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Process development for cellulose production from *in vitro* shoots of *Juncus rigidus* treated with $CaCl_2$ and NaCl - a potential source of high quality cellulose

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Abstract

Novel method has been developed to efficiently produce high-quality cellulose for biomaterial production. This study introduces *Juncus rigidus* shoots derived from callus as a new cellulose source. Application of $CaCl_2$ and NaCl significantly enhanced shoot proliferation and elongation of *J. rigidus*, leading to a notable improvement in cellulose content (25.1%). XRD (X-Ray diffraction) and FTIR (Fourier transform infrared spectroscopy) confirmed the presence of characteristic bands associated with crystalline structure of cellulose. Cellulose-related peaks appeared fused in the absence of $CaCl_2$ and NaCl. FTIR spectra of tissue culture-derived cellulose from $CaCl_2$ and NaCl-treated samples (JT4) exhibited distinct bands at 3440 cm^{-1}, 2926 cm^{-1}, 1300 cm^{-1}, 1440 cm^{-1}, 1163 cm^{-1}, and 1040 cm^{-1}. These bands serve as fingerprints of pure cellulose components. The JT4, TGA (Thermogravimetric analysis) analysis revealed highest thermal stability with the greatest mass loss (72.73%) and lowest char residue (1.34%), indicating high cellulose purity and crystallinity. Additionally, DTG (Derivative Thermogravimetry) of JT4 exhibited a peak at 342°C, which falls within the typical cellulose degradation range (330°C–370°C). The DSC analysis of JT4 exhibited a strong exothermic peak at 389.4°C, indicating high crystallinity and thermal stability. While others samples showed degradation-associated endothermic transitions, influenced by cellulose quality and salt treatments. Thermogravimetric analysis revealed $CaCl_2$ and NaCl-treated cellulose exhibited greater thermal resistance. To the best of the authors' knowledge, this study pioneers the use of *J. rigidus* callus regenerated shoots for producing high-quality cellulose for biomedical applications.

Keywords: *Juncus rigidus, In vitro* cultures, Cellulose, Sodium chloride, Callus

Introduction

Plant tissue culture was mainly explored for propagation in the past but in the recent past, it has also been employed to identify and isolate bioactive phytocompounds. These compounds have medicinal properties, making them valuable for producing pharmaceuticals and nutraceuticals as they possess high value characteristics. The application of *in vitro* culture methods for the development of bioengineered products and valuable plant-derived metabolites with medicinal or commercial significance is well-established. As the vast amount of available information is not possible to include as all studies are not within the scope of this article. We extend our apologies to the authors whose work could not be referenced. This article offers extraction of the compound formed by plant cells, tissue culture for biomedical application. According to Fischer et al. (2015), plant cell culture is now an effective method of producing a number of useful natural compounds. These include pigments and anti-inflammatory agents such as rosmarinic acid and berberine, and anti-cancer compounds like paclitaxel and podophyllotoxin. One notable development is the enzyme taliglucerase alfa, marketed as by two companies to cure Type 1 Gaucher disease. In 2014, regulatory agencies approved it as the first commercially available human recombinant therapeutic protein produced from plants developed under controlled conditions (Pfizer 2014; Pastores et al. 2016). Meanwhile, several other plant-based products are undergoing clinical trials pending approval. Companies such as Icon Genetics, Ventria Bioscience, and Greenovation Biotech have the technological capabilities to develop a wide range of plant-derived pharmaceuticals. However, the progress in this field has been relatively slow, primarily due to several challenges such as low production yields, undesired glycosylation of products, difficulties in purification, and complications in downstream processing (Tuse et al., 2014; Yao et al., 2015). Nevertheless, plant pharmaceuticals' the supply has frequently lacked consistency and uniformity. Plant tissue culture is one option for creating significant plant active chemicals since it removes the requirement for wild plants and ensures independence from geographic conditions. Additionally, plant transformation makes it possible to use plants in the future to produce synthetic chemicals like vaccines and various medications. The key bioactive substances that are currently produced by plant tissue culture (PTC), as well as the basic techniques and plants used to manufacture them. Bacterial cellulose (BC) production by various genera, including *Komagataeibacter, Gluconacetobacter, Enterobacter, Pseudomonas, Achromobacter, Alcaligenes, Aerobacter, Azotobacter, Agrobacterium, Burkholderia, Dickeya, Escherichia, Rhizobium, Salmonella*, and *Sarcina*, has been extensively documented for a range of applications (Klemm et al., 2001; Zhu et al., 2011; Maneerung et al., 2008; Cauerhf and Castro, 2013; Lee et al., 2014; Augimeri et al.,

2015; Cacicedo et al., 2016; Rastogi et al., 2018). BC is biodegradable, non-toxic, and characterized by high crystallinity and a high degree of polymerization (Hsieh et al., 2008; Kaewnopparat et al., 2008; Szymańska-Chargot et al., 2011). Several experimental factors influence BC synthesis, including: (1) the composition of the culture media, particularly the sources of carbon, nitrogen, and essential micro- and macronutrients; (2) cultivation conditions such as temperature, pH, dissolved oxygen levels, and cultivation methods (Ramana et al., 2000; Tompson & Hamilton, 2001; Chawla et al., 2009; Pa'e et al., 2011; Matsuoka et al., 1996); and (3) the evaluation of various carbon sources for BC synthesis (Hestrin & Schramm, 1954; Park et al., 2003; Hungund et al., 2013). *Cannabis sativa* (industrial hemp) is considered an excellent bioenergy source. Its studies on callus cultures derived from different parts of *in vitro*-grown hemp seedlings have demonstrated its potential for use in the Hydrothermal Liquefaction process, highlighting their role as a next-generation energy crop. The integration of plant tissue culture with thermochemical processes presents a promising approach for biofuel production and the synthesis of value-added chemicals. *In vitro*-grown biomass hydrothermally liquefied can be used for bio-crude production (Norouzi et al., 2022).

Plant fibers are known as lignocellulosic fibers, and since long used for various applications like development of different types of composites. They are accessible, recyclable, inexpensive, eco-friendly, abrasion-free, biodegradable, and have respectable mechanical performance. (Guimarães et al., 2009, Hamza et al., 2013). *Juncus rigidus*, or stiff rush, is a potential source of cellulose and can grow in saline environments (Singh et al., 2019, Vyas and Singh, 2024). Sulphated and carboxylated cellulose nanocrystals (CNC) have been produced from *Juncus* (Kassab et al., 2020). *Juncus* fiber characteristics were similar to those of some synthetic fibers and can be suitable for producing cellulose derivatives (Naili et al. (2017). Despite the advantages of *in vitro* culture for both basic and applied research compared to traditional methods, only a limited number of recent studies have focused on the generation of *in vitro* cultures of halophytes (Joshi et al., 2012; Kulpa et al., 2020; López-Corona et al., 2019; de Jesus Raposo & de Morais, 2021; Oliveira et al., 2016; Papafotiou et al., 2014; Regalado et al., 2020; Sedun et al., 2021; Singh et al., 2015; Xiong et al., 2019 ; Vyas et al., 2021; Yao et al., 2021; Jurado-Mañogil et al., 2024). Plants are the primary source of cellulose, and their cultivation typically involves the use of insecticides and pesticides. In cotton farming 24% of insecticides and 11% of pesticides are used (Bhad, 2022), posing significant risks to both human health and the environment (Bhad, 2022). As a result, cellulose derived from such sources may contain harmful pesticide residues (Bhad, 2022). Therefore, producing plant-based cellulose through *in vitro* cultures offers a pesticide- and insecticide-free

alternative, making it suitable for biomedical applications. In recent past, plants *in vitro* cultures are explored for various application (Pfizer 2014; Pastores et al. 2016) other than propagation techniques. Till now no studies were carried out on generation of cellulose and their characterization using plant cultures, except few (Vyas and Singh, 2021). In previous study cellulose was extracted from *J. rigidus* using low cost clonal propagation method with 80-150 shoot buds and there is a need to further minimize the culture cost by increasing number of shoot bud using liquid medium and characterize the cellulose obtained from modified medium for appropriate valorization.

The study was carried out to investigate the effect of different culture medium supplements ($CaCl_2$ and NaCl) on the structural, thermal, and crystalline properties of cellulose extracted from *Juncus in vitro* cultures, with the goal to identify its suitability for sustainable biomaterial applications. The study includes cellulose extraction from treated *in vitro*-grown tissues, analyzing thermal behaviour using TGA (Thermogravimetric analysis), DTG (Derivative Thermogravimetry), and DSC (Deferential scanning calorimetry), evaluating chemical composition through FTIR (Fourier transform infrared spectroscopy), and determining crystallinity using XRD (X-Ray diffraction). The study further aimed to compare the impact of different treatments on cellulose purity, crystallinity, and assessing the potential of plant tissue culture as a scalable method for producing high-quality, pesticide and insecticide free cellulose for customized products.

Materials and methods

Plant material and culture initiation

Surface sterilized (Vyas et al., 2021) shoots of 1.0-1.5 cm sized were cut and kept on MS (Murashige and Skoog) medium supplemented with 3.0 mg/L TDZ (Thidiazuron) for callus development. These callus was cultured on 0.5 mg/L TDZ and 0.5 mg/L IAA (Indole-3-acetic acid) for shoot bud initiation and elongation for 30 days in a liquid culture medium. These shoot buds were used for further experiments.

Effect of additives on shoot multiplication and elongation for cellulose extraction

To examine the impact of various additives on shoot buds, explants were inoculated into the Ms medium supplemented with $CaCl_2$ -200.00 mg/L and 400.0 mg/L, NaCl – 100mM, and 200mM. Buds were also inoculated on MS media. Sucrose concentration (3%), pH value (5.8), and PGRs (Plant Growth Regulators) (IAA 0.3g/L + BAP (6-Benzylaminopurine) 0.5g/L) were similar in all treatments. Results were recorded after 70 days.

Cellulose extraction

To isolate cellulose fiber, dried tissue culture shoots samples were cut into pieces and then digested using 5 M NaOH solution at 100º C for 3 hours. Followed by bleaching for 1 hour at 95º C using hydrogen peroxide (H_2O_2) (10ml/L) solution. Sodium Silicate (3%) was added to H_2O_2, it was used as a stabilizer for H_2O_2. Sample solution ratio was 1:3 (w/v). At every stage of the various treatments, the insoluble residue was thoroughly washed with demineralize water till 7 pH was achieved, oven-dried at 50º C for two days, and their weight was recorded. After NaOH treatment the solution was collected and treated with H_2SO_4 for lignin estimation.

XRD characterization

The PANalytical Empyrean apparatus was used to perform X-ray powder diffraction research in order to evaluate the crystalline structure of cellulose fibres. The 2θ range of 0° to 80° was used to analyse the diffraction pattern. The peak height approach as per Singh et al. (2019), was used to calculate the crystallinity index (CI). The CI was calculated using the following empirical equation (Segal et al., 1959):

$$CI\ (\%) = (I_{200} - I_{am}) / I_{200} * 100$$

The most intense diffraction peak, known as I_{200}, corresponds to the (200) crystallographic planes and typically appears between 22° and 23°, and reflects crystalline and noncrystalline area. The intensity of the noncrystalline region denoted as I_{am}, generally occurs between 18° and 19°. In this study, diffraction maxima for $I_{am,}$ I_{200}, and the crystallinity index (CI) were noted at 2θ angles around 16°, 22°, and 34°, respectively.

FTIR characterization

FTIR (Perkin Elmer, GX-FTIR) spectroscopy was performed to examine for the Sample JT-C (Control), JT1-$CaCl_2$ 200, JT2-400, JT3-$CaCl_2$ 200 + 100mM NaCl, and JT4- $CaCl_2$ 200+ 200mM NaCl) in the 400–4000 cm^{-1} ranges. The KBr pellet method was used for the analysis, wherein 1 mg of sample was mixed with KBr to pelletize the sample.

Cellulose water and other solvents solubility

Cellulose solubility was systematically assessed in water, various organic solvents (methanol, isopropanol, acetone, butanol and chloroform), and distilled water mixture of NaOH-urea- (6% and 4%) was prepared.

Thermal analysis

Thermogravimetric analysis (TGA)

Thermal strength analysis was performed by a Netzsch TGA machine. This technique measures changes in sample weight as temperature increases, under a precise heating rate in an inert condition. Approximately, 10 mg sample was subjected to heating at 10 °C per minute up to 900 °C under a nitrogen atmosphere

Derivative Thermogravimetry (DTG)

The Netzsch model determines DTG. This process involves plotting the changes in mass of a sample as a function of temperature or time.

Differential Scanning Calorimetry (DSC)

A Phoenix device with Netzsch software was used for DSC measurements. First thermal scan was performed using a heating rate of 10 °C /min, 6 to10 mg sample was heated at -30 °C to 200 °C temperature. A second thermal scan was then performed after the materials had been quickly chilled by submersion in liquid nitrogen. The second scan was conducted -30 °C to 450 °C at a heating rate of 2 °C /min.

Experimental Design

All the experiments were laid down according to completely randomized block design (CRBD). Each experiment was repeated twice with a minimum of 3 replicates per treatment. Observations on culture response and the number of shoot buds were scored after a regular interval. Numbers of shoot buds were counted per culture vessel.

Results and discussion

Effect of $CaCl_2$, and NaCl on the number of shoot buds

In tissue culture, the medium plays a key role on the culture's growth and development (Fig. 1 A-H). Hence, addition of additional $CaCl_2$ and NaCl were evaluated for the same. Exposure to 200 mM NaCl the growth and development of *Juncus rigidus* enhanced, in addition to it salinity increased cellulose content (Vyas et al., 2024). *J. rigidus* cultures were able to tolerate 200 mM NaCl treatment. Moreover, liquid culture medium proved to be cost-effective and promoted better culture growth (Vyas et al., 2021). In the present study, 3-6 cm long shoot bud numbers considerably increased in 200mg/L $CaCl_2$ (JT1) treatment followed by 400mg/L (JT2). Among all the treatments, 400 mg/L $CaCl_2$ has a higher number of shoot buds of 0.5-2.0 cm length, whereas, 3-5 cm shoots was similar to control.

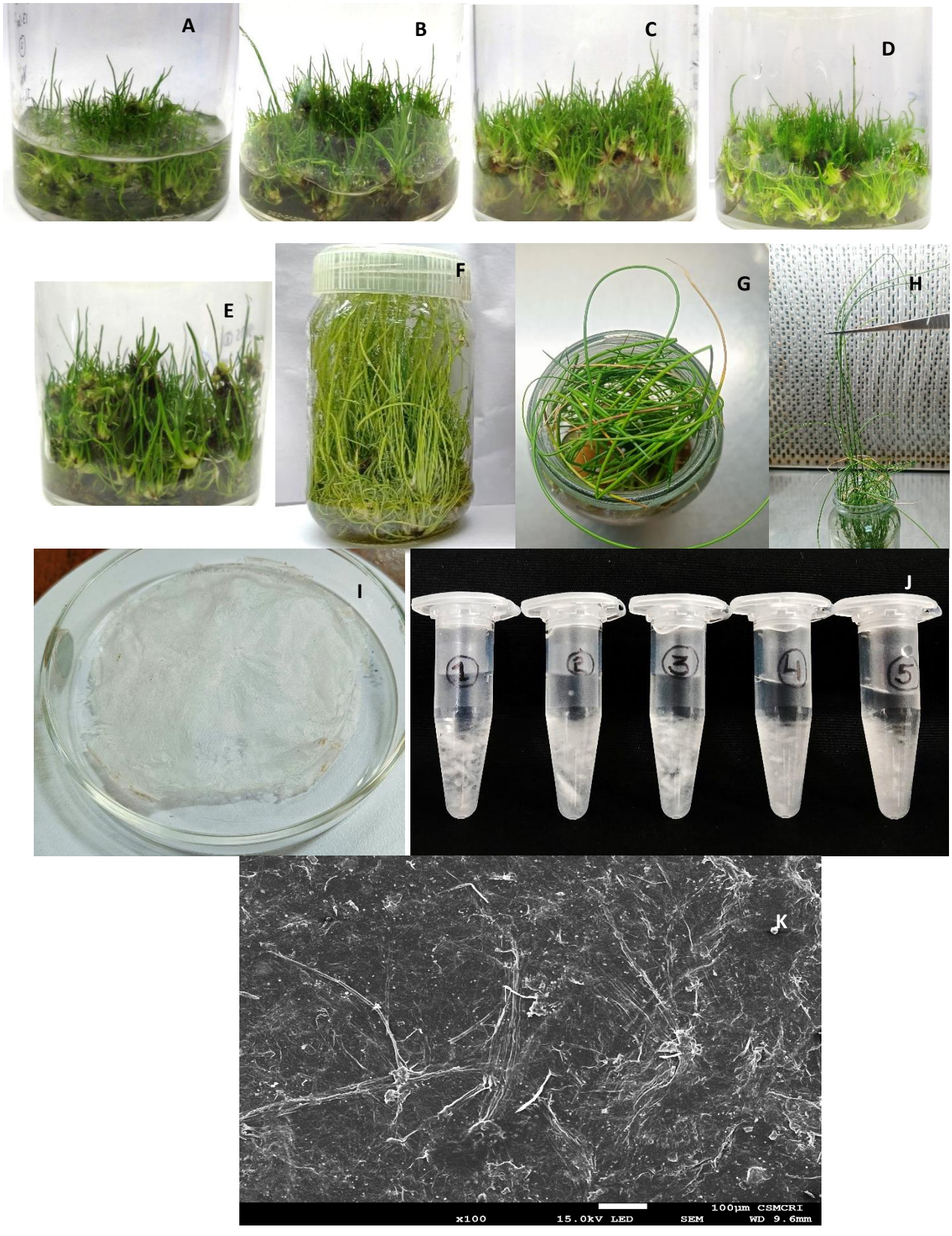


Fig. 1. Effect of different type of media on *Juncus rigidus* culture generation in JT-C MS medium BAP+ IAA (A), addition of $CaCl_2$ 100mg/L (B), JT1-$CaCl_2$ 200mg/L (C), JT2-$CaCl_2$ 400mg/L (D), JT3- $CaCl_2$ 200mg/L +NaCl 50 mM (E), JT4-$CaCl_2$ 200mg/L + NaCl 100mM 70 days old (F-G-H), Cellulose film (I), cellulose in organic solvents (J), SEM image of cellulose film depicting network of microfibers of cellulose (K).

Highest Ca content has bad impact on shoot elongation as only 6 shoots were noted of 8-14cm length in JT2 (Fig. 2). Results reveals that high Ca restricts shoot elongation. Total number of shoot buds were less JT2 (987) compared to Ca 200 JT1 (1296). Shoot buds cultured in media containing $CaCl_2$ with different NaCl concentrations demonstrate moderate changes in shoot bud development (Fig. 1). The total number of buds were highest in the JT1 treatment compared to the control and comparable with JT3 and JT4 treatments. In contrast, salinity in the nutrient solution led to a significant reduction in both shoot and root yield in barley cultivars (Morales, 1992). Similarly, Akhavan-Kharazian et al. (1991) observed that increasing NaCl concentrations inhibited plant growth and various physiological functions. In different rice genotypes, salinity stress caused a substantial decline in pigment biosynthesis (chlorophyll a, chlorophyll b, and total chlorophyll) and overall plant yield, including root and shoot dry weight (Ali et al., 2004). The present study was in corroborate with *Salicornia* culture growth that remained unaffected by the salinity stress (Singh et al 2015).

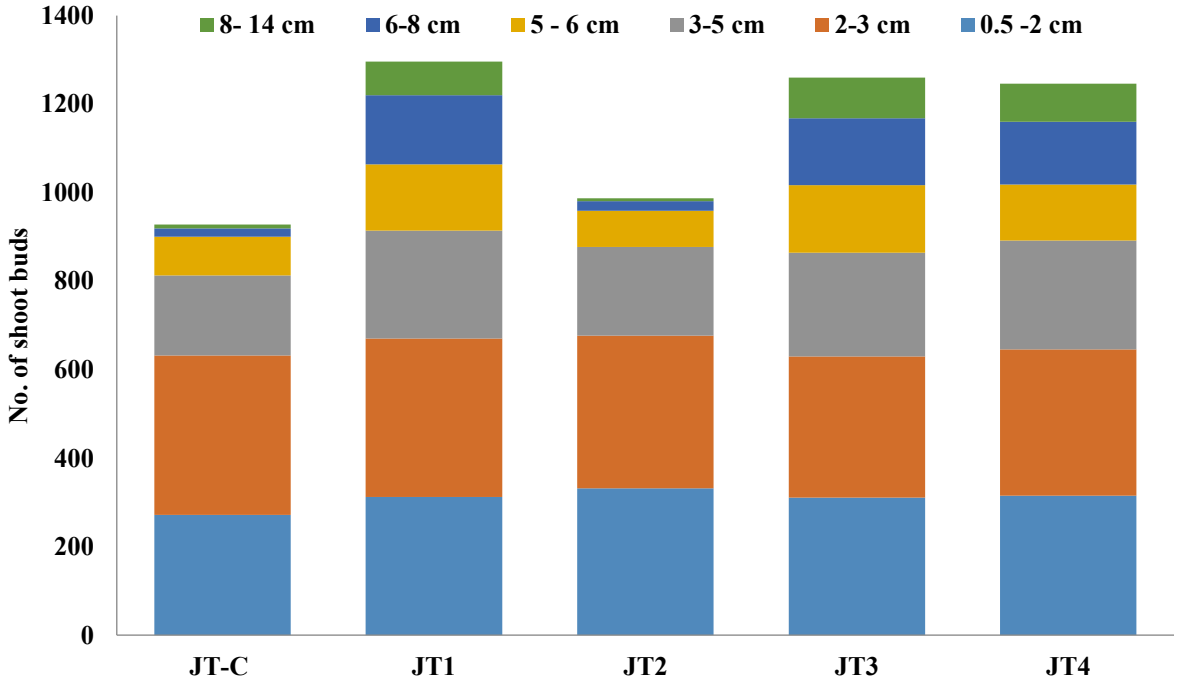


Fig. 2. Effect of $CaCl_2$, and NaCl on the shoot proliferation and elongation of *Juncus rigidus*.

Effect of $CaCl_2$ and NaCl on cellulose content

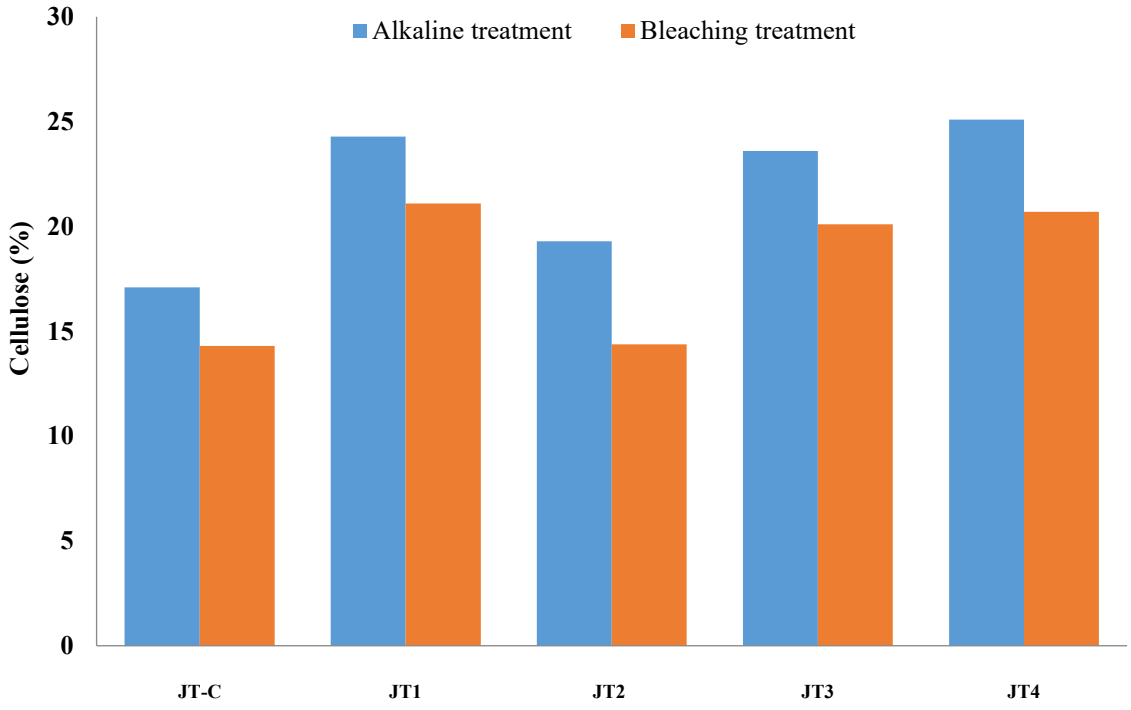


Fig. 3. Effect of $CaCl_2$, and NaCl on cellulose content in *Juncus rigidus in vitro* shoots

In the present study, 17.1 % crude cellulose was noted in the control JT-C, addition of 200mg/L $CaCl_2$ to the medium enhanced cellulose content. The modified medium increased the content of BC (Almihyawi et al 2024). However, addition of 400mg/L $CaCl_2$ to the medium did not further improve the cellulose content. The highest crude cellulose (25.1 %) content was obtained from JT4 (Fig. 3). The results reveal that $CaCl_2$ enhanced cellulose content. Calcium-dependent protein kinase 32 (CPK32) plays a key role in regulating cellulose biosynthesis in Arabidopsis thaliana (Xin et al 2023). In plants, cellulose synthase (*CesA*) complexes situated on the plasma membrane play a key role in controlling both cellulose production and salinity tolerance (McFarlane et al., 2014; Schneider et al., 2016). The first cellulose synthase gene in plants was discovered in cotton, leading to the identification of the broader *CesA* superfamily (Pear et al., 1996, Wang et al., 2010; Li et al., 2013) along with Cellulose Synthase-Like D (CSLD) cellulose synthases (Yang et al., 2020) has been characterized across various plant species. The expression of *PvCesA4* and *PvCesA6* genes directly influences cellulose percentage and CI (Mazarei et al., 2018).

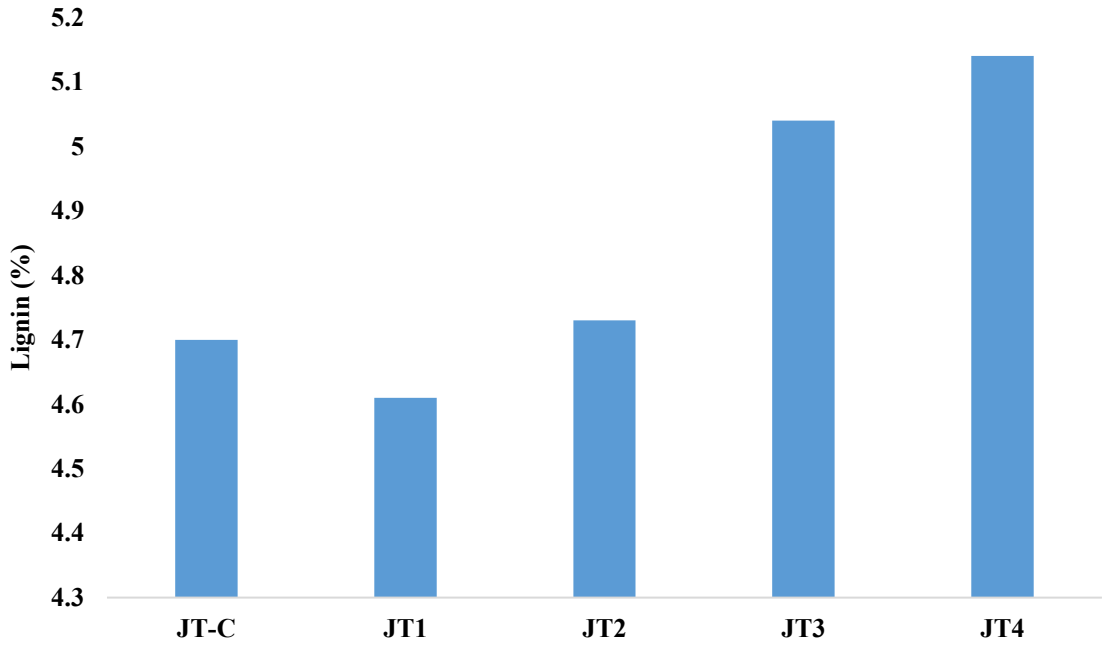


Fig. 4. Effect of $CaCl_2$, and NaCl on lignin content in the *in vitro* shoots of *Juncus rigidus*

Additionally, the Cellulose Synthase-Like D5 (CSLD5), a protein related to *CesA* enhances abiotic stress tolerance in *Arabidopsis* (Chen et al., 2005; Kang et al., 2008; Zhu et al., 2010). Similar to our study, modification in the HS medium with molasses, molasses + ethanol, and antioxidants increased the yield of BC compared to control (Park et al. 2003; Son et al. 2003, Keshk 2014a, b). The successive batch culture increased BC content in 56 days (Gomes et al., 2023). The lignin content was not considerably different (Fig. 4).

Physical properties of cellulose.

The cellulose film was white (Fig. 1I) and odourless.

Cellulose solubility in water and other solvents

Cellulose solubility assessment test in water, various organic solvents, and a NaOH-urea-distilled water mixture confirmed that cellulose remained insoluble in water and organic solvents methanol, isopropanol, acetone, butanol and chloroform (Fig. 1J), and there by consistent with its inherent properties, however, with successfully dissolved in the NaOH-urea-distilled water mixture. This insolubility in water and organic solvents is attributed to its strong intermolecular hydrogen bonding and high polarity (El-Naggar et al., 2023).

Scanning electron microscopy (SEM)

Spectrophotometry characterization of cellulose obtained from *in vitro* shoots showed compact arrangement of cellulose micro-fibrils (Fig. 1K) similar to BC (Jozala et al 2015). The energy dispersive X-ray spectra reveal the presence of Na, Cl, N, O, and C. Carbon and oxygen are derived from natural polymers, while sodium and chlorine result from the treatment process. In BC, the higher atomic percentages of calcium and phosphorus in BC/HA-A indicate that the agitation method enhances mineralization efficiency compared to the static technique. During static fermentation, hyaluronic acid (HA) has minimal interaction with BC throughout the production process. As a result, the calcium (Ca) and phosphorus (P) ratios are higher under agitated conditions compared to those observed in static conditions, as indicated by the X-ray spectra (Saleh et al 2024).

X-ray diffraction (XRD)

The crystallographic structure of the extracted cellulose (from *in vitro* shoot on medium supplements) developed peaks in the XRD diffractogram at 2θ values of 16.4°, 22.55°, and 34.83°in all the samples except JT4 (Fig. 5). To analyze the XRD patterns originPro software program was used for baseline correction. The peak observed at around 16.4 degrees (2θ) in the XRD pattern of cellulose is typically attributed to the (110) plane, which indicates the presence of crystalline regions within the sample and peaks around 22 degrees often attributed to the (200) plane. This specific diffraction peak is a known signature of the native cellulose Iβ allomorph and is indicative of well-ordered cellulose structures found in plant-derived materials. However, Barud et al. (2007) observed diffraction peaks at 16° and 22.5°, corresponding to the cellulose Iα and Iβ phases in BC. 34.8° is typically assigned to the (004) crystallographic plane of crystalline cellulose, especially in Cellulose Iβ and sometimes in Cellulose II depending on structure and was absent in JT4. The absence of the peak may be due to the medium optimization. The 2θ values changed with change in the BC medium composition (Saleh et al 2024). Numerous techniques employed to ascertain the crystallinity of cellulose, and it is widely known that the outcome depends on the approach employed (Park et al., 2010). One important property of cellulose is its crystallinity, which determines its mechanical and physical properties and significantly influences the nanoparticles' ultimate use (de Souza et al., 2020). A basic crystalline structure present in a wide variety of cellulosic fibres is cellulose I. For hydrogel synthesis with enhanced mechanical qualities, cellulose I can be utilised (Mahmud et al., 2019). Cellulose I is a crystalline form of cellulose composed of parallel chains and is naturally synthesized by various organisms, including bacteria, algae, and plants (Wada et al. 2010; Seddiqi et al 2021). Thermodynamic metastability allows the cellulose I structure to change into cellulose II or III.

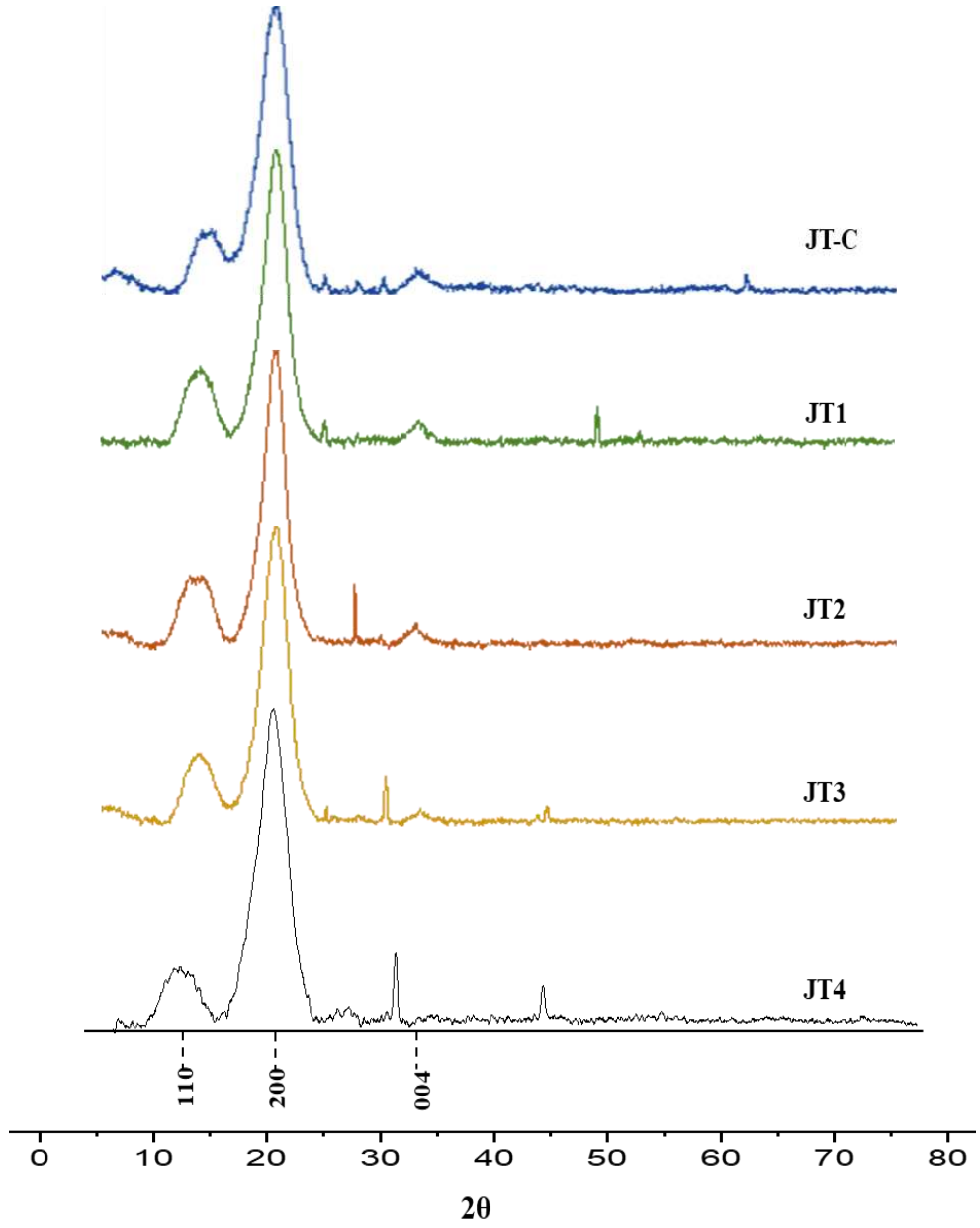


Fig. 5. XRD pattern of cellulose film extracted from *in vitro* shoots of *Juncus rigidus*

The Crystallinity Index (CI) of JT4 sample was highest 51.6% followed by JT3 (Fig. 6). The medium composition affected the CI of the extracted cellulose test samples. In corroborate with our results, the BC crystallinity was 73.5% in medium containing molasses + ethanol under static conditions, while molasses + ascorbic acid medium produced lower crystallinity cellulose under same conditions (Shezad et al., 2010).

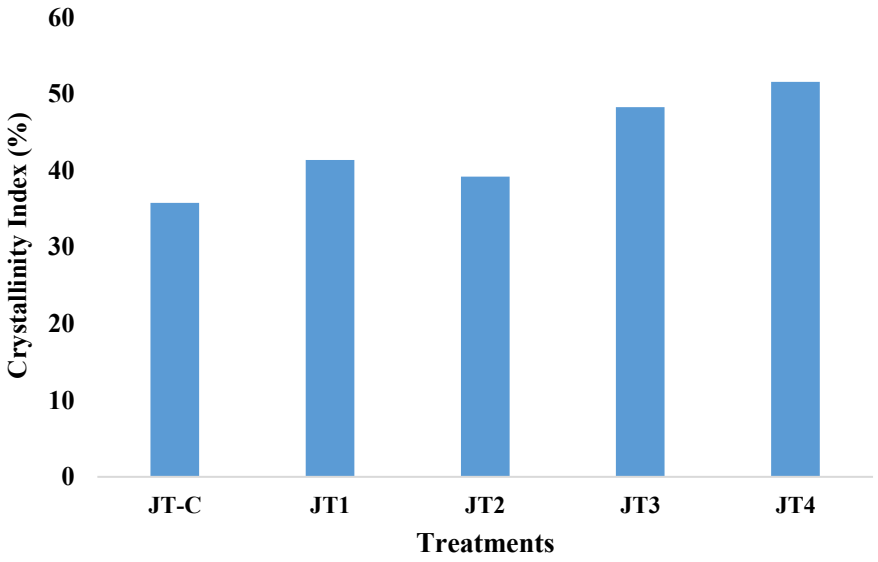


Fig. 6. The percent crystallinity index of cellulose extracted from *in vitro* shoots of *Juncus rigidus*

FTIR analysis

The characteristics of cellulose are influenced by multiple factors (Szymańska-Chargot et al. 2011). In the present study, cellulose crystallinity was affected by culture media treatments that developed changes in the FTIR spectra. The spectra of *Juncus* tissue culture cellulose (JT) extracted from treatment samples, control (JT-C), $CaCl_2$ 200 (JT1), $CaCl_2$ 400 (JT2), $CaCl_2$ 200+ 100Mm NaCl (JT3), and $CaCl_2$ 200+ 200mM NaCl (JT4) in the wavenumber range of 4000-400 cm^{-1}. To compare the FTIR spectral functional groups in JT, a reference material, BC was also analyzed. As illustrated in Fig. 7, the JT cellulose exhibited all the characteristic bands of cellulose, similar to BC, confirming that *Juncus* cellulose possessed a comparable cellulose structure. FTIR spectra of BC grown in different media showed bend shifts related to the valence vibrations of hydroxy groups. The level of cellulose crystallinity plays a crucial role in defining BC characteristics. Molecular structure of plant and BC were similar, they vary notably in their crystalline structures, which are related to the various cellulose polymorphs (I–IV). Cellulose types II, III, and IV are produced from cellulose I through chemical modification (Brett & Waldron, 1996; Szymańska-Chargot et al., 2011). In the FTIR spectra of JT4, a band at 3340 cm^{-1} corresponds to O-H stretching vibrations in cellulose, while bands between 2919–2893 cm^{-1} are associated with C-H stretching (Vyas et al., 2024; Singh et al., 2019). Additionally, symmetric and asymmetric C-H stretching vibrations produce bands around 2918 cm^{-1} and 2893 cm^{-1}. Notably, band at 1730 cm^{-1}, typically linked to acetyl and ester groups in hemicelluloses and lignin (Reddy et al 2014) were absent in JT4 (Fig. 7).

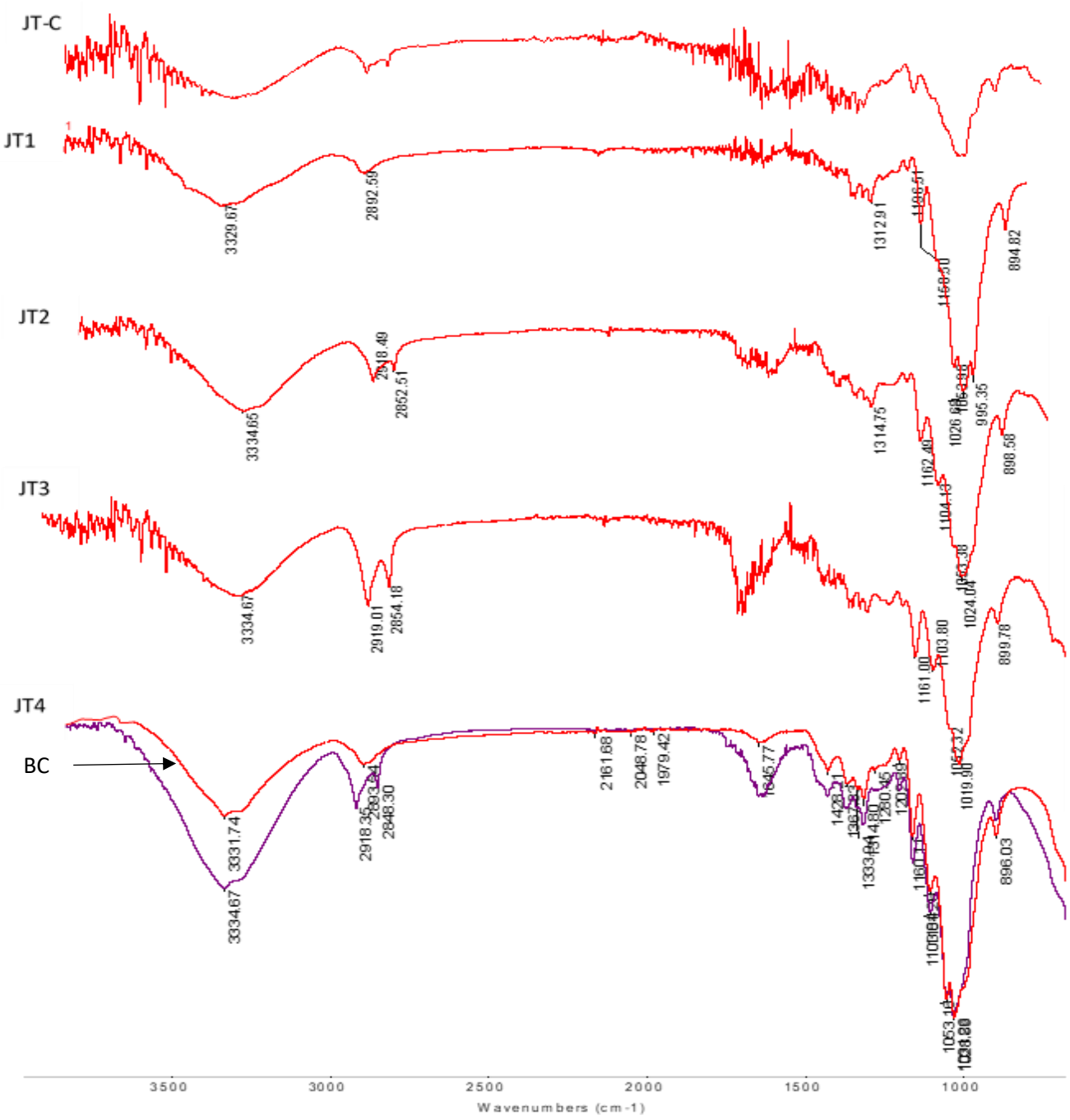


Fig. 7. FTIR peaks of cellulose extracted from *in vitro* shoots of *Juncus rigidus*

The band at approximately 1640 cm^{-1} represents H-O-H bending for absorbed water, while a sharper and more intense band at 1644 cm^{-1} in JT4 indicates a strong carbonyl (C=O) bond. In contrast, band shifts observed at 1655 cm^{-1} and 1669 cm^{-1} in other treatments appeared weaker and merged. The presence of this band reflects potential water adsorption properties (da Silva et al., 2018). The band at 1645 cm^{-1} corresponds to the glucose carbonyl group (C=O) (He et al., 2020), while this band at 1645.77 cm^{-1} was either absent or weak in other treatments. Additionally,

the absorption bands around 1506 cm^{-1} and 1238 cm^{-1} were absent in all samples, indicating the effective removal of hemicellulose and lignin (Vyas et al., 2024). The band at 1367 cm^{-1} corresponds to C-H deformation vibration (Maheswari et al 2012). The absorption band at 1423 cm^{-1} was attributed to the $–CH_2$ bending vibration of cellulose. In the JT4 treatment, a well-defined band appeared at 1428 cm^{-1} (CH_2), indicating the presence of highly crystalline cellulose as this band signifies crystalline absorption (Poletto et al., 2014). However, the weak band in JTC, JT1, and JT2 shows lower crystallinity compared to JT3 and JT4. These findings align with our XRD data (Fig. 5). The absorption band at 1450 cm^{-1}, corresponding to C–H deformation in lignin's methyl and methylene groups were absent in JT4 and BC, while weak fused bands were observed in other samples. Additionally, the lignin band at 1269 cm^{-1} was detected (Maheswari et al 2012). According to Naili et al. (2017), the samples did not exhibit the stretching vibration of C-O. These findings indicate that lignin and hemicellulose, pectin and phenols were absent (Kacurakova et al., 2000). This implies that the chemical treatment has caused the noncrystalline components to be gradually eliminated. Additionally, cellulose did not exhibit any structural alterations as a result of salt stress. According to these investigations, cellulose's hydroxy group and chains have not changed in response to salinity. Furthermore, 1158 cm^{-1} and 1103 cm^{-1} bands indicated the C-O-C deformation vibration and C-C bending, respectively (Nikonenko et al., 2000). The bands observed at 1158 cm^{-1} were assigned to the C-O-C asymmetric stretching vibrations associated with the β (1→4)-glycosidic linkage observed in JT4 and MCC samples attributed to β-D glucose. The absorption bands detected in the 1160–1030 cm^{-1} range in both spectra correspond to C-O stretching and C–H deformation vibrations within the pyranose ring structure of cellulose (Reddy et al 2014). The bands between 1028–1030 cm^{-1} correspond to the stretching vibrations of C-O. The band at 1030 cm^{-1} observed in all cellulose samples was associated with the C-O stretching vibrations (Maache et al., 2017), except for JT-C, where only a very weak band developed. The arabinose band at 975 cm^{-1} was present in JT1 but absent in BC and JT4, indicating the lack of this sugar in the later samples. The band at 896 cm^{-1} originates from the β-glycosidic linkages between sugar units in the cellulose structure. Additionally, the band shift observed at 896 cm^{-1} in JT4 and BC corresponds to the C–O–C vibrations of the β-glycosidic linkages connecting sugar units in cellulose (Dalmis et al., 2020). The absorption band at 894 cm^{-1} corresponds to β-glucosidic linkages (Maheswari et al 2012). FTIR spectroscopy is commonly employed to analyze cellulose microfibers, offering a simple and effective way to directly assess their chemical composition. The high-intensity bands in JT4 may be attributed to the growth and development of shoot buds, influenced by modifications in calcium (Ca) and sodium (Na) levels in the medium.

Pyrolysis characteristics

TGA

The TGA analysis of the extracted cellulose revealed a three-stage pyrolysis process. The initial stage, occurring between 150–200°C, was observed in all samples and was primarily attributed to the removal of moisture and other volatile components. The cellulose samples tested contained approximately 8–13% moisture, as indicated by the relatively minor initial weight loss in the TGA curve (Fig. 8). Recent studies have emphasized that medium composition and ionic supplements significantly influence cellulose thermal stability by altering crystallinity and hydrogen bonding networks (Parajuli et al., 2021; Poletto et al., 2014). In particular, divalent cations such as Ca^{2+} are known to induce ionic crosslinking and promote alignment of cellulose microfibrils, thereby delaying the onset of thermal decomposition (Parajuli et al., 2021; Chen et al., 2022). The second stage, taking place between 200-600°C, corresponded to active pyrolysis (devolatilization), while the final stage occurred at temperatures exceeding 600°C. A distinct degradation in JT-C started at ~ 154 ºC and continued till 500 ºC, during this the mass loss occurred in two stage with 35 %. Whereas, JT1 sample exhibited distinct degradation at 176 ºC and continued until 500 ºC during this the mass loss was 58.72 %. In JT2 sample, degradation started at 195 till 500 C and in JT3 sample, pyrolysis started at 195 till 500 ºC with a mass loss 53.10 %. While in JT-4 sample, pyrolysis started at 209.5 till 600 C with the highest mass loss at 72.73 % (Fig. 8). Similar results were reported in BC, The BC decomposition produced by the isolate on HS medium ranged from 260 to 415°C, reaching approximately 98.2% and with whey medium was 43 % (Kumar et al., 2021). Furthermore, the peak decomposition temperature was 310 °C for BC produced using the alternative medium and 290 °C for BC derived from the HS medium (Costa et al., 2017). Our results were in corroborated BC results revealing that medium composition/supplements affect cellulose thermal stability. The addition of $CaCl_2$ and NaCl to the medium enhances the thermal stability and crystallinity. The tightly packed structure of cellulose, resulting in increased crystallinity, serves as a barrier against thermal degradation. Intramolecular hydrogen bonding plays a key role in improving cellulose thermal stability by minimizing thermal expansion (Poletto et al., 2014). During the 3rd phase of scan, a large fraction of sample is converted into volatile compounds and solid char. The slower weight loss at this stage is attributed to the aromatization reaction, which breaks down the carbonaceous content in the char residue, accompanied by the cleavage of C-H or C-O bonds (Wang et al., 2007). A higher temperature decreases char residue, likely due to removal of volatile substances from a solid, liquid, or gaseous material (Peterson et al., 2022).

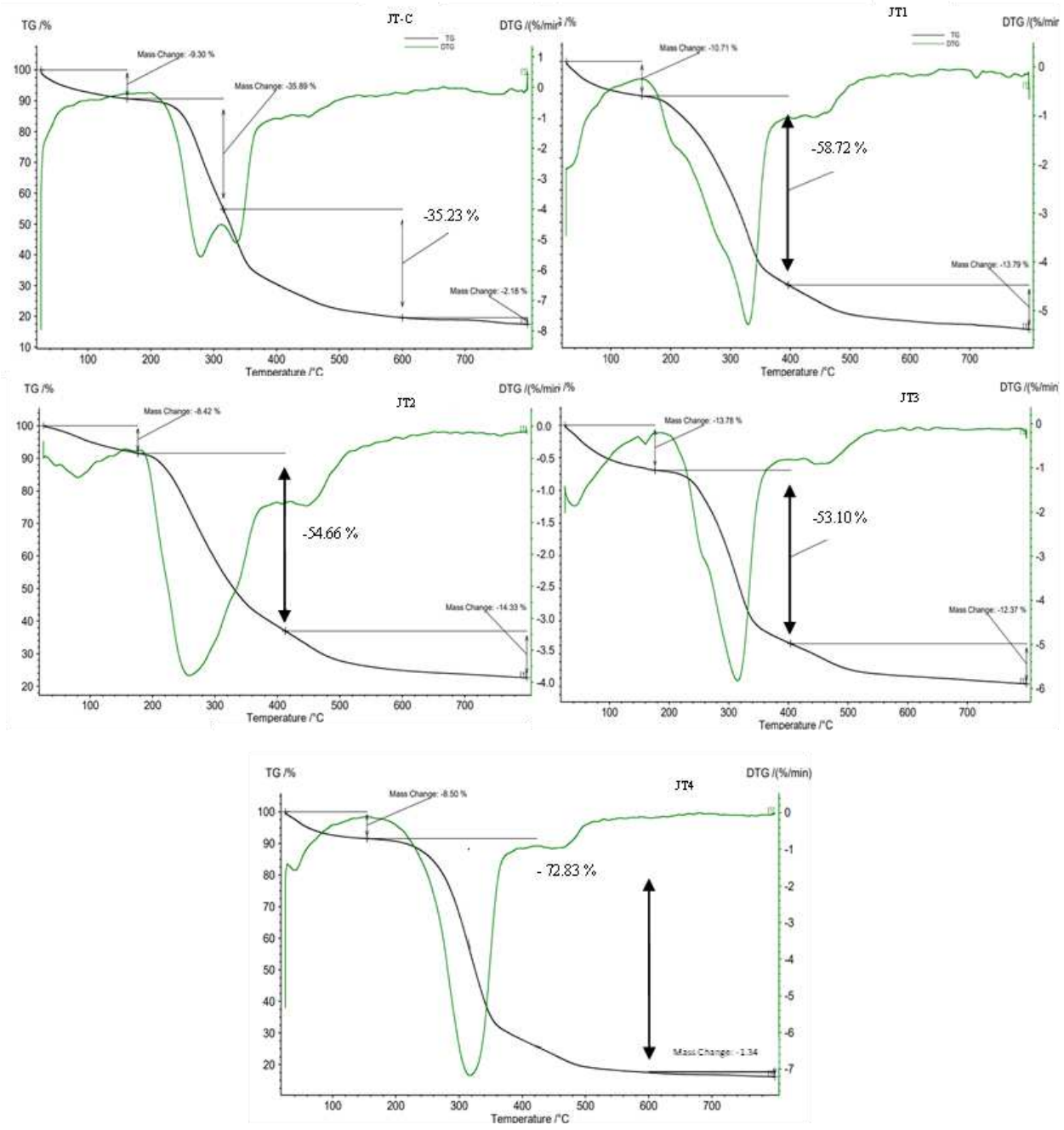


Fig. 8. TGA-DTG analysis of cellulose extracted from *in vitro* shoots of *Juncus rigidus*

The solid residue (biochar) obtained at approximately 800°C varied across samples, measuring 2.18%, 13.79%, 14.33%, 12.37%, and 1.34% for JT-C, JT1, JT2, JT3, and JT4, respectively. A lower char residue percentage, as observed in JT4, indicates higher cellulose purity, whereas other samples had higher char content, aligning with reports that optimal processing reduces thermally labile impurities and enhances cellulose quality (Peterson et al., 2022; Wang et al., 2007). The lowest remaining weight among all samples was recorded in JT-C and JT4 likely due to optimal

delignification (Devnani & Sinha, 2019). JT4 exhibited the highest thermal stability among all tested samples (Fig.5). The charred ashes accounted for approximately 37% of the BC produced and 42% of that obtained using the alternative modified HS media, with mass loss rates remaining low above 700°C (Costa et al., 2017).

DTG

Differential Thermogravimetric Analysis (DTG) provides insights into the heat reaction type, distinguishing between exothermic and endothermic processes. The maximum degradation temperature (T_{max}) for JT1 was observed at 273.51°C and 331.49°C, while JT4 exhibited a T_{max} of 261.3°C (Fig. 8). The peak at 331.49°C corresponds to cellulose decomposition, whereas the temperatures of 273.51°C and 261.3°C are associated with hemicellulose degradation (Fig.8). The results were in corroboration with Rosli et al., (2013). Low T_{max} represents presence of hemicellulose in the sample. Cellulose degradation occurred at a Tmax of 322.49°C in JT2, 261.39°C in JT2, and 310.49°C in JT3. Notably, the higher thermal stability observed in JT2 (Tmax 322.49°C) and JT3 (Tmax 310.49°C) suggests an increase in crystallinity, which enhances heat resistance and further improves the thermal stability of cellulose (Rosli et al., 2013). The results reveals that the elimination of non-cellulosic material enhances crystallinity, which in turn improves thermal stability and heat resistance. Crystallinity, defined as the degree of molecular order within cellulose chains, plays a crucial role in analyzing the material's thermal properties. Consistent with previous studies, Treatment-B exhibited the highest crystallinity index (CI) and the greatest thermal stability based on DTG analysis. Additionally, its higher Tmax value indicates superior heat resistance (Poletto et al., 2012; Jiang et al., 2020). The DTG findings align with TGA analysis of same type of cellulose (Vyas et al 2024).

DSC of cellulose

The DSC thermograms of JTC, JT1, JT2, JT3 and JT4 samples was carried out to evaluate the changes that occurred in the cellulose by the addition of $CaCl_2$ and NaCl in the culture medium. Also, the DSC curve reflects the energy consumption properties of cellulose. All samples exhibited an initial endothermic peak between 59–88°C, attributed to removal of water (Fig. 9). The first transformation peak, occurring between 10 and 200°C, varied significantly among the initial BC samples due to differences in water evaporation (Auta et al., 1996). Likewise, George et al. (2005) observed a transformation associated with the melting of the crystalline phase of cellulose occurring at temperatures ranging from 80 to 140°C.

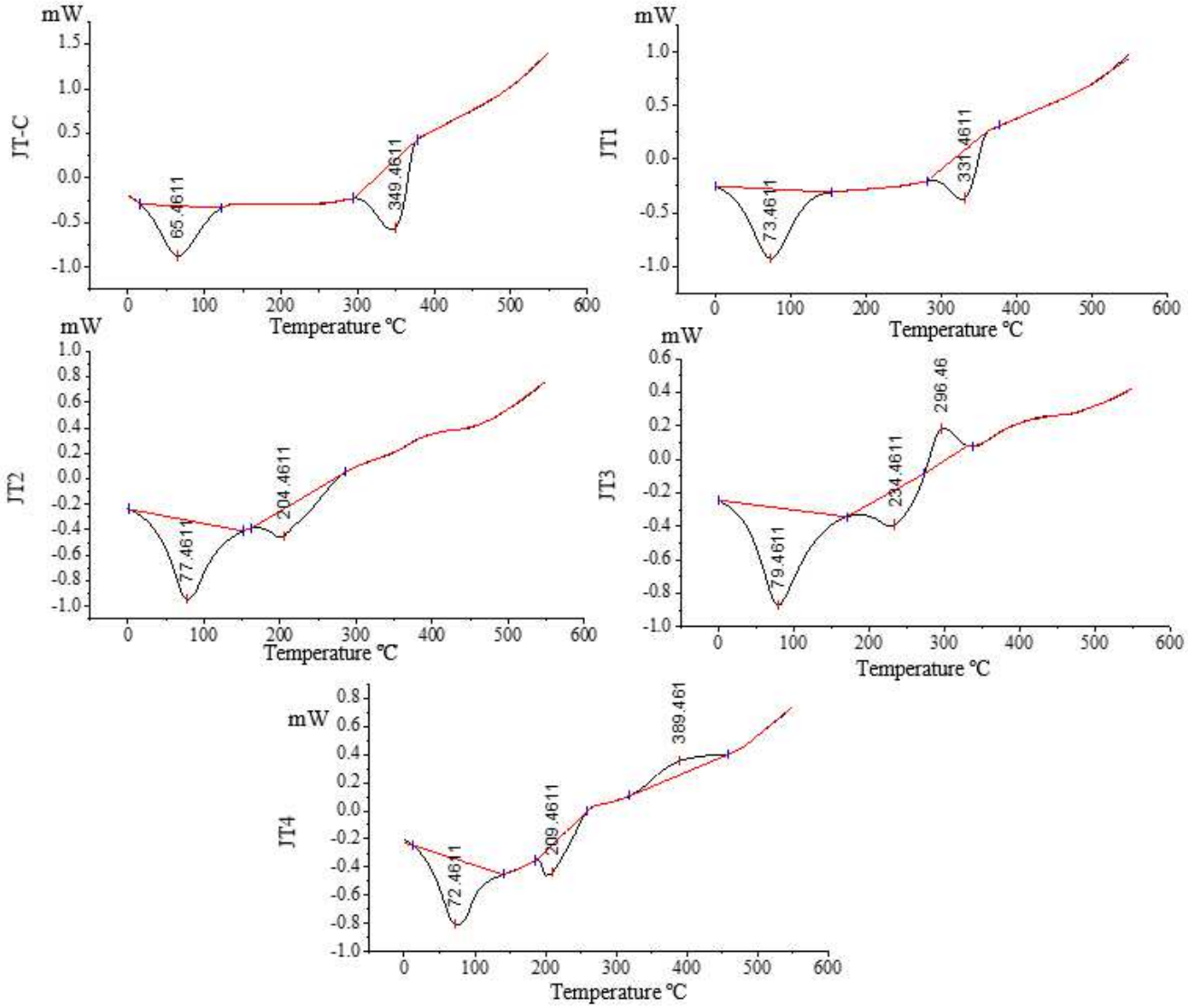


Fig. 9. DSC analysis of cellulose extracted from *in vitro* shoots of *Juncus rigidus*

Interestingly, the DSC thermograms of JT-C, JT1, and JT2 displayed sharp second endothermic peaks between 198–358°C, while no exothermic peaks were observed (Fig. 9). This suggests the presence of amorphous regions within the cellulose structure, potentially due to insufficient $CaCl_2$ and NaCl content in the medium. Xin et al. (2023) reported that calcium-dependent protein kinase 32 (CDPK32) plays a regulatory role in cellulose biosynthesis in *A. thaliana*. Additionally, CDPKs are essential for plant growth regulation and responses to abiotic stress (Shi et al., 2018). The DSC thermogram clearly displays an early endothermic peak, with changes occurring between 59.5°C and 87.9°C across all samples. This minor enthalpy shift results from moisture desorption as

trapped water within the polysaccharide structure is released. This behaviour is inherent to cellulosic materials due to interactions between water molecules and nonsubstituted hydroxy groups. In this study, the presence of a strong exothermic peak at 389.4°C indicates a high degree of crystallinity in the cellulose structure of JT4, which is further supported by XRD results. In BC second endothermic peak around 216.26°C was attributed to melting, while the third exothermic peak at 298.21°C corresponds to the crystallization. As temperature increases, a gradual and reversible transition occurs in the solid phase, shifting from a rigid, glassy state to a more flexible, rubbery state—this phenomenon is known as the "glass transition." According to Mishra et al. (1999), as the temperature of an amorphous solid polymer increases, its viscosity decreases. At a certain point, known as the crystallization temperature, polymer particles undergo an exothermic transition, reorganizing into a crystalline solid and gaining mobility. This enhanced stability likely results from the compact nature of the crystalline structure, which resists thermal degradation by limiting intramolecular interactions before the eventual breakdown of cellulose (Merkel et al 2014). The low thermal stability of JT2 cellulose might be due to poor cellulose development or non-cellulosic constituents with poor thermal stability, such as hemicellulose (amino acids, sugars, and protein (Çakar et al., 2014) in *invitro* shoots. The $CaCl_2$ and NaCl treated cellulose samples, JT3 and JT4 exhibited exothermic peaks (representative of Tc for crystallinity) at around 296.4 and 389.4 °C, respectively. Despite this, the rest of the samples showed more prominent endothermic peaks within the 197 and 390 °C temperatures, which represent a strong degradation phenomenon during thermal analysis.

Conclusion

This study highlights the potential of *in vitro*-cultured shoots of *J. rigidus* as a novel and sustainable source of cellulose. By optimizing culture conditions shoot proliferation, cellulose yield and quality can be improved considerably. The structural and thermal analyses (XRD, FTIR, TGA, DTG, and DSC) collectively revealed that cellulose extracted from *Juncus in vitro* cultures were influenced by the addition of $CaCl_2$ and NaCl. XRD analysis confirmed this with sharp peaks at $2\theta = 16.4°$, and 22.55° representing the cellulose I crystalline form, and the highest Crystallinity Index (CI) in JT4 (51.6%). FTIR spectra of JT4 showed the absence of lignin and hemicellulose peaks, presence of strong O–H, C–H, and β-glycosidic linkage bands, and a distinct 1428 cm^{-1} band, indicating high crystallinity and structural integrity. DSC thermograms JT4 exhibiting a strong exothermic peak at 389.4°C, suggesting enhanced crystallinity. TGA and DTG confirmed a three-stage degradation process in JT4 showing the highest thermal stability, greatest mass loss (72.73%), and lowest char residue, indicating higher cellulose purity. These findings demonstrate

that optimized salt supplementation in the culture medium enhances cellulose purity, crystallinity, and thermal stability that makes it a promising candidate for applications in development of biopolymer based biomaterials in medical and drug delivery fields. The findings suggest that *in vitro* cultivation of *J. rigidus* under optimized conditions not only improves biomass production but also enhances cellulose extraction efficiency. This approach offers an environmentally friendly method for producing high-quality, pesticide and insecticide free cellulose. To the best of our knowledge, this study is the first to demonstrate the successful extraction and characterization of cellulose from callus based *in vitro*-grown *J. rigidus* shoots. Exploring a broader range of abiotic factors or stress conditions on *in vitro* cultures may influence cellulose characteristics and content. Future research can focus on scaling up the production process for developing cellulose with desired properties by further modifying culture medium compositions, exploring the genetic and molecular mechanisms underlying cellulose biosynthesis. This could support the development of nanocellulose-based biomaterials and identify new high-cellulose plant sources. Integrating plant tissue culture offers a sustainable alternative to traditional methods, meeting the growing demand for eco-friendly, chemical-free biomaterials.

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Credit author statement

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