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RESEARCH ARTICLE

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Extraction and Characterization of Two Algal Polysaccharide based Microbial Inoculum Beads Prepared from *Gracilaria salicornia*

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ABSTRACT

Background: Thallophytes are marine plants biochemically possessing beneficial compounds aiding human and animal life in various forms, such as medicine, food, polymers, composites and so on. The present investigate aim to study the influence of extractants on the characteristics of two polysaccharides. The two algal polysaccharides were purified from *Gracilaria salicornia*.

Methods: Current work focuses on the extraction, characterization of two polysaccharides and preparation of floridean starch and cellulose nano crystalline-based microbial inoculum complex (MIC beads) encapsulating three important agricultural microorganisms in one bead, which might help farmers with cost-cutting in crop management.

Result: Here *Tricoderma viride* (bio-fungicide), *Azospirillum* (growth promoter) and *Bacillus megaterium var. phosphaticum* (phosphate solubiliser) are encapsulated and treated over brinjal plants, thus aiding with the bio-fungicide effect over phomopsis blight with simultaneous phosphate solubilization and growth improvement.

Key words: Cellulose nanocrystals, Fesem, Floridean starch, Mic bead, Raman spectroscopy, Tg-Dta.

INTRODUCTION

Algae are thallophytic organisms, including a large member ranging from macroscopic to multicellular marine organisms. For marine-inhabited forms, they provide as a source of food and shelter. The three primary families of seaweeds, commonly referred to as macroalgae, are green, brown and red algae. The genus *Gracilaria* is the most prolific and productive resource for the production of agar and has significant cost-effective value as an agarophyte. According to Radiah *et al.* (2011), it has around 150 species, most of which are found in temperate and subtropical regions. Agar has been reported to come from various species of *Gracilaria,* including *G. edulis*, *G. corticata, G. millardetii, G. debilis* (formerly *G. fergusonii*) and *G. salicornia*, in the Indian Ocean and Tanzania (Jaasund, 1976; Kappana and Rao, 1963; Guiry and Guiry, 2021). Red algae are reddish or purplish primitive forms among the three, finding their application in varied industrial areas exclusively for their polysaccharides as agar, carrageenan, nanocellulose, starch and so on. All these polysaccharides find their applications in food, pharmaceutical, industrial and laboratory fields. The aquaculture sector makes extensive use of some plant extracts, like Astragalus spp polysaccharides, due to their inherent anti-inflammatory, pro-immune and antioxidant qualities. Additionally, these polysaccharides have antioxidant enzyme activity and are naturally occurring free radical scavengers (Yajun *et al.,* 2025).

Red algae include a special type of starch called floridean starch, which makes up 80% of the large red algal cell volume and contains amylopectin and a 1,4-glycosidic linked glucose homopolymer with a 1,6-branch that acts as a carbon and energy source in the cells (Yu, 1992).

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Floridean starch is predominant among red algae classes such as Bangiophyceae and Florideophyceae (Mc Cracken and Cain, 1981). The use of a UDP-glucose donor for glycogen synthesis as the primary source for floridean starch production rather than the use of an ADP donor for synthesis like that of higher plants, means that its metabolism happens in the cytosol rather than the chloroplast, catalyzed by 1,4- glycan lyase for 1,5-anhydro-D- Fructose synthesis (Yu *et al.,* 2002).

Cellulose is an abundant and finest biopolymer discovered with enormous properties such as tensile strength, stiffness, biodegradability, light weightiness, high surface area and low-cost production (Lam *et al.,* 2012). Nanocellulose is used in more versatile industries such as pharmaceuticals, food packaging and papermaking (Bettaieb *et al.,* 2015). In the past, cellulose nanocrystals (CNC) were extracted from rice husk, wheat straw and banana trash (Johar *et al.,* 2012; Kaushik *et al.,* 2010).

This was done in response to the growing need for green composite materials with a variety of uses and characteristics. Cellulose nanocrystals as a reinforce agent for combine application with improved, proficient extraction procedures are of recent interest among researchers (Savadekar *et al.,* 2012; Sheltami *et al.,* 2012). Seaweeds are significant cellulose nanocrystals for the properties they possess. They have low natural physicochemical barriers like those of lignocellulosic biomasses, so no chemicals are required for the treatment and extraction of cellulose for biopolymer production, making seaweeds sustainable, eco-friendly and biodegradable nanomaterials as packaging materials. Commercial petroleum-based materials cause environmental pollution with harmful additives (Ma *et al.,* 2016). Extraction of nanocellulose has been reported in members of red algae like *Gelidium elegans* (Chen *et al.,* 2016) and *Gellidiella aceroso* (George, 2012).

In the current work, floridean starch extraction was done by cold extraction with a sequential centrifugation process. The extraction of CNC was done by the ultra-sonication method in a series of steps including dewaxing, alkali treatment, complete delignification and sonication. The polysaccharides extracted were characterized by gelling temperature by TG-DTA, surface topography by FESEM, spectroscopic analysis by Raman, UV spectral analysis for sulfur and anhrogalactose content and gelling strength under a texture analyzer. Further preparation of floridean starch (FS) and cellulose nanocrystal (CNC) MIC beads encapsulating three agriculturally significant microbes and treatment of brinjal plants, measurement of growth rate over a 15- day interval and recording of NPK levels. Seaweed often has a low yield for polysaccharide extractions, but new techniques are being developed to help and recover the extraction processes. Enhancing the physicochemical characteristics of specific polysaccharides, such as gelling, thickening and emulsifying, is the main focus of current research on seaweed polysaccharides. It has been suggested that the construction of small units may result in improved molecules that are also appropriate for biomedical use, even if many of the many bioactivity reports are still just at the research stage and require clinical validation before being commercialized.

MATERIALS AND METHODS

Sample collection and preparation

The species *Gracilaria salicornia* was gathered from the shoreline of Pudupattinam in the Tamil Nadu district of Ramanathapuram (Fig 1) in the month of December 2022. The experiment was conducted in Department of Botany, PSGR Krishnammal College for Women, Peelamedu, Coimbatore during the year 2023-2024. To stop UV light from deteriorating the chemicals, samples were thoroughly cleaned with seawater, followed by fresh water and then sealed in black polybags. After a week of shade drying, the samples were ground into a consistent, fine powder in a kitchen tool known as an analytical mill. After passing through a 60- mesh filter, the seaweed powder was kept in sample bags at 20°C until it was further investigated.

Extraction of floridean starch

Floridean starch extraction (Fig 2) involved the grinding of a 100- gram dry sample using a pestle and mortar under cold conditions. The pulverized sample was combined with 400 mL of 50 mm citrate buffer (pH 6.5) and then filtered using cheesecloth. The final filtrate was centrifuged for 10 minutes at 2000 rpm to extract the pellet containing the starch granules. For the removal of pigments and lipid contaminants, the pellet was centrifuged at 2000 rpm for 5 minutes using 100% ethanol. Subsequent purification was done using 500 mL of 100% ethanol and the excess solvent was evaporated. The final pellet was dried at room temperature for 30 minutes to obtain a dry powder (Prabhu *et al.,* 2019).

In order to further refine the starch granules, 600 ml of 0.165% sodium hydroxide (w/v) was mixed with dry powder

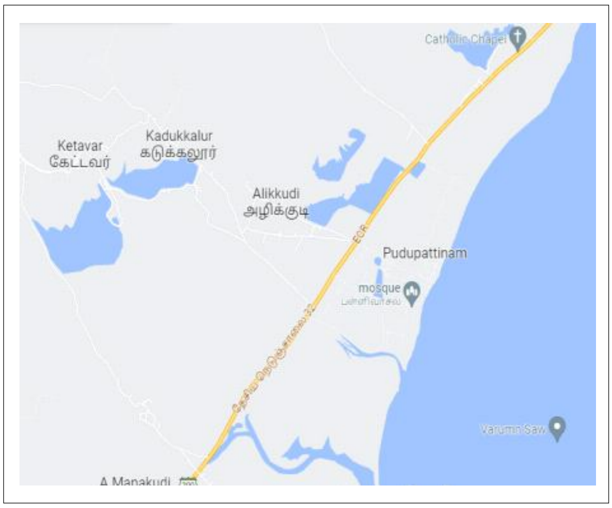

Fig 1: Map of collection site, Pudupattinam coast.

and the mixture was agitated for an hour at 25°C at maximum speed. The mixture was centrifuged for ten minutes at 5000 rpm. In order to get rid of the proteins, the centrifugation and NaOH treatment processes were repeated twice. Following centrifugation, distilled water (DW) and 100% ethanol were used to wash the particle twice. The cellulose was broken down by dissolving the dried starch powder in 200 milliliters of 50 milliliters of sodium acetate buffer (pH 4.5). Following a 10-minute centrifugation at 2000 rpm, the pellet was ethanol-washed and the final floridean starch was dried at 40°C.

Extraction of cellulose nanocrystals

Algal nanocellulose extraction, slightly modified (Fig 3) (Singh *et al.,* 2017). For 30 minutes, the powdered seaweed samples were processed with 2.5 M NaOH in a water bath set at 55°C. Biomass is dewaxed by microwave heating (Brum *et al.,* 2009). After being allowed to cool to ambient temperature, the resulting slurry was filtered through Whatman filter paper. Hot distilled water was used several times to cleanse the filtrate. 55°C is used to oven-dry the sample. The alkali-pretreated sample was bleached for four hours at 55°C using 32% hydrogen peroxide to

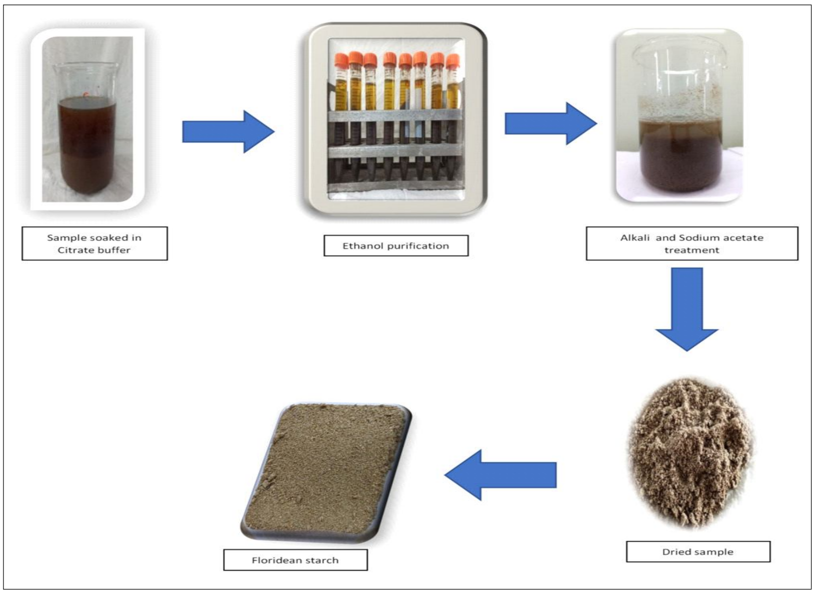

Fig 2: Shows various extraction stages involved in the extraction of floridean starch.

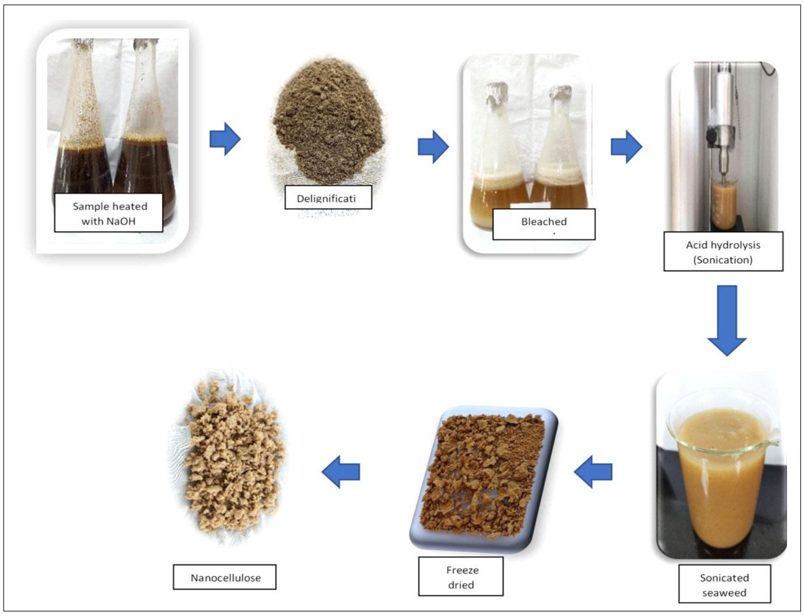

Fig 3: Extraction stages of cellulose nanocrystals from *Gracilaria salicornia.*

ensure full delignification. After being continuously cleaned with hot distilled water, the final sample was oven-dried at 55ºC and stored in an airtight container for additional characterization. Using a 500 W Sonics Vibra Cell Psg Tech, Bionest Step Lab ultrasonicator, the bleached sample was hydrolyzed with 1 M sulfuric acid for 20 minutes at 95°C. The collected samples were pulverized, freeze-dried and kept in storage.

Characterization of floridean starch and cellulose nanocrystals

Microscopic observation of starch granules

The *G. salicornia* section of the thallus showed starch granule accumulation within their cells, which was observed under a trinocular microscope (Model: Optika) under magnifications of 10 and 40 times.

Field emission scanning electron microscopy (FESEM)

FESEM is an advancement that captures the topography of the sample under high-vacuum electron bombardment technology and provides microstructure video-scan images. Two polysaccharides were analyzed under the sigma model from Carl Zeiss (USA) with Gemini at 1.5 nm column resolution and three detectors (in lens, SE2, BSD) in PSG TECHS COE INDUTECH.

Differential thermal analysis using thermogravimetric methods (TG-DTA)

Samples were scanned from 32°C to 299°C at a rate of 15°C min-1 using a modulated differential thermal analyzer (Model: TGA 1000) to perform thermal analysis. Curves and a sequential reduction of weight were noted.

Raman spectroscopy

Raman spectroscopy is an analytical technique that provides details on the chemical and structural data of a compound through principle of the Raman scattering effect, where the scattered light measures the vibrational energy of the compound. Raman spectroscopy analysis was performed at Nanoscience and Technology, TNAU Coimbatore.

Gel strength and gelling temperature

A texture analyzer (Model TA-DEC) was used to determine the gel strength. The measurements were performed an overnight-treated polysaccharide solution.

Sulfur content

The sulfur concentration of the isolated polysaccharide was evaluated using the turbidometric method (Susan *et al.* 1979; Marinho-Soriano and Bourret, 2003). 0.5% barium chloride and 0.02% agarose were combined to prime a barium-agarose reagent. After adding 1.2 ml of 8% trichloroacetic acid to each polysaccharide sample along with the produced reagent, the samples were incubated for 30 minutes. Using sodium sulfate as the control, absorbance was measured at 500 nm.

Content of anhydro galactose (AG)

To create a stock solution, 130 mg of resorcinol was dissolved in 100 milliliters of pure ethyl alcohol to create a resorcinol reagent. A resorcinol reagent was then created by mixing 10 ml of stock solution with 100 mL of 12 M hydrochloric acid. For five minutes, two milliliters of each polysaccharide solution and ten milliliters of resorcinol reagent were placed in a cold bath. The tube was heated to 80 degrees Celsius for 10 minutes, then cooled in a frost bath and the absorbance at 500 nm was measured Yaphe (1984).

Preparation of polysaccharide MIC beads

The preparation of polysaccharide MIC beads involves a combination of Floridean starch/cellulose nanocrystal, calcium chloride and sodium alginate, making the encapsulation bead's outer shell. The inner complex of the bead is *Tricoderma viride* (biofungicide), *Azospirillum* (growth promoter), *Bacillus megaterium var. phosphaticum* (phosphate solubiliser). The MIC beads are characterized by treatment over brinjal plants.

Experimental layout and characterization of polysaccharide MIC beads efficiency over the brinjal plant

The Brinjal on a limited scale, plant saplings were taken from the nursery and placed in grow bags filled with nursery soil that had been supplemented with nutrients. According to Sultana *et al.* (2022), the physical and chemical characteristics of the soil were characterized before the application of Polysaccharide MIC beads by monitoring parameters like pH, electric conductivity (ds/m), organic matter (%), total nitrogen (%), phosphorus (ug/g soil) and potassium (mg/100 g soil). Growth measurements following MIC bead application were taken at 15-day intervals starting on the day of inoculation and continuing on the 15th, 30th and 45th days. Plant height (cm), the number of branches per plant and the number of leaves per plant were among the physical metrics used to measure growth. The adoption of NPK was also observed on the 45th day and tabulated.

Statistical analysis

Data were analyzed using ANOVA, which was expressed as mean± standard deviation with at least three analyses and a significant difference at $p<0.05$.

RESULTS AND DISCUSSION

According to reports, seaweeds can contain up to 76% of their dry weight in carbohydrates. Water-soluble and highly hydrophilic are the polysaccharides found in seaweed. The presence of other polysaccharides in the cytoplasm and chloroplast, such as laminarin starch and floridean starch, may vary depending on the kind of seaweed. Comparable to human glycogen, seaweed's stored carbohydrates serve as its main energy source. According to Dibya *et al.* (2024), the food industry widely uses seaweed polysaccharides known as xylans, agar, carrageenan, or alginates as clarifying, gelling, emulsifying, stabilizing, thickening and flocculating agents in a variety of food products, including ice cream, yogurt, candies, meat products and beverages. Proteins, fats, polysaccharides, minerals, vitamins and enzymes are all abundant in seaweeds. Based on thallus color, seaweed can be divided into three groups: brown, red and green. Additionally, they differ in a number of ultrastructural and biochemical characteristics, such as storage compounds, photosynthetic pigments, cell wall composition and flagella presence or absence. Na, K, Mg, Cl, S, P, I, Fe, Zn, Cu, Se and Mo are abundant in seaweeds. Seaweeds improved ruminant growth rate and feed conversion efficiency when added to livestock feed. Numerous macroalgal species contain antimicrobial, antiviral, antioxidant and anti-inflammatory qualities that enhance the health and functionality of animals (Bomalee *et al.,* 2022). The current study provides a thorough review of seaweed's nutritional

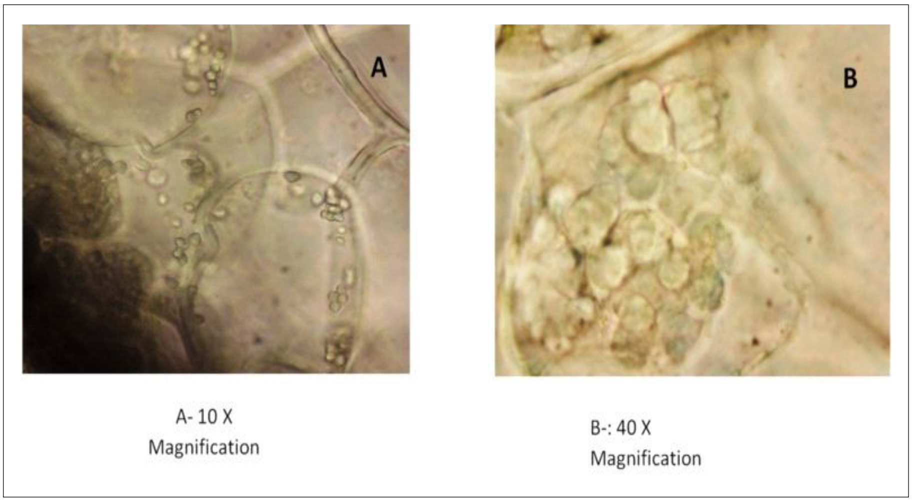

Fig 4: Starch granules under 10x and 40x Magnification.

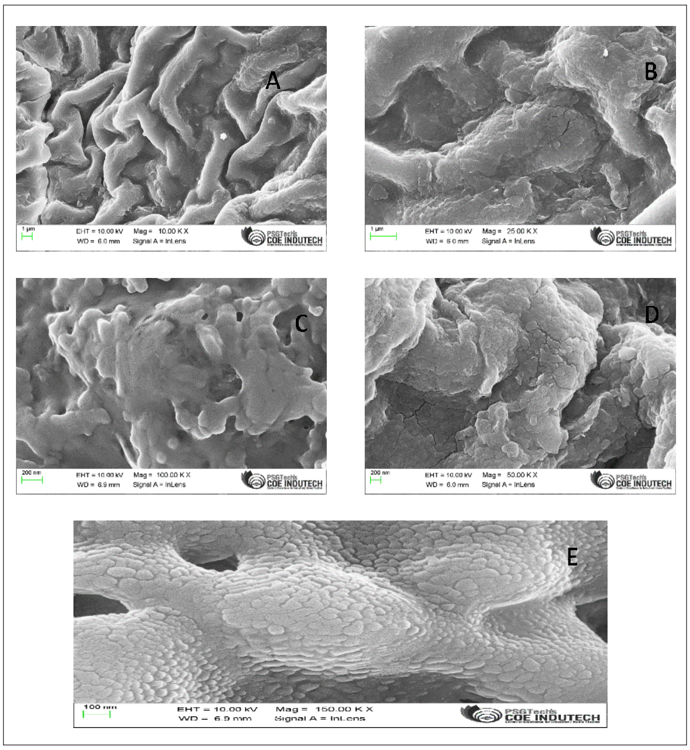

Fig 5a: Represent floridean starch topography of *G. salicornia* in A-10x, B-25x, C-50x, D-100x, E-150x magnification of micro and nanoscale under FESEM.

makeup, health advantages, variety of applications, growth techniques and possibilities for creating processed goods for the food and agriculture industries.

Microscopic observation of starch granules

Trinocular microscopic examination of *G. salicornia* thalli exposed starch deposition on the cell wall of algae (Fig 4). The result obtained was under 10 and 40 power magnification, where 10x shows two adjacent cells containing a few granules onto their cell wall, while 40x shows a single cell enclosing their granules, which was in accordance with starch deposition (Prabhu *et al.,* 2019) under various microscopes such as light, confocal, phase contrast and TEM in a green algal member.

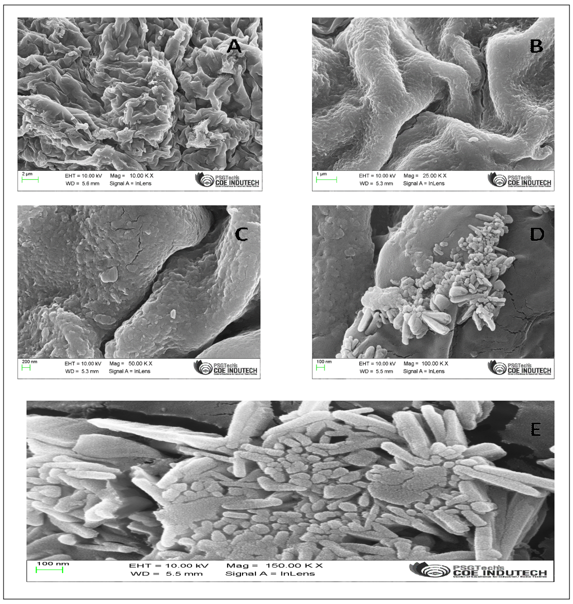

Fig 5b: Represent cellulose nanocrystal topography of *G. salicornia* in A-10x, B-25x, C-50x, D-100x, E-150x magnification of micro and nanoscale under FESEM.

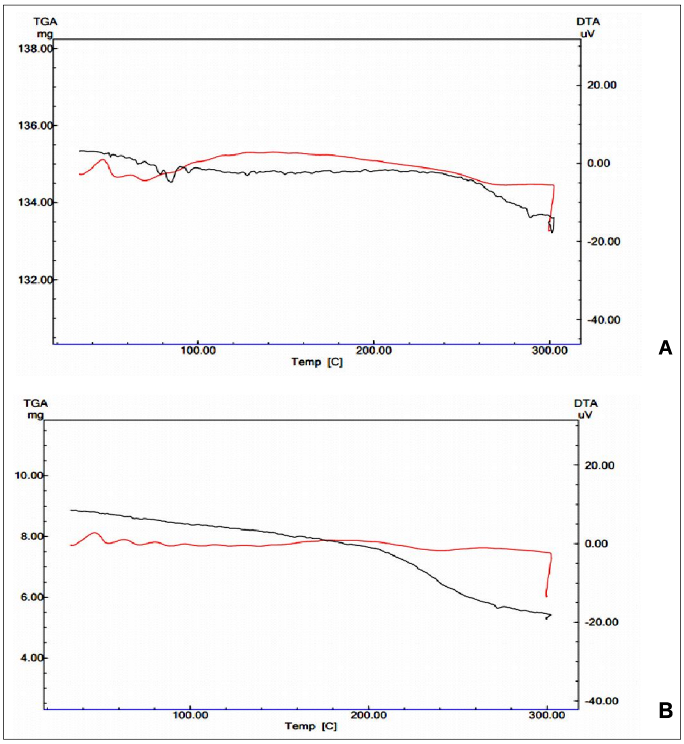

Fig 6: A- TG-DTA analysis of *G. salicornia* floridean starch; B- Cellulose nanocrystal.

FESEM for floridean starch and cellulose nanocrystal

FESEM Microscopic analysis revealed that the polysaccharides obtained from *G. salicornia* under various magnifications such as 10x, 25x, 50x, 100x and 150x and sizes 1 µm, 200 nm and 100 nm all appear rigid, polymeric and starchy Fig 5a, 5b in comparison to starch extracted from *Gracilariopsis lemaneiformis* sps by Yu, 1992 and, green macroalgae *Ulva ohnoi* by Prabhu *et al*. (2019).

Thermo gravimetric differential thermal analysis (TG-DTA)

Thermal analysis of the floridean starch and cellulose nanocrystal (Fig 6) revealed sequential weight loss (mg) from 0.111 mg to 0.467 mg with a percentage rate of 0.082% to 0.345% through the entire screening with an increased temperature from 32°C to 299°C for the simultaneous TGA-DTA curve. The overall weight loss was 0.647% for *G. salicornia* while and 60.73% for green algae in a similar temperature range (Prabhu *et al.,* 2019).

Raman spectroscopy

The spectrum is depicted in a Raman spectroscopy graph in Fig 7a and 7b. Table 1 shows that the polysaccharides of *G. salicornia* have anhydrogalactose concentration, sulfur, gel strength and gelling temperature that are all higher than the usual range (Marinho- Soriano and Bourret, 2003). The species *G. salicornia* (Said and Vuai, 2022) exhibits the highest gel strength of 458 15.5 g cm^2 (treated) and 394.4 16.4 g cm^2 (untreated), followed by species *G. corticata* 259.4 16.4 g cm^2 (treated) and 229.2 28.3 g cm^2 (untreated). However, *G. edulis* species recorded the least amount of gel strength, 133.8 64.4 gm^2 s^2 (treated).

The gelling temperature of 29.05±3.34 observed was much lower than the temperature observed in two other

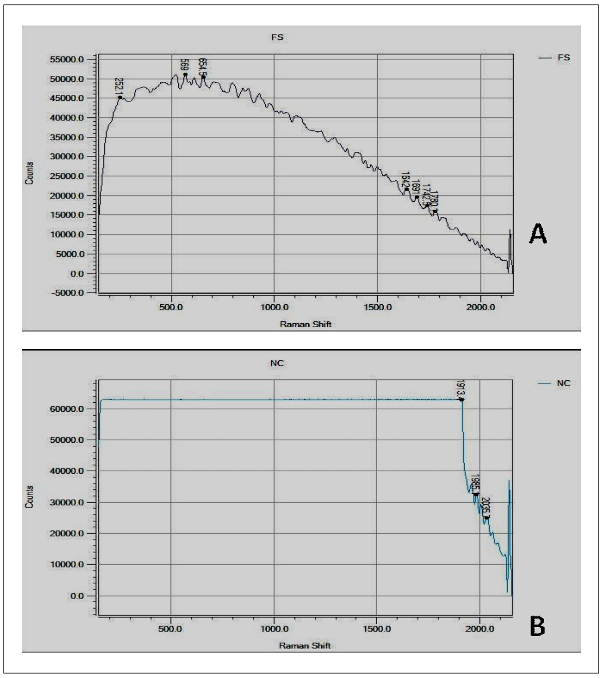

Fig 7: Raman spectroscopy for floridean starch and cellulose nanocrystal of *G. Salicornia.*

Fig 8: A- Showing polysaccharide MIC beads; B- Showing brinjal plant inoculated with MIC Beads.

red algal members by (Yu, 1992) suggesting starch from *G. salicornia* to be a fast gelling compound. The sulphur and AG content, in extracted Carrageenan isolated from *Hypnea sps* (Rafiquzzaman *et al.,* 2016) was 20-25% for sulphur and 30-35% for AG content which were higher than current observations, while the sulphur and AG content in extracted agar from two red algae (Marinho-Soriano and Bourret, 2003) was similar to current findings in Table 1.

Sulfated polysaccharides derived from red algae are known as carrageenans and agarans. These polymers' rheological characteristics are crucial to their industrial uses. These two polymers properties make them important thickeners and gelling agents, primarily in the food industry (Jiao *et al.,* 2011). Sulfated polysaccharides also exhibit a number of biological functions, such as antioxidants (Godard *et al.,* 2009; Qi *et al.,* 2012; Shao *et al.,* 2013; Souza *et al.,* 2011). Algal polysaccharide depositions, such as carrageenan, agar and floridean starch, can be compared. Approximately 65% of the 7.5 tons of agar generated annually worldwide come from red algae belonging to the genus *Gracilaria,* which are also important in the production of phycocolloids (Martin *et al.* 2013) (Niu *et al.,* 2013). Sulfated polysaccharides derived from *Gracilaria* species include 3-linked-β-D-galactopyranose (G unit) and 4-linked-3,6-anhydro-á-L-galactopyranose (LA unit) (Rodríguez-Montesinos *et al.,* 2013; Maciel *et al.,* 2008; Lahaye *et al.,* 1988; Araki, 1966). The hydroxyl groups are replaced by ester sulfate, methyl groups and pyruvic acid (Maciel *et al.,* 2008; Lahaye *et al.,* 1988; Araki, 1966). On the north eastern Brazilian coast, gracilaria is extremely common and its extraction has emerged as a viable option for some of the province's residents to profitably integrate (Barros *et al.,* 2013; Vidotti *et al.,* 2004). Good degrees of encapsulation were demonstrated by the produced polysaccharide beads. The MIC beads were tested on brinjal plants for their potency and the nursery soil used for planting was characterized for physical and chemical properties both prior to and after bead treatment. The parameter was obtained as shown in Table 2 in comparison with the critical value suggested by (Sultana *et al.,* 2022). Table 3 summarizes the results of polysaccharide beads, whose values were found to be higher than both the control and critical values. The polysaccharide MIC beads treated brinjal plant also showed improved growth when monitored over a 15-day interval Fig 8. The obtained results were compared with those presented by (Sultana *et al.* 2022) and both values seem greatly higher and satisfactory. The bacteria that produce EPS aid in retaining free phosphorous and circulating vital nutrients for the plant. In interactions between plants and microbes, extra cellular polysaccharides producing bacteria shield plants against desiccation, invasion and defense. One macronutrient found in plants is phosphorus. Inorganic phosphorus is dissolved by PSB from insoluble

Table 1: Showing various analyzed parameters.

Parameter	MEAN±SD			
	Gel strength	Gelling temperature	Sulphur content	Anhydrogalactose content
Floridean starch	379.21±176.13	29.05±3.34	1.27±0.09	1.27±0.09
Cellulose nanocrystals	350.14±155.09	23.15±2.89	1.04±0.05	0.45±0.01
Average range	229.5 to 828	37.5 to 48.5	1.14 to 1.61	0.63 to 0.55

Table 2: Physical and chemical characteristics of soil before and after polysaccharide MIC Bead application.

Parameter	Value before treatment		Value after treatment		Critical limit
	FS	NC	FS	NC	
pH	7.1	7.1	7.2	7.1	-
Electric conductivity (Ds/M)	2.03	2.01	3.8	3.3	-
Organic matter (%)	3.1	2.9	4.3	4.1	-
Total nitrogen (%)	0.39	0.34	0. 47	0. 44	0.12
Phosphorous (Mg/G Soil)	13	11	16	13	8.0
Potassium (Mg/100 g Soil)	0.44	0.41	0.50	0.47	0.1

Table 3: Measurement of growth.

Treatment	Plant height (cm)			No. of branches/plant			No. of leaves/plant		
	15	30	45	15	30	45	15	30	45
Control	10.3	17.2	20.1	1	1	2	5	7	11
FS MIC Beads treated	12.1	18.5	23.4	1	1	2	6	9	14
CNC MIC Beads treated	12.0	18.1	23.3	1	1	2	7	8	13

substances (Mugip *et al.* 2025). The extra cellular polysaccharides protects themselves against unfavorable environmental conditions.

CONCLUSION

Although the commercialization of high-value chemicals made from seaweeds has increased recently, there are still molecules that need to be discovered. The sole structure of many seaweed polysaccharides determines their biological (biocompatibility, cell adhesion, cell proliferation, gel-forming capacity, collagen matrix formation) and technological (emulsification, gelation, foaming) characteristics. In light of the increasing need for “natural” and “functional” substances for use in food, medicine and pharmaceuticals, seaweed polysaccharides are of particular interest. In this study, polysaccharide was profitably extracted from the *G. salicornia* species. The floridean starch extraction exhibited low gelatinization and pasting temperatures with high transparency. Cellulose nano crystals (CNC) were extracted by advanced methods of ultrasonication, which reduced the extraction period by half, suggesting that this method is greener for the synthesis of CNC. The characteristics of CNC rigidity and gel strength suggest that it can be a greener alternative as a polymer composite material. The polysaccharide MIC bead showed good encapsulation of three agriculturally potent microbes and treatment of the brinjal plant showed a great growth rate and NPK levels. The Brinjal plants to disease incidence was critically low. Thus, MIC beads could be a versatile future tool for farmers, aiding in agricultural crop management. Additionally, extraction yields need to be improved for industrialized awareness because they might occasionally be extremely low.

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Disclaimers

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Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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