged from 0.23 ± 0.01 to 1.09 ± 0.36 mg C L^{-1} hr^{-1} (Table 2). The highest (1.09 ± 0.36 mg C L^{-1} hr^{-1}) NPP rate was observed in strain VER0321219 (F= 4.64, p<0.0001). The GPP ranged from 0.536 ± 0.14 to 1.40 ± 0.31 mg C L^{-1} hr^{-1} (Table 2).

The highest (1.40 ± 0.31 mg C L^{-1} hr^{-1}) GPP was observed in strain VER0321219 (F= 3.86, p<0.0001). Antioxidant compounds are complex molecules that display their role in survival and defense mechanisms as seaweeds are exposed to environmental stresses (Sampath-Wiley *et al.,* 2008). In stress conditions, algae produce metabolites for growth while some pigments and antioxidants are essential for defense and survival (Vieira *et al.,* 2022). During the development of seaweeds, ROS are enhanced under environmental stress conditions like exposure to high light intensity and temperature. The stress conditions are assessed by monitoring various environmental parameters such as temperature, salinity, humidity, light intensity etc. The effects of variations in these parameters are studied on the seaweeds for growth, biochemical analysis and photosynthesis (Kameyana *et al.,* 2021). The primary source of oxygen radicals is photosynthesis, a superoxide molecule that acts as ROS. Antioxidants produced during stress conditions are crucial in scavenging ROS free radicals (Bisch of and Rauntenberger, 2012). To overcome such effects, photosynthetic organisms, evolved to increase antioxidative activity (Savchenko and Tikhonov, 2021). Antioxidants are reported as critical agents to help red algae fight against stress from fluctuations in light intensity, temperature and nutrient levels (Kannaujiya *et al.,* 2017). Antioxidant capacity is highly associated with productivity, possibly related to higher photosynthesis in all strains.

Variations were found in the agar quantity of all the strains. The agar content ranged from 1.28 to 6.78 % FW (Table 2). The highest agar (6.78 %) observed was extracted from the sample ADI0121195. The findings of this study indicate variations in agar content across all the strains. Similar discrepancies have been observed in *Gracilaria birdiae* strains (Ursi *et al.,* 2013) and *G. tikhvahiae* cultivated in outdoor tanks (Cote and Hanisak, 1986). The average agar yield was 4.5 % (based on fresh weight), within the range of 3.4% to 5.02% found in wild and farmed populations of *G. dura* (Sambhwani *et al.,* 2022). Intraspecific variations, environmental conditions, and developmental stages can influence the agar yield in *Gracilaria* (Armisen, 1995). Variations in agar yield have also been observed among different

Table 1: Growth (DGR), regeneration and pigment content (Chl-a, R-PE and R-PC) of 38 *G. dura* strains

Strains	DGR (% per day)	Regeneration (Shoots per fragment)	Chl-a (µg g^{-1})	R-PE (µg g^{-1})	R-PC (µg g^{-1})
VER0719011	2.35±0.9	4.2±2.1	26.2±0.7	46.9±1.7	23.3±3.9
VER0719026	2.43±1.0	3.0±1.4	31.0±0.5	110.3±3.0	50.0±2.2
VER0719033	1.20±0.1	5.2±1.3	49.6±1.6	210.2±5.8	55.3±4.9
VER1219041	1.61±0.8	5.4±1.9	50.4±3.7	153.2±2.8	64.2±3.5
VER0220087	0.48±0.3	3.0±0.7	29.9±3.1	112.2±4.1	41.4±1.6
VER0220090	3.50±1.3	5.2±1.6	48.6±4.2	177.7±2.8	80.9±2.5
VER0220092	1.48±0.7	3.6±0.5	49.6±6.5	184.0±3.8	69.2±2.2
VER0220102	2.65±0.8	5.3±3.4	38.2±4.5	183.8±5.4	55.1±2.4
VER0220104	3.03±1.6	2.8±1.5	41.9±2.9	214.2±5.5	75.4±3.5
VER0121141	2.09±0.4	5.6±2.2	43.0±2.8	150.9±2.1	51.9±1.6
VER0121142	2.38±0.6	7.8±1.8	47.8±3.5	208.4±3.3	72.1±1.7
ADI0121194	1.12±0.5	2.2±1.1	56.2±1.6	267.6±5.6	86.8±2.6
ADI0121195	2.99±0.6	5.0±0.3	29.8±0.7	112.8±3.8	56.9±15.3
VER0121196	2.93±1.2	4.8±0.3	42.2±4.7	164.1±13.8	53.7±2.7
VER0121197	1.38±0.2	3.0±0.8	40.0±7.8	129.9±10.5	46.9±3.6
VER0121198	1.88±1.0	5.3±2.9	25.5±0.5	92.9±27.0	36.7±10.2
VER0121199	2.03±0.6	3.6±1.5	29.9±3.4	130.9±8.9	69.8±7.4
VER0121200	2.17±0.5	4.6±0.5	36.8±2.8	228.6±3.7	115.3±2.1
ADI0221201	2.00±1.2	4.8±0.3	44.8±5.9	193.0±5.5	91.8±1.9
ADI0221202	2.74±0.6	2.8±1.0	29.3±2.1	203.0±3.5	75.2±9.5
ADI0221203	2.08±0.9	3.0±1.6	34.6±2.0	85.2±4.8	41.3±1.8
ADI0221204	1.76±0.3	5.8±01.3	35.6±1.4	91.3±1.6	49.6±1.4
ADI0221205	2.26±0.5	1.5±0.6	62.9±8.6	172.3±3.0	86.0±2.4
VER0221206	2.71±0.7	8.6±2.1	43.2±0.8	135.0±2.3	61.4±2.6
VER0221207	2.26±0.3	2.4±0.5	38.6±3.8	126.0±2.7	52.7±1.6
VER0221208	2.07±0.6	5.8±1.4	47.4±2.2	202.8±4.3	77.1±4.0
VER0221209	1.58±0.5	4.4±1.3	38.1±4.3	150.2±2.3	58.4±2.3
VER0221210	1.01±0.7	3.2±0.4	48.9±0.9	183.7±6.2	72.2±2.0
ADI0321211	2.45±1.2	3.6±2.2	43.8±1.0	126.6±3.5	33.6±1.8
ADI0321212	0.85±0.3	5.8±1.3	33.3±1.1	138.0±1.8	69.0±0.5
ADI0321213	1.51±0.4	4.0±1.2	31.8±0.9	148.0±14.3	75.5±1.4
ADI0321214	1.64±0.5	3.2±1.3	33.4±2.3	95.1±2.7	54.1±1.3
ADI0321215	2.02±0.9	4.6±2.2	41.8±1.0	156.9±7.5	66.1±4.3
VER0321216	2.23±0.7	2.2±0.8	44.7±2.1	156.0±3.0	57.8±1.1
VER0321217	1.64±0.9	5.0±1.7	38.5±2.3	140.8±6.8	64.4±4.4
VER0321218	2.67±0.8	3.0±1.6	36.7±1.1	239.7±13.0	110.4±9.4
VER0321219	1.80±0.5	4.5±1.7	24.2±1.3	108.1±5.1	60.3±2.6
VER0321220	1.42±0.2	1.8±1.5	26.9±1.0	142.5±3.8	52.5±1.7

Values are mean of 5 replicates ± S.D.

strains of *Gracilaria birdiae* cultivated in aquaculture (Ursi *et al.,* 2013). The agar content depends on the productivity and is a significant factor for commercial purposes. ^{1}H NMR spectra were acquired after NMR analysis. The peaks on the spectra were compared with the peaks obtained from the previous NMR studies on *G. dura*. Metabolites were identified after the comparison with these previous studies by Gupta *et al*., (2013). Both the spectra were compared and twelve metabolites showed common peaks between them. These metabolites were identified based on the previous studies (Gupta *et al.,* 2013). Major carbohydrate metabolites found were Glucose (δ 3.75), Galactose (δ 4.0 s), Sucrose (δ 5.4 d), whereas amino acid compounds were Alanin (δ 1.5 d), Aspartate (δ 2.57 d). Other compounds were Lactate (δ 1.33 d), Hypotaurine (δ 2.7 d), L-Cysteinsulfunic acid (δ 4.1 t), Ethanolamine (δ 3.37 d), Creatine (δ 1.5 d), Isethionic acid (δ 3.15 t), and Coniferaldehyde (δ 6.85 d). NMR spectroscopy revealed 12 compounds present, including ethanolamine and isethionic acid as unique compounds found only in *G. dura* from previous studies (Jaiswar *et al.,* 2021). Nitrogen-containing compounds in the 1.55−3.00 ppm region of the NMR spectra are crucial for growth and development of macroalgae. In *G. lemaneiformis,* the nitrogen rate corresponds to the growth rate (Wang *et al.,* 2018).

Similar to the previous studies in *G. dura*, amino acids were identified in the samples (Jaiswar *et al*., 2021). As

Table 2: Total antioxidant capacity(TAC), cupric reducing antioxidant capacity (CUPRAC), Primary productivity (Respiration, NPP and GPP) and agar content of 38 *G. dura* strains

Strains	TAC (mg g^{-1})	CUPRAC (mg g^{-1})	Respiration (mg C_2 L^{-1} hr^{-1})	NPP (mg C_2 L^{-1} hr^{-1})	GPP (mg C_2 L^{-1} hr^{-1})	Agar (% f.wt.)
VER0719011	0.67±0.03	1.0±0.05	0.30±0.05	0.24±0.11	0.54±0.14	2.79
VER0719026	0.75±0.02	0.7±0.05	0.18±0.07	0.87±0.21	1.05±0.18	6.22
VER0719033	0.57±0.04	0.7±0.22	0.41±0.11	0.44±0.23	0.85±0.33	2.41
VER1219041	0.53±0.02	0.7±0.16	0.20±0.07	0.47±0.12	0.67±0.14	4.14
VER0220087	0.58±0.04	0.7±0.1	0.19±0.09	0.63±0.03	0.82±0.09	1.28
VER0220090	0.47±0.01	0.7±0.09	0.21±0.02	0.51±0.08	0.71±0.10	1.57
VER0220092	0.48±0.01	0.6±0.08	0.28±0.12	0.84±0.11	1.12±0.22	4.76
VER0220102	0.64±0.1	0.6±0.1	0.14±0.04	0.65±0.07	0.79±0.08	2.83
VER0220104	0.47±0.01	0.7±0.14	0.32±0.11	0.44±0.22	0.76±0.33	3.44
VER0121141	0.37±0.01	0.6±0.05	0.10±0.05	0.51±0.37	0.60±0.40	4.54
VER0121142	0.32±0.01	0.5±0.03	0.22±0.07	1.05±0.54	1.26±0.61	2.51
ADI0121194	0.50±0.01	0.8±0.11	0.28±0.05	0.43±0.13	0.71±0.15	5.52
ADI0121195	0.42±0.02	0.6±0.04	0.34±0.05	0.63±0.07	0.97±0.05	6.78
VER0121196	0.64±0.02	1.0±0.02	0.34±0.05	0.92±0.19	1.25±0.16	2.87
VER0121197	0.65±0.06	1.1±0.03	0.26±0.06	0.81±0.20	1.06±0.24	4.17
VER0121198	0.47±0.02	0.7±0.02	0.21±0.08	0.99±0.35	1.19±0.36	5.93
VER0121199	0.60±0.02	0.9±0.07	0.31±0.09	0.53±0.27	0.84±0.31	5.69
VER0121200	0.39±0.01	0.6±0.01	0.24±0.11	1.08±0.43	1.32±0.43	5.10
ADI0221201	0.60±0.1	1.0±0.06	0.30±0.07	0.78±0.24	1.08±0.25	6.02
ADI0221202	0.49±0.02	0.8±0.01	0.29±0.06	0.65±0.14	0.94±0.13	5.53
ADI0221203	0.48±0.01	0.8±0.01	0.34±0.04	0.29±0.17	0.63±0.20	4.97
ADI0221204	0.42±0.01	0.6±0.01	0.31±0.04	0.66±0.27	0.97±0.26	4.63
ADI0221205	0.44±0.02	0.8±0.06	0.23±0.02	0.56±0.13	0.79±0.12	4.56
VER0221206	0.46±0.02	0.7±0.05	0.21±0.05	0.64±0.13	0.85±0.10	5.59
VER0221207	0.60±0.02	0.8±0.04	0.25±0.01	0.69±0.04	0.94±0.05	6.59
VER0221208	0.47±0.02	0.8±0.03	0.37±0.07	0.54±0.26	0.91±0.26	6.52
VER0221209	0.38±0.02	0.7±0.05	0.44±0.17	0.86±0.39	1.30±0.26	5.60
VER0221210	0.46±0.03	0.6±0.08	0.52±0.18	0.53±0.19	1.04±0.33	5.64
ADI0321211	0.43±0.01	0.7±0.10	0.35±0.05	0.92±0.14	1.28±0.16	5.55
ADI0321212	0.44±0.01	0.5±0.01	0.42±0.06	0.79±0.73	1.21±0.75	5.11
ADI0321213	0.47±0.02	0.6±0.03	0.31±0.04	0.94±016	1.24±0.20	5.11
ADI0321214	0.40±0.02	0.7±0.06	0.48±0.04	0.29±0.23	0.77±0.21	5.97
ADI0321215	0.42±0.01	0.7±0.01	0.37±0.05	0.41±0.16	0.78±0.16	2.77
VER0321216	0.45±0.01	0.5±0.05	0.47±0.03	0.24±0.11	0.71±0.11	2.66
VER0321217	0.35±0.01	0.3±0.02	0.35±0.04	0.83±0.15	1.19±0.16	4.85
VER0321218	0.48±0.01	0.7±0.02	0.41±0.04	0.32±0.06	0.74±0.04	4.04
VER0321219	0.44±0.02	0.5±0.02	0.31±0.09	1.09±0.36	1.40±0.31	3.30
VER0321220	0.40±0.01	0.3±0.02	0.32±0.10	0.37±0.15	0.69±0.21	4.99

Values are mean of 5 replicates ± S.D.

macroalgae are known to be rich in amino acids, they play an important role in nitrogen transport and serve as storage molecules (Bolton, 2009). At the cellular level, sugar molecules are source of energy. The presence of glucose, galactose, sucrose marks these strains are rich in energy which is helpful in growth. Previous studies have indicated the suitability of using the ^{1}H NMR spectra as a chemo-taxonomic tool (Jaiswar *et al.,* 2021). In the strain selection process, the common metabolites observed in both the strains with higher growth may be considered as growth-based biomarkers. The dissimilarities in growth, agar and biochemical characteristics in different strains were observed when grown in similar culture condition. Strains VER0220090, VER0220104 and ADI0121195 had the highest growth rates in open sea farm while ADI0121195, VER0221207 and VER0221208 has higher agar content. These strains with higher growth and agar can be used to develop new strains by breeding methods such as hybridisation. Three strains namely, VER0220090, ADI0221201 and ADI0221202 were selected with higher growth and having maximum variables in common. These variables were agar content, regeneration, pigment content, antioxidant capacity and primary productivity. These strains might be the potential strains for seedling production in *G. dura* using clonal propagation. Three strains namely, VER0220090, VER0220104 and ADI0121195 with higher growth and three

strains namely, ADI0121195, VER0221207 and VER0221208 with higher agar content were also selected. Developing new strains of *G. dura* by hybridization could be a promising approach to improve growth, agar yield, pigments and antioxidants. These strains with desirable traits can be targeted for breeding and developing hybrids. In red macroalgae, attemps were made to develop inter-species hybrids of *Pyropia* (Gu *et al.,* 2018). An improved strain with hybrid recombinant advantages was developed from inter-species hybridization between *Pyropia* sp. from India and *P. haitanensis* from China (Ding *et al.,* 2018).

The growth rate and photosynthetic pigment contents were more significant than their parental strains, indicating that breeding new strains through hybridization is feasible. Growth and agar content are essential factors for the economic value of *G. dura,* as higher growth rates and agar yields can increase the profitability of seaweed industry. Based on the higher significant growth in open sea farm, 10 strains namely, VER0220090, VER0220104, ADI0121195, VER0719011, ADI0221201, VER0121196, ADI0221202, ADI0321215, VER0321216 and VER0221206 were selected. Further, these strains were compared with regeneration, agar content, pigments, antioxidant capacity and primary productivity. The number of common variables with growth were noted for each strain. Strain ADI0221201 had 6 variables in common, namely agar content, antioxidant capacity (TAC and CUPRAC) and pigments (Chl-a, R-PC and R-PE). Strain VER0220090 had 3 variables in common, namely, regeneration, Chl-a and R-PC. Strain ADI0221202 had 3 variables in common, namely, antioxidant capacity (CUPRAC) and pigments (R-PE and R-PC). These selected 3 strains with highest common variables were analysed by Spearman's correlation coefficient for their relationship with all the variables (Laboratory and field growth, regeneration, agar content, pigments, antioxidant capacity and primary productivity).

The major variables positively associated were agar content, pigments, productivity and antioxidant capacity. Variable laboratory growth showed moderate positive correlation with field growth (0.5) and R-PC (0.5), while a perfect positive correlation with regeneration (1) and Chl-a (1). Variable agar content showed perfect positive correlation with GPP (1), NPP (1), Respiration (1), TAC (1) and CUPRAC (1) while moderate positive correlation with R-PC (0.5) and R-PE (0.5). Variable field growth showed moderate positive correlation between field growth with regeneration (0.5) and Chl-a (0.5). Variable regeneration showed perfect positive correlation with Chl-a (1) while moderate positively correlated with R-PC (0.5). Variable Chl-a showed moderate positive correlation with R-PC (0.5). Red pigment R-PE showed moderate positive correlation with TAC (0.5), CUPRAC (0.5), Respiration (0.5), NPP (0.5) and GPP (0.5). Variable R-PC showed moderate positive correlation with TAC (0.5), CUPRAC (0.5), Respiration (0.5), NPP (0.5) and GPP (0.5). Variable TAC showed perfect positive correlation with CUPRAC (1), Respiration (1), NPP (1) and GPP (1). Variable CUPRAC showed perfect positive correlation which had strong positive association with Respiration (1), NPP (1) and GPP (1). Variable net primary productivity showed perfect positive correlation with GPP (1). For the growth of seaweeds, various macromolecules are essential, such as proteins, chlorophyll, antioxidants and amino acids. Nitrogen is a fundamental element for these macromolecules. Hence, variations in these nitrogenous macromolecules are directly related to the growth (Chen *et al.*, 2023). The correlation analysis in our results showed positive correlations between the regeneration and growth. Considering these macromolecules in seaweeds, a decline in growth under nitrogen-deficient conditions in *G. lemaneiformis* have been observed in the previous study (Liu *et al.*, 2019). Pigments also act as potential antioxidants in *Gracilaria* species because, when present in lesser quantity, antioxidant activity is observed to be reduced (Sedjati *et al*., 2018). Positive associations within these variables describe their importance in the growth and development of *G. dura*.

This study uncovered that the strains of *G. dura,* despite sharing same environment, exhibited differences in functional traits associated with growth and survival. The strains of *G. dura* selected through a functional trait-based approach show potential for future commercial cultivation. Moreover, six strains were selected based on their growth and agar yield for breeding and hybridization, aiming to enhance favourable traits. These findings contribute to the advancement of *G. dura* utilization, establishing guidelines for optimizing biomass yield or production of specific commercially valuable compounds.

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