

Effect of shrimp farm effluent (SFE) on growth, survival, regeneration, and biochemical composition in indigenous red seaweed *Gracilaria corticata var. cylindrica*

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Abstract

The growth and biochemical profile of the red seaweed Gracilaria corticata var. cylindrica was evaluated after in vitro cultivation with effluent from a shrimp Litopenaeus vannamei farming pond. Seaweeds were cultivated for 21 days in sterilized seawater enriched with shrimp effluent with 5 different concentrations (0.1, 1, 10, 50, and 100 mL L^{-1}). Fragments with 50 and 100 mL L⁻¹ showed a higher significant growth rate of $3.53 \pm 0.08\%$ and $3.7 \pm 0.55\%$ respectively compared to sterilized seawater as control ($2.5 \pm 0.15\%$). Survival and regeneration of fragments were higher in the treatments 10, 50 and 100 mL L^{-1} . A 100% survival was observed in 10 and 100 mL L^{-1} , while 100-mL L^{-1} showed 100% regeneration in fragments. The number of branchlets developed per fragment was observed higher (2.33 ± 0.52) and (2.23 ± 0.05) in 50 and 100 mL L⁻¹ respectively. A 50 mL L⁻¹ showed the highest $(124.45 \pm 9.28 \ \mu g \ g^{-1})$ chlorophyll-a, and 100 mL L⁻¹ showed the highest $(54.5\pm5.42 \ \mu g \ g^{-1})$ chlorophyll-b content. Agar yield $(2.8\pm0.32\% \ FW)$, total sugar $(72.6 \pm 2.7\%)$, and 3,6-anhydrogalactose $(8.77 \pm 0.63\%)$ was observed highest in 100 mL L⁻¹ treatment of shrimp effluent, while sulphate content ($1.5 \pm 0.8\%$) found higher in 50 mL L^{-1} . Results showed that effluent treatments positively affected G. corticata var. *cylindrica* growth and biochemical composition, especially 50 and 100 mL L^{-1} , which could be considered a good bio-stimulator for the making of the seedling. This work presents the potential use of effluents from shrimp cultivation as fertilizer, improving the growth and seedling production for sustainable cultivation of endemic red seaweed Gracilaria corticata var. cylindrica.

Keywords Agarophyte · Gracilaria · Biofertilizer · Shrimp farming effluent · Growth

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Introduction

Gracilaria species contributed about 10.67% of the total world aquaculture production of aquatic algae (FAO 2020). This species is the main source of agar production especially food-grade agar because it has been successfully cultivated in Chile, Indonesia (Qin 2018) and India (Veeragurunathan et al. 2015). Agar manufacturing industries produced 14,500 t of agar globally including bacteriological and technical grade agar (Porse and Rudolph 2017). Gracilaria is cultivated on large scale in countries like China, Malaysia, Indonesia, the Philippines, Spain, Portugal, Chile, Peru, and Argentina (Bixler and Porse 2011). Krishnamurthy (1991) reported 32 species of *Gracilaria* in the Indian region. Gracilaria corticata var. cylindrica is endemic to the Indian coast. Native agar reported from Gracilaria corticata showed gel strength below 100 ± 6.19 g cm⁻² (Meena et al. 2008) which makes them extremely important for pharmaceutical applications where low gel strength agar is needed. The agars with low gel strength are used for medical formulations and preparation of special food for cancer survivals and also to impart slimy texture to the products. (Dawange and Jaiswar 2020). The global dysphagia diet thickening agents market was worth US\$ 415 million in 2018 and is expected to witness a CAGR (compound annual growth rate) of 5% from 2019 to 2029. The prevalence of dysphagia is relatively high among the geriatric population, and it has been estimated that around 10-20% of the geriatric population across the globe have swallowing difficulties (Persistence Market Research, 2022). The extremely low gel strength agarophytes are thus crucial, but their existence in nature is limited, and thus there is an immediate requirement to develop cultivation practices for such species. Since Graci*laria* biomass is propagated through vegetative means, development of elite seed material for large-scale cultivation has substantial importance.

Marine shrimp farming is well known aquaculture business; presently, shrimp farming is mainly cultivated in brackishwater for human consumption. Though traditional shrimp farming activity has been carried out in Asia for more than centuries, actual commercial shrimp farming began in the 1970s, and production grew steeply, to match the market demands of region like USA, Japan, and Western Europe. If we see in terms of global trade, China and the USA are the top-most importing countries of shrimp, whereas India is the largest exporting country. More than 40% of all shrimp imported into the USA came from India worth 4426.19 USD million during 2020 – 2021 (https:// mpeda.gov.in/?page_id=438). During the full harvest, wastewater is generated which significantly pollutes the water (Ge et al. 2019). Deprived farm management practices such as littering the farm with wastes resulting from dead shrimps can lead to pollution load into the cultured water. The major problem of this cause is the discharged water effluent from the shrimp farms. The discharged water effluent contains a huge load of nutrients and organic matter. If this water is left untreated, it can cause serious spoiling to the surrounding water bodies and expressively reduce the environmental carrying capacity (https://thefishsite.com/articles/how-to-manage-water-effluent-from-shrimpfarms). Since shrimp farm effluents contain a high amount of nutrients, they could be effectively used for rearing the small filaments of Gracilaria corticata var. cylindrica and also reduce an environmental load of pollution. The use of shrimp farm effluent as a growth medium for seaweed gives remarkable growth and quality to plants (Martins et al. 2020). The present study aims to evaluate the effect of shrimp farm effluents as a source of fertilizer for the development of seedling material and also its evaluation by studying growth, survival, regeneration, pigments, and agar contents.

Materials and methods

Collection of samples

Healthy growing samples of *Gracilaria corticata* var. *cylindrica* were collected from the intertidal area of the Mithi Viradi coast of Gujarat ($21^{\circ} 47 '11.28'' N$, $72^{\circ} 24' 56.69'' E$). Samples were immediately brought to the CSMCRI laboratory in seawater. Samples were cleaned with filtered seawater to remove and clean the attached unwanted particles, sand, and epiphytes. Samples were acclimatized for 5 days in controlled laboratory conditions (35 PSU salinity, $25 \,^{\circ}C$ temperature, $40 \,\mu$ mole light intensity, and $12:12 \, (L:D)$ photoperiod) (Dawange and Jaiswar 2020). *Litopenaeus vannamei* shrimp effluent was collected from a farm pond located at Mannar ($21^{\circ}24'55.3''N \, 72^{\circ}10'08.3''E$) village (Supplementary Fig. 1). The effluent was brought to the laboratory in sampling bottles and filtered through glass microfiber filter (GFC, Whatman, $1.2 \,\mu$ m pore size). It was stored in cool condition for further use.

Study of daily growth rate, survival, and regeneration

Approx. 0.5 cm fragments were excised randomly and acclimatized in seawater for a day. Ten fragments were placed in a 500-mL flask (Stocking density 5 g/L) containing one set of sterilized seawater as control (n=3) and 4 sets (n=3) with different concentrations of filtered Shrimp farm effluent (SFE), i.e., 0.1, 1.0, 10, 50, and 100 mL per liter in sterilized filter seawater with 35 PSU salinity. Standard controlled laboratory conditions were maintained with temperature (25 °C), Light (40 µmole photons m⁻² s⁻¹) and photoperiod 12:12 h (light:dark). Media was replenished every 5 days interval, and fragments were cleaned with a soft brush to reduce fouling. The experiment was carried out in 3 replicates (n=3). After 21 days, fragments were weighed and the daily growth rate (DGR) (% d⁻¹) was estimated by using the formula: DGR(% d⁻¹) = In $\left[\frac{\frac{W_2}{W_1}}{t}\right] * 100$, where W_1 is the initial weight recorded, W_2 is the final weight recorded, and *t* is the total number of days (Dawes et al. 1993).

The survival rate was calculated by the number of initial fragments and final remaining fragments. Regeneration was calculated manually by counting the number of fragments developing new branchlets and the number of branchlets.

Estimation of pigments

R-phycoerythrin (PE) and R-phycocyanin (PC) contents were determined by homogenizing 100 mg fresh sample in mortar and pestle using liquid nitrogen. A 0.8 mL 0.1 M phosphate buffer (6.8 pH) was added to the homogenate and incubated overnight at 4 °C. After incubation, the homogenate was vortex, centrifuged at 13,000 rpm at 4 °C, and supernatant was collected in a 1.5-mL tube. To the pellet, 0.2 mL phosphate buffer was added for the complete extraction of pigments. Absorbance scan was recorded on a dual-beam UV spectrophotometer (EPOCH/2 Biotek, USA). For extraction of chlorophyll-a, a similar process was carried out using 90% acetone as a solvent. The amount of R-PE and R-PC content was calculated according to Sampath-Wiley and Neefus (2007), while Chl-a content was calculated according to Jeffrey and Humphrey (1975).

Agar characterization

Extraction of agar

Native agar was extracted as per the process described by Sambhwani et al. (2022). A 5 g of fresh biomass was dipped in 50 mL of distilled water and kept overnight. It was then cooked at 121 °C for 1 h. The cooked solution was then filtered using a muslin cloth. The filtrate was added to the 2 volumes of prechilled isopropanol for the precipitation of agar. Precipitated agar was collected, then dried, and crushed to a fine powder with liquid nitrogen. The agar was collected in the pre-weighed tube for estimation of total agar content. Total sugar, 3,6-anhydro-galactose and sulphate content was estimated using the native agar.

Estimation of total sugar

Approximately 10 mg native agar was added to $250 \ \mu\text{L}$ of concentrated sulphuric acid and kept for 30 min. After incubation, the volume was made up to 7 mL by adding distilled water and was kept at 121 °C for 1 h. The solution was neutralized by adding calcium carbonate till the effervescence stopped. The solution was centrifuged at 7500 rpm for 10 min. The collected supernatant was used for further total sugar estimation. To the sample, 5% phenol and concentrated sulphuric acid was added and after incubation of 25 min, OD was measured at 390 nm and plotted against glucose as standard (Dubois et al 1956).

Estimation of 3,6-anhydrogalactose

Approximately 10 mg agar was taken to estimate the 3,6-anhydrogalactose content with fructose as standard. In an ice bath, samples were cooled, and a resorcinol acetal mixture was added to it. The mixture was then heated at 80 °C for 10 min before transferring to an ice bath. The violet-pink color developed was read at 555 nm. Conversion of fructose to 3,6-anhydrogalactose, the final values were multiplied by a factor of 1.087 (Yaphe and Arsenault 1965).

Estimation of sulphate content

Approximately 10 mg agar was hydrolyzed in 2.0 N HCl at 100 °C for 1 h in a sealed tube, the hydrolysate was centrifuged at 7000 rpm for 20 min, and the supernatant was used for analysis. Four percent TCA was added to each aliquot followed by the addition of barium chloride (BaCl₂)-Gelatin solution. Potassium sulphate (K_2SO_4) was used as a standard and UV absorbance was measured at 360 nm (Sambhwani et al. 2022).

Statistical analysis

All the experiments were performed in triplicates and the values are expressed as mean \pm standard deviation. The data analysis was carried out by one-way analysis of variance (ANOVA) with Tukey as a post hoc test that was applied at the significance difference level of confidence (α =0.05) with the help of software Infostat (Di Rienzo et al. 2018). Correlation between DGR and survival, regeneration, and no. of branchlet per segment of different concentrations of SFE was carried out using the parametric Pearson's coefficient correlation, with a minimum significance level of *P*<0.05 being accepted.

Results

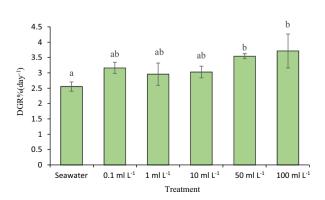
Growth, survival, and regeneration

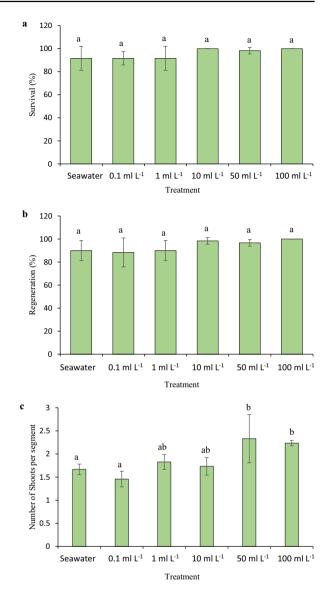
After 21 days of culture, the growth rate (DGR), survival, and regeneration were observed. Highest DGR $(3.7 \pm 0.55\% \text{ d}^{-1})$ was observed in the samples with the treatment of 100 mL L⁻¹ of shrimp farm effluent compared to seawater $(2.54 \pm 0.15\% \text{ d}^{-1})$ culture medium while no significant difference was found in 0.1 mL L⁻¹, 1 mL L⁻¹, and 10 mL L⁻¹ treatments (Fig. 1).

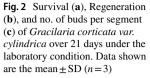
Considering the survival of the fragments, there was no significant difference observed in all the treatments including the control. One hundred percent survival rate was found in treatments 10 mL L^{-1} and 100 mL L^{-1} (Fig. 2a).

No significant difference was observed in the regeneration of new fragments. The 10 mL L⁻¹ and 100 mL L⁻¹ showed the highest regeneration with $98.33 \pm 2.88\%$ and 100% regeneration respectively (Fig. 2b). The number of branchlets developed per segment was found significantly higher in treated fragments compared to seawater. The highest $(2.28 \pm 0.45\%)$ branchlets per segment were observed in treatment with 50 mL L⁻¹ while lowest $(1.33 \pm 0.15\%)$ in 0.1 mL L⁻¹ (Fig. 2c). Correlation between DGR and survival, regeneration, and no. of branchlet per segment of different concentrations of SFE during the study was significant. Estimation of chlorophyll-a, chlorophyll-b, R-phycoerythrin, R-phycocyanin, total agar yield, total sugar content, 3,6-anhydrogalactose content, and sulphate content of native agar was performed for concentration 50 mL L⁻¹ and 100 mL L⁻¹ as it shows highest growth rate.

Fig. 1 Daily growth rate (%) of *Gracilaria corticata var. cylindrica* over 21 days under the laboratory condition. of 25 °C temperature, 40 µmol photons $m^{-2} s.^{-1}$ light intensity, 12-h light period, and 35 PSU salinity. Data shown are the mean \pm SD (n = 3)

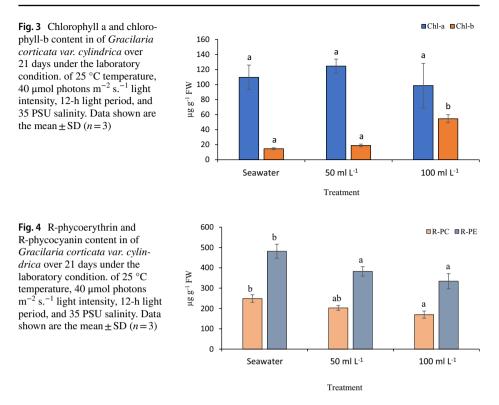






Pigment analysis

Pigment analysis of Chl-a, Chl-b, R-phycocyanin (R-PC), and R-phycoerythrin (R-PE) pigments was performed. No significant difference was observed in Chl-a content, while a significant difference was observed in Chl-b content. Chl-a ranged from 98.57 ± 29.5 to $124.45 \pm 9.28 \ \mu g \ g^{-1}$, while Chl-b found ranging 14.11 ± 8.9 to $54.50 \pm 5.42 \ \mu g \ g^{-1}$. The highest Chl-a content was observed in 50 mL L⁻¹, while the highest Chl-b content was observed in Chl-b content (Fig. 3).



R-PE and R-PC content was found significantly different. Higher R-PE ($481.64 \pm 18.6 \ \mu g \ g^{-1}$) and R-PC ($248.96 \pm 12.11 \ \mu g \ g^{-1}$) were observed in the control compared to fragments treated with shrimp effluent. A decrease in red pigment content was observed as the effluent concentration was increased (Fig. 4).

Agar yield, total sugar, 3,6-anhydrogalactose, and sulphate content

No significant differences were observed in the agar yield and its biochemical analysis. Highest native agar content $(2.85 \pm 0.8\%$ FW) (Fig. 5a), total sugar $(72.65 \pm 7.6\%)$ (Fig. 5b), and 3,6-anhydrogalactose content $(7.8 \pm 0.6\%)$ (Fig. 5c) were observed in the fragments with 100 mL L⁻¹ treatment. Sulphate content was higher $(1.5 \pm 0.8\%)$ in treatment with 50 mL L⁻¹ compared to the control (Fig. 5d).

Discussion

The results confirmed that the effect of shrimp farm effluent on the growth and development of *Gracilaria corticata var. cylindrica* was significant. Seaweeds are known to use nitrogen and phosphorous as primary nutrients for their growth and development (Harrison and Hurd 2001). These two important nutrients were present in sufficient quantity in SFE. Seaweeds have an excellent capacity as biofilters because of their ability to uptake and convert dissolved nutrients for somatic growth (Xu et al. 2008).

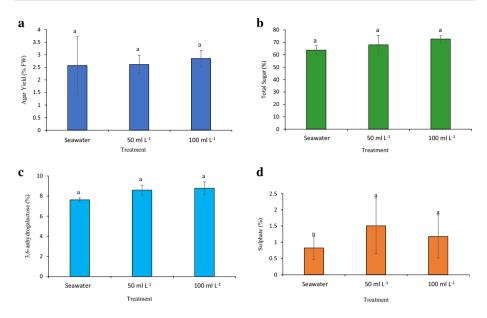


Fig. 5 Total agar yield (**a**), total sugar content (**b**), 3,6-anhydrogalactose content (**c**), and Sulphate content (**d**) of native agar from *Gracilaria corticata var. cylindrica* over 21 days under the laboratory condition. of 25 °C temperature, 40 μ mol photons m⁻² s.⁻¹ light intensity, 12-h light period, and 35 PSU salinity. Data shown are the mean ± SD (*n*=3)

Gracilaria tenuistipitata growth, survival performance, and immune potential were carried out in a biofloc-based Recirculating Aquaculture System (RAS) (Das et al. 2022). Nutrients removal efficiency from aquafarm discharge waters by Gracilaria verrucosa was studied by Devi and Gowri (2007). Macroalgae were found to effectively treat fishpond effluents producing useful algal biomass and recorded a daily growth rate of $1.17 \pm 0.4\%$ and $1.05 \pm 0.25\%$ for *Gracilaria crassa* and *Ulva reticulata* respectively (Msuya and Neori 2002). Highest DGR was found in the plant with the highest concentration of SFE; this may be due to the ability to absorption of a high rate of nitrogen from the medium as in the case of G. changii (Badraeni et al. 2020). Marinho-Soriano et al. (2002) studied SFE as a source of nutrients for the growth of Gracilaria sp., growth rate varied from 8.8% day⁻¹ to 1.8% day⁻¹, whereas in our study, it ranged between 2.38% day⁻¹ and 4.15% day⁻¹ with 42.65% increased as compared to control and this was statistically significant. Marinho-Soriano et al. (2009) again studied the utilization of shrimp farming wastewater as nutrients for evaluation of the growth rate in Gracilaria caudata; his observation revealed that the highest growth rate of 2.28% day⁻¹ which was almost 50% less as compared to the growth of Gracilaria corticata var. cylindrica in our study; however, they were able to achieve the growth rate of 3.32% day⁻¹ by using the tubular net methodology of cultivation. The low growth rate in control fragments shows the requirement of nutrients for the seaweed. Under laboratory conditions, commercially available medium is widely used for the growth and development of seaweed; costly medium does not permit the rearing of seedling on large scale; therefore, use of SFE could be an effective alternative for the development of nursery culture for sustainable seedling availability for large scale seaweed cultivation. The observation of a higher growth rate in fragments treated with SFE indicates

Table 1Elemental compositionof the Shrimp farm effluent water	Element (SFE)	$mg L^{-1}$
	Sodium (Na)	36.7
	Phosphate (PO_4^{-3})	3.00
	Potassium (K)	5.17
	Calcium (Ca)	4.91
	Zinc (Zn)	0.65
	Nitrate (mg N-NO ₃ ⁻ L^{-1})	60.80
	Nitrite (mg N-NO ₂ ⁻ L^{-1})	18.62

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that seaweed was probable with low contents of nitrogen and phosphorous internally. The nutritional state, light intensity, biomass density, water motion, age of the seaweed, structure and surface area of the thallus, initial nutrient concentration, etc. depend on the nutrient absorption by the seaweed to determine the uptake rate at a range of concentrations (Hurd et al. 2014; DeBusk et al. 1986; Zhou et al. 2006; Mao et al. 2009); therefore, seaweed with low nitrogen and phosphorous contents rapidly absorb those nutrients from the surrounding area and consequently exhibited fast growth during the growing period. The efficiency of SFE increased with increasing dose due to the presence of nitrate and phosphate contents at high concentrations (Table 1) which was clearly demarcated in survival, regeneration, and number of branchlets per segment observations. This may be due to the ability to absorb nitrates in water as stated by Badraeni et al. (2020). Since Gracilaria corticata var. cylindrica shows a good growth rate and effective utilization of nutrients from SFE, it could be efficiently utilized in IMTA (Integrated Multitrophic Aquaculture) approach based on this species. In the present study formation of the new branchlet was observed during regeneration on the 5th day, whereas the formation of a new axis in the same seaweed was observed on the 3rd day itself by applying Ascophyllum marine plant extract powder (Dawange and Jaiswar 2020). The regeneration in seaweed is found to be in the form of filamentous callus or bud or branchlet formation. The direct regeneration from the mother tissue makes the propagation of studies plant more cost-effective and economical as it avoids the process of callus induction followed by the requirement of excising and further culturing procedures. It may be noted that the number of Branchlets per segment was highest in 50 mL L⁻¹ and 100 mL L⁻¹ of SFE, and it was statistically significant. The plant obtained by this technique prefers to have a cost-effective strategy for large-scale cultivation. The concentration of chlorophyll a and b pigments increased in the plant treated with SFE since the frequency of nutrients concentration increased by making nitrogen available in the cultivation environment similar results were revealed by De Martino et al. (2021) and this was also observed by Hayashi et al. (2008) and made hypothesis stronger that the enriched thalli accumulated nutrients in chlorophyll form (Dodds et al. 1997), however, it was decreased in case of R-Phycoerythrin and R-Phycocyanin this may be due to the high amount of sodium in SFE similar observation was made by Mihriban et al. (2018) in case of Gracilaria gracilis were PE and PC contents were decreased in higher concentration of salinity. Under laboratory conditions, the chlorophyll concentration was stable with no significant differences. Although the reduction in phycobiliproteins was significant as compared to the control (seawater), it did not affect the seaweed's photosynthetic ability, as it was observed in respective daily growth rates. The average agar yield was observed higher at 100 mL L^{-1} of SFE as compared to the control; however, it was not statistically different from control and 50 mL L⁻¹. The quality of agar is an important aspect, but it remains unchanged in our study; nevertheless, agar yield was increased SFE treated plant which would be considered for economic profitability while considering the seaweed for large-scale cultivation. Since *Gracilaria corticata var. cylindrica* was reported to produce low gel strength agar, their yield was also less compared to other agarophytes like *Gelidium elegans grown* in shrimp broodstock effluent (SBE) that reported higher yield of agar which was significantly (p < 0.05) higher than that in the seawater (Rabiei et al. 2016). It is worth mentioning that the agar contents in seaweeds varies with species, location, season, extraction methods, and environmental condition during culture (Martín et al. 2013; Santelices 1987). Higher sulphate content and higher 3,6-anhydrogalactose value was an indication that it contains inferior quality of agar whereas lower sulphate content ($2.51 \pm 0.21\%$), and higher 3,6-anhydrogalactose value ($31.21 \pm 2.02\%$) was reported from *Gracilaria dura* known for the superior quality of agar (Sambhwani et al. 2022).

Conclusion

Finally, as per the results of this study, we can conclude that development of cultivation technology by applying SFE as nutrients for early seedling development. However, the growth and performance of seedlings need to be assessed at open sea cultivation to confirm the feasibility of using endemic red seaweed *Gracilaria corticata var. cylindrica* for large-scale cultivation. Effective utilization of SFE leads to the development of low-cost treatment techniques and conversion of wastewater into resource. However, further research needs to be done for the essential elemental composition and make necessary adjustments before utilizing SFE in cultivation technique. If we could able to cultivate this species on large scale, it would be a surprisingly add-on to the requirement of low gel strength agar for the formulation of special food for people suffering from dysphasia.

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Author contribution Chetna M. Zala conducted the experiments; Pankaj S. Dawange and Nikunj Balar wrote the manuscript and data analysis; Santlal Jaiswar conceptualized and planning of the work, data analysis, interpretation, and manuscript preparation. All authors reviewed the manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval No animals were used in this study.

Consent for publication All authors agree to give their consent for publication.

Conflict of interests The authors declare no competing interests.

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