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RESEARCH ARTICLE

Immobilization of *Kluyveromyces lactis* for xylose production using different lignocellulosic materials as carbon sources

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Abstract - Lignocellulosic biowaste is a renewable and abundant resource with significant potential for the production of value-added products such as xylose which is a major intermediary in the biofuel, bioplastic, and pharmaceutical sectors. However, problems such as inefficient bioconversion and instability of free-cell fermentation systems limit its industrial application. This research investigates the use of immobilized recombinant Kluyveromyces lactis for xylose production using various lignocellulosic biowastes such as sugarcane bagasse, corn stover, rice straw, oil palm fronds, and banana leaves. The biowastes were washed, dried, ground, and pre-treated with 1% sodium hydroxide (NaOH) solution. K. lactis cells were cultivated in yeast peptone dextrose (YPD) medium and immobilized in 2% sodium alginate beads cross-linked with 0.1 M calcium chloride (CaCl2). Fermentation was carried out at 30 °C and 150 rpm for 24 hours using 1 g of pre-treated biomass and immobilized yeast beads in 100 mL of YPD medium. Reducing sugar concentration was determined using the dinitro salicylic acid (DNS) method, while yeast growth and pH changes were monitored. Results demonstrated that immobilized K. lactis could produce xylose production across all biowaste types, with sugarcane bagasse and corn stover yielding the highest reducing sugar concentrations. However, a further optimization is needed to increase the enzyme production from the immobilized cell systems. The findings highlight a cost-effective and environmentally friendly approach for improving industrial xylose production through K. lactis immobilization on lignocellulosic biowastes.

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1. Introduction

The growing demand for sustainable energy has accelerated research into renewable biofuels as viable alternatives to fossil fuels. Among the various feedstocks, lignocellulosic biomass, which contains cellulose, hemicellulose, and lignin, stands out for its availability, low cost, and potential for value-added products. Lignocellulosic biowastes such as rice straw, wheat straw, and sugarcane bagasse are often dumped, burned, or left to rot, thereby polluting the air, degrading the soil, and increasing greenhouse gas emissions. However, these materials contain hemicellulose, which can be hydrolysed into xylose, a five-carbon sugar with huge potential for producing biofuels, bioplastics and pharmaceuticals [1]. Despite this potential, efficient xylose fermentation remains a major difficulty due to the poor performance of free-cell yeast systems. The free yeast systems suffer from drawbacks such as instability, low reusability, and sensitivity to inhibitory compounds produced during biomass pre-treatment [2]. These limitations reduce fermentation efficiency and increase production costs due to cell loss during fermentation, thereby hindering large-scale production [3]. Recent advancements in genetic engineering have expanded the fermentative capabilities of various yeast strains, including *K. lactis*, which has traditionally been used in dairy industries for lactose fermentation, to encompass xylose fermentation. Through genetic modification, *K. lactis* has been introduced with essential enzymes such as xylanase, helping it to convert xylan, which is available in the hemicellulose of plant cell walls, into a reducing sugar, specifically xylose [4]. Furthermore, *K. lactis* has shown enhanced tolerance to common inhibitors in lignocellulosic hydrolysates, such as acetic acid, furfural, and phenolic compounds, supporting its potential for industrial fermentation processes even in the presence of toxic compounds that often hinder other yeast strains [5]. Nonetheless, most previous studies involving recombinant *K. lactis* have relied on free-cell systems or synthetic immobilisation supports, which are typically constrained by low stability, reduced cell reusability, and high operational expenses [6], [7]. While some investigations have explored alternative immobilisation materials, limited attention has been given to optimising established methods, such as calcium alginate encapsulation, specifically for recombinant *K. lactis* in lignocellulosic enzymatic degradation. This highlights a gap in the development of robust, cost-effective, and scalable immobilised systems optimised for recombinant strains in lignocellulosic bioconversion.

In response to these challenges, this study investigates the use of recombinant *K. lactis* cells immobilised in calcium alginate beads for lignocellulosic degradation. Five types of lignocellulosic biowastes, including sugarcane bagasse, corn stover, rice straw, oil palm fronds, and banana leaves, were selected. Calcium alginate was used to immobilise yeast cells, which is a well-known encapsulation approach that provides good cell retention, inhibitor protection, and operational stability [8]. The approach, which combines recombinant yeast in an immobilised system with lignocellulosic biowaste as the fermentation substrate, aims to improve xylose production efficiency in a cost-effective and environmentally friendly manner, offering advantages over conventional free-cell fermentation systems [5]. By addressing the constraints of free-cell systems and utilising recombinant yeast technology, this study hopes to benefit ongoing efforts in renewable energy generation and agricultural waste valorisation [9] - [10].

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2. Materials and Methods

2.1 Preparation of lignocellulosic biowaste

Lignocellulosic biowastes such as corn stover, oil palm fronds, rice straw, and banana leaves were collected from local agricultural areas, while sugarcane bagasse was obtained from a sugarcane juice vendor. The materials were washed with distilled water, oven-dried at 60 °C for 48 hours, and ground into fine powder. For alkaline pre-treatment, the ground biowastes were soaked in 1% (w/v) sodium hydroxide (NaOH) solution for 2 hours with gentle agitation. The pre-treated biomass was neutralised with distilled water to a pH of 6.5-7.0, oven-dried, autoclaved, and stored in sterile containers at room temperature until use [11].

2.2 Preparation of recombinant *K. lactis* culture

Recombinant *K. lactis* expressing xylanase [12] was revived from glycerol stock by streaking onto YPD agar plates, which contained 1% yeast extract, 2% peptone, 2% glucose, and 2% agar and incubated at 30 °C for 48 hours. The YPD broths with inoculated recombinant *K. lactis* were incubated at 30 °C with shaking at 150 rpm. Growth was monitored every 4 to 6 hours by measuring optical density at 660 nm (OD_{660}). Once cultures reached exponential phase (OD_{660} = 0.6–1.0), the colony-inoculated culture was selected for further use. Cells were harvested by centrifugation at 6000 rpm for 15 minutes at 4 °C; the yeast pellet was washed twice with sterile distilled water and resuspended to an OD_{660} of 0.6–1.0 for encapsulation [13].

2.3 Encapsulation of recombinant *K. lactis*

A 2% (w/v) sodium alginate solution was prepared by dissolving sodium alginate in distilled water and sterilised by autoclaving. The yeast suspension (OD_{660} = 0.6–1.0) was mixed with the sodium alginate solution at a 1:1 (v/v) ratio. The mixture was added dropwise into sterile 0.1 M calcium chloride ($CaCl_2$) solution under gentle stirring to form alginate beads via ionic gelation. Beads were cured in the $CaCl_2$ solution for 30 min at room temperature, then washed with sterile distilled water to remove unbound calcium ions and free yeast cells. The immobilised yeast was stored at 4 °C until use [7].

2.4 Fermentation Process

Fermentation was conducted in 500 mL fermentation flasks containing 1 g of pre-treated sugarcane bagasse, corn stover, rice straw, oil palm fronds and banana leaves and 100 mL of YPD medium, which contained 1% yeast extract, 2% peptone and 2% glucose, adjusted to a pH of 5.5 to 6.0. The medium was sterilised by autoclaving prior to use. A total of 300 alginate-encapsulated *K. lactis* beads were introduced into each flask. Fermentation was conducted at 30 °C with shaking at 150 rpm to ensure aeration and uniform substrate suspension. Samples were collected and stored at 4 °C prior to analysis. A control experiment using free *K. lactis* cells was performed under identical conditions to evaluate the performance of the immobilised system. Fermentations were conducted in duplicate [14].

2.5 Analytical Methods

Yeast growth was monitored by measuring optical density at 660 nm (OD_{660}) using a UV-Vis spectrophotometer, with YPD medium as the blank. 1 mL samples were taken every targeted hour to monitor the growth of recombinant *K. lactis* in both free and immobilised conditions [15]. Xylose production was identified by reducing sugar detection using the dinitrosalicylic acid (DNS) method. After centrifugation, 1 mL of the supernatant was mixed with 1 mL of DNS reagent, boiled in a water bath for 10 minutes, cooled, and absorbance was measured at 540 nm. A standard glucose curve ranging from 0.1 to 1.0 g/L was used for quantification [16]. The pH of the fermentation medium was measured at regular intervals using a calibrated digital pH meter. Calibration was performed using standard buffer solutions at pH 4.0, 7.0, and 10.0. pH values were recorded to check whether they were within the optimal range of 5.0–6.5 for recombinant *K. lactis* [17] - [18].

2.6 Statistical analysis

All experiments were conducted in single or triplicate as appropriate. Data from reducing sugar quantification (dns method) and od_{660} measurements were collected in triplicate and expressed as mean ± standard deviation (sd). Graphs illustrating changes in reducing sugar concentration, OD_{660}, and pH during fermentation were generated using GraphPad Prism (version 8.0.2).

3. Results and Discussion

3.1 Optical density (OD) measurement

To assess growth dynamics during fermentation, the optical density at 660 nm (OD_{660}) was measured at regular intervals for free *K. lactis* systems. As the immobilised cells were encapsulated in bead form, their growth was controlled and was not measured using optical density. Optical density measurements are collected regularly and in a timely manner to assess cell density during cultivation and monitor cell growth [19]. As shown in Figure 1, the culture began a lag phase from 0 to 12 hours, characterised by a slow rise in OD_{660} values as the cells adapted to the new YPD medium. This is referred to as the lag phase, the time it takes for yeast cells to multiply and populate the medium [20]. From 12 to 71 hours, *K. lactis* entered the exponential growth phase, with a steady and pronounced increase in cell density. During this phase, the increase in biomass concentration is normally proportional to the original biomass concentration, driven by favourable nutrient availability and optimal environmental conditions [21].

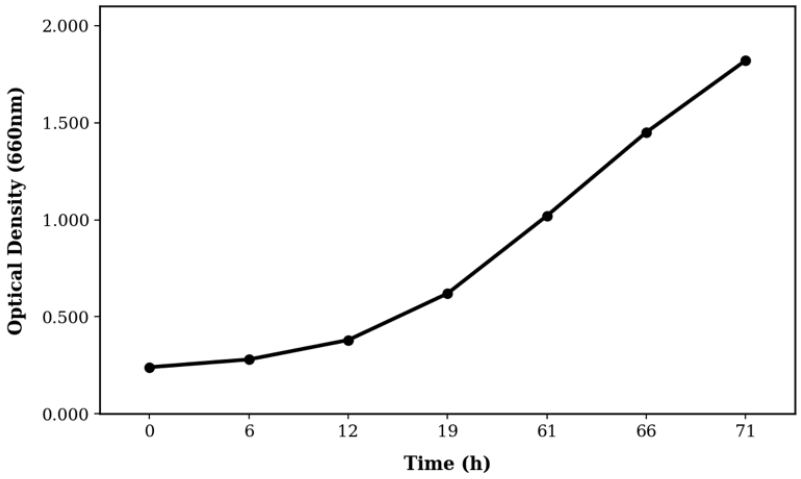


Figure 1. Growth profile of free *K. lactis*

No stationary or death phase was observed within the 71-hour period, as OD_{660} continued to rise without reaching a plateau. This implies that nutrient depletion or inhibitory metabolite build-up had not yet reached levels that could impede growth. In typical yeast physiology, the onset of the stationary phase is associated with nutrient exhaustion and stress-related cellular changes, such as cell wall thickening, glucose accumulation, and the development of thermotolerance [22]. Furthermore, no decrease in OD_{660} values was observed, indicating the absence of cell death, which often results from ineffective stress responses under prolonged nutrient limitation [23].

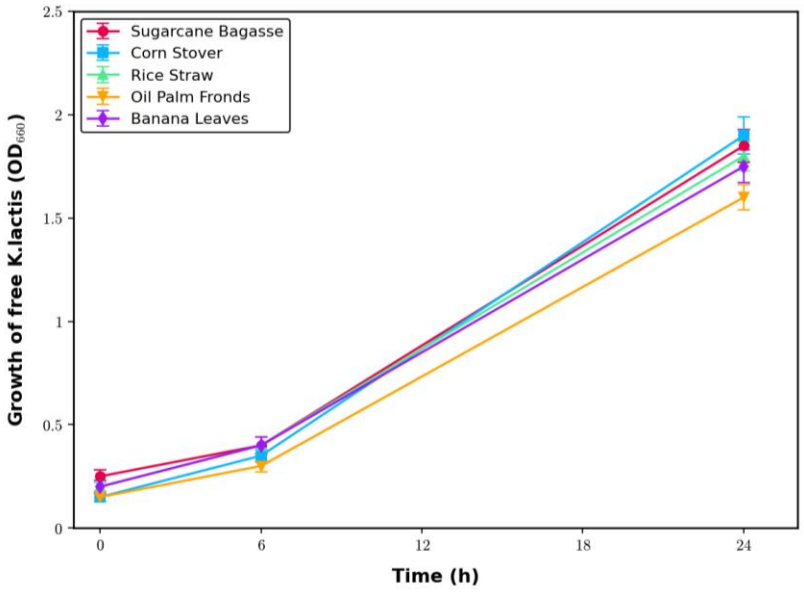


Figure 2. Growth profile of free *K. lactis* on different lignocellulosic materials over time

To compare cell growth, observations were made on free *K. lactis* cells in the presence of different lignocellulosic materials. As shown in Figure 2, the growth of *K. lactis* was consistent with the presence of different types of lignocellulosic materials. No inhibition seems to occur that can disturb the growth of *K. lactis*, where corn stover gives the highest OD_{660} value of 1.921, followed by sugarcane bagasse and rice straw with $OD_{660\ values}$ of 1.843 and1.859, respectively. Banana leaves resulted in an OD_{660} of 1.735, and oil palm fronds had the lowest OD_{660} at 1.572. As illustrated in Figure 2, the OD_{660} values increased consistently over the fermentation period, indicating active yeast cell proliferation. In addition, the growth rate of *K. lactis* was higher in the presence of lignocellulosic material, where it needed only 24 hours to reach OD_{660} 1.5-2.0, whereas growth without lignocellulosic material took 71 hours. This is most likely due to their structural porosity and good surface characteristics, which encourage yeast adherence and nutrient accessibility [24] - [25]. Free-cell systems consistently demonstrated higher OD_{660} values due to the direct exposure of free cells to nutrients and the absence of physical barriers [26]. Overall, the consistent rise in OD_{660} values affirms that *K. lactis* remained metabolically active under free-cell conditions. These findings demonstrate the efficacy of lignocellulosic biowastes as cost-effective, environmentally acceptable matrices for yeast immobilisation, with implications for scalable, sustainable bioprocessing systems.

3.2 pH Monitoring

The pH of the fermentation medium was measured during fermentation to assess metabolic activity and acid production by recombinant *K. lactis* using both free and immobilised cells. As shown in Figure 3, the pH trend decreased over time, indicating active fermentation and organic acid accumulation. As shown in Figure 3, immobilised *K. lactis* resulted in greater acidification compared to free cells. Figure 3(a) showed the pH profile of free *K. lactis*, which ranged from pH 6 to pH 5, while in the immobilised system (Figure 3(b)), the pH decreased from 5 to 3. These findings suggest enhanced metabolic activity in the immobilised systems, likely due to increased cell retention and stability conferred by the support matrices. Immobilisation techniques often improve cell density and prolong metabolic activity, contributing to greater acid production [8]. This pattern of decrement fits all types of lignocellulosic waste used, indicating delayed but prolonged acid generation, possibly due to the slow release of fermentable carbohydrates or favourable surface properties for yeast adherence and activity. The observed acidification trends are consistent with typical yeast fermentation behaviour, in which metabolic by-products such as lactic acid, ethanol, and citric acid contribute to a drop in pH, thereby promoting greater acid production. These findings align with the research by [27], which concluded that the production of citric acid by immobilised yeast cells was substantially higher than that of the freely suspended yeast cells.

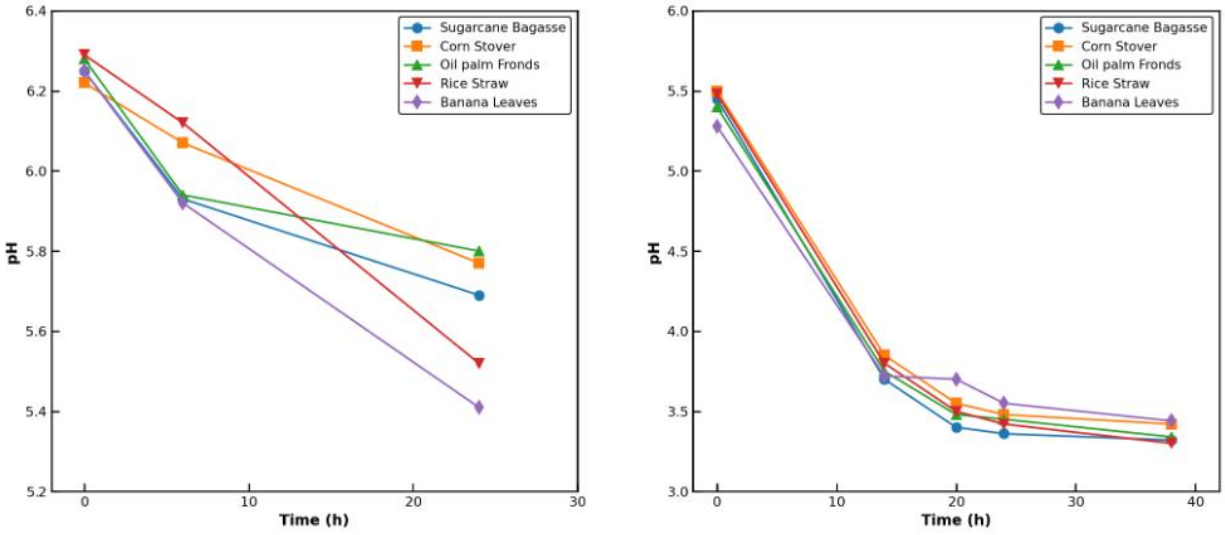
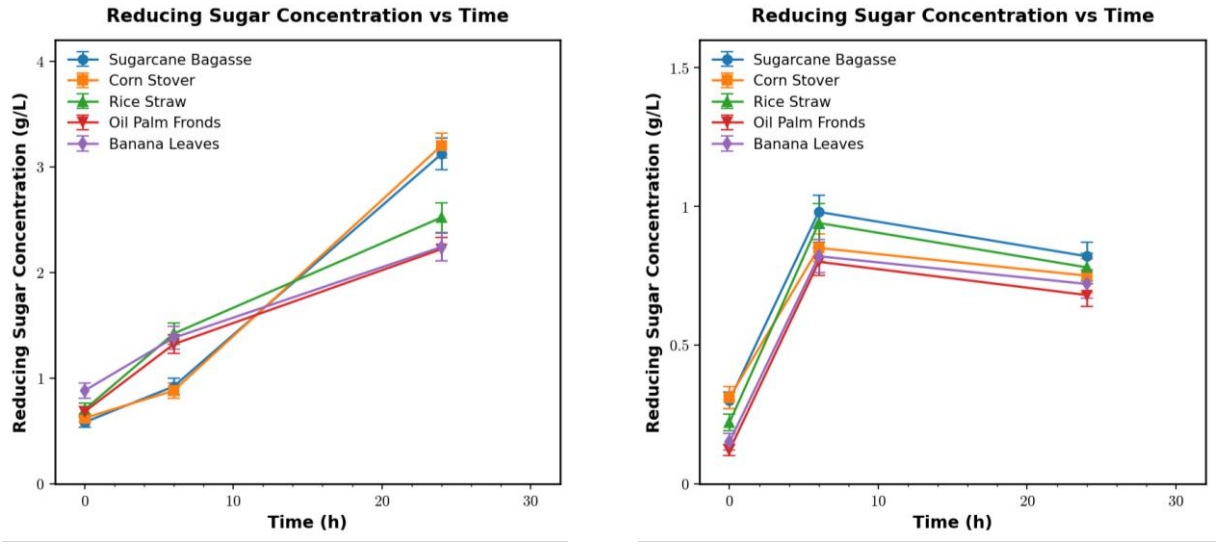


Figure 3. pH profile during fermentation using free (a) and immobilised (b) *K. lactis* containing different types of lignocellulosic material

3.5 Reducing Sugar Quantification

For the breakdown of lignocellulosic material into xylose (reducing sugar) by xylanase produced by recombinant *K. lactis*, reducing sugar detection was used. The reducing sugar concentrations during fermentation were estimated using the dinitro salicylic acid (DNS) method, with a glucose standard curve confirming linearity and reliability at 540 nm [16]. This method offers a rapid, non-specific estimation of reducing sugars in complex hydrolysates. Figure 4 illustrates the reducing sugar concentrations during fermentation using both free and immobilised *K. lactis* across various lignocellulosic substrates in two batches, showing temporal trends and variations in sugar release efficiency. Figure 4(a) shows the reducing sugar concentration obtained in the free cell system. The reducing sugar concentration increases steadily over 24 hours for all substrates. Corn stover shows the highest reducing sugar concentration (~3 g/L), followed by oil palm fronds and rice straw, while banana leaves and sugarcane bagasse show slightly lower values. The consistent upward trend suggests active hydrolysis or enzymatic breakdown of complex carbohydrates into reducing sugars by free K. lactis cells. This indicates effective sugar release over time as the yeast remains metabolically active and free in the medium. In Figure 4(b), the immobilised system shows a sharp increase at the early stage (up to 6 h), followed by a plateau or slight decrease in reducing sugar concentration up to 24 h. The maximum sugar concentration achieved is around 1.0 g/L, significantly lower than in the free cell system. This suggests restricted enzymatic activity or substrate diffusion limitations due to the immobilisation matrix, which may limit contact between cells and substrates. The early increase implies initial release of sugars possibly from easily hydrolysable fractions, while the later stagnation indicates reduced enzymatic efficiency over time. Both systems show rapid sugar release within the first 6 hours, indicating active enzymatic hydrolysis and substrate conversion by *K. lactis* in the early stage. At 6 hours, reducing sugar concentrations in the free cell system (~1.0–1.3 g/L) are higher than those in the immobilised system (~0.8–1.0 g/L), reflecting better substrate accessibility and enzyme activity in the free system. However, the free cell system consistently produces slightly higher reducing sugar concentrations across all substrates, suggesting more efficient substrate–cell interaction. In the immobilised system, sugar production appears to reach an early plateau by 6 hours, indicating possible mass transfer limitations or restricted substrate diffusion within the immobilisation matrix. This implies that while both systems are active initially, free cells exhibit faster and more extensive conversion at this intermediate stage.



(a) (b)

Figure 4. Reducing sugar concentration for (a) free and (b) immobilised *K. lactis*

Finally, the comparative research shows that free *K. lactis* is better suited for short-term fermentations requiring rapid, high initial sugar release due to its greater substrate accessibility and enzymatic activity. However, sugar concentrations tend to decline over time, limiting their use in extended processes. Immobilised K. lactis, on the other hand, produces more stable and sustained sugar release over longer periods, making it suited for continuous or batch-fed fermentation operations that require cell reuse and longevity [28]. Although the immobilised cell system is more stable for long-term use, it suffers from diffusion constraints that lead to lower enzyme release, thereby reducing interaction between lignocellulosic biomass and the sugar conversion process. Thus, optimising factors such as bead porosity, substrate pre-treatment, or agitation rate could improve the performance of the immobilised system. As studied by [29], optimisation using response surface methodology would help identify the significant parameters for increasing enzyme production, thereby indirectly increasing sugar release. These results underline the importance of selecting the appropriate fermentation strategy and substrate type for efficient bioconversion of lignocellulosic biomass.

4. Conclusions

This study successfully demonstrated the potential of immobilised recombinant *K. lactis* cells to produce xylose from a variety of lignocellulosic biowastes, including sugarcane bagasse, corn stover, rice straw, oil palm fronds, and banana leaves. Immobilisation of *K. lactis* in calcium alginate beads improved the operational stability of the fermentation process and reduced cell loss when compared to free-cell systems. Among all lignocellulosic biowastes, sugarcane bagasse and corn stover had the highest reducing sugar concentrations, likely due to their higher hemicellulose and cellulose content. Even though the immobilisation of K. lactis was successful, further optimisation is needed to increase enzyme release for interaction with lignocellulosic material for sugar production, specifically xylose. Overall, the findings support the feasibility of using lignocellulosic biowaste as low-cost, sustainable immobilisation matrices in industrial fermentation processes, providing a viable path for sustainable bioprocess development.

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Declaration of Competing Interests

The authors declare no conflicts of interest.

CRediT Author Contribution Statement

D. Chandran: Conceptualization; Investigation; Formal analysis; Methodology; Data curation; Writing – original draft)
M.A. Jenol: Resources, Writing manuscript
S. A. Abdul Manaf: Visualisation; Supervision; Resources; Writing manuscript

Availability of Data and Materials

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

Ethics Statement

This research involved extraction and characterisation of biomass-derived materials and did not involve human participants, animal subjects, or sensitive personal data. Therefore, ethical approval and informed consent were not required for this study.

Generative Artificial Intelligence Disclosure

AI-based tools were used solely for language editing and grammar refinement. No AI content generation or data interpretation was performed. All intellectual contributions are original and solely attributable to the authors.

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