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Isolation of Cellulose Nanocrystals from Rice Husks using Natural Deep Eutectic Solvent

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ABSTRACT

Cellulose nanocrystals (CNCs) are highly crystalline, rod-shaped nanoparticles derived from cellulose, commonly found in biomass such as rice husks. Rice husks, an agricultural waste rich in cellulose, can be utilized for CNC production. In this study, CNCs were isolated from rice husks using a natural deep eutectic solvent (NADES), an environmentally friendly solvent. The objective was to examine the effects of temperature and reaction time on CNC solubility during dissolution with NADES. The one-factor-at-a-time (OFAT) method revealed that the optimal conditions were at temperature of 120°C and a reaction time of 8 hours. Morphological analysis using microscopy showed that raw rice husks had a rough, solid, brown appearance, while alkaline-treated rice husks appeared smoother and more porous. Bleached rice husks exhibited a very smooth, white, and fluffy appearance, and CNCs appeared as transparent solids. Fourier transform infrared (FTIR) analysis indicated the presence of β-glycosidic linkages in all three samples (CNCs, alkaline-treated, and bleached rice husks), suggesting that the cellulose structure remained intact during pretreatment. Antibacterial activity was evaluated using the disc diffusion method, confirming that raw, alkaline-treated, and bleached rice husks, as well as CNCs isolated from rice husks, exhibited antibacterial properties against both gram-negative bacteria (E. coli) and gram-positive bacteria (B. subtilis). This study successfully isolated CNCs from rice husks using NADES, demonstrating the potential for further improvements to enhance production efficiency.

Keywords: Cellulose nanocrystals, Deep eutectic solvents, Rice husks, Solubility, Antibacterial.

1. INTRODUCTION

Cellulose nanocrystals (CNCs) are rod-shaped nanoparticles composed of highly ordered cellulose. Typically less than or equal to 100 nanometers in length (Li et al., 2018), CNCs are noted for their high aspect ratio, low density, exceptional tensile strength, and high biodegradability. These characteristics have garnered significant interest. Common methods for CNC preparation include acid hydrolysis, and various mechanical and chemical techniques (Lu & Hsieh, 2012), with sulfuric acid hydrolysis being the most widely used.

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Rice husks, an agricultural byproduct of rice milling, is a protective layer surrounding rice grain. It contains hard substances like lignin and silica to protect the seed during growth. Composed of about 35% cellulose, 25% hemicellulose, 20% lignin, 17% silica, and 3% wax (Samsalee et al., 2023), rice husks presents disposal challenges and is often underutilized or burned for basic energy. Due to its high cellulose content, rice husks is a suitable raw material for CNC production. Acid hydrolysis is a conventional method for separating CNC from amorphous cellulose (Wulandari et al., 2016), but it generates significant inorganic salts and contaminated water, posing environmental concerns, and requires lengthy reaction times and high temperatures (Wang et al., 2023b).

Natural deep eutectic solvents (NADES) offer an eco-friendly alternative for producing anionic and non-derivatized nanocelluloses. NADES are formed by combining two or three components that create a eutectic mixture through hydrogen bonding, resulting in a lower melting point than the individual components (Wang et al., 2023a). A common NADES formulation uses choline chloride (ChCl) as the hydrogen bond donor (HBD) and citric acid as the hydrogen bond acceptor (HBA). The eutectic mixture remains liquid at room temperature due to hydrogen bonding (Xiong Chang et al., 2021).

CNCs exhibit antibacterial activity against both gram-positive and gram-negative bacteria, although efficacy varies among bacterial types (Firmanda et al., 2023). The precise antibacterial mechanism is not fully understood but may involve membrane damage, cell wall synthesis interference, or metabolic disruption. This study aims to evaluate the effect of temperature and reaction time on the CNC solubility, as well as to examine the antibacterial activity of CNCs produced from rice husks using NADES.

2. MATERIALS AND METHODS

2.1 Preparation of NADES

NADES was prepared by mixing the citric acid with choline chloride at 1:2 mole ratio. The mixture was stirred at 80° C for 1 hour until homogeneous solution is obtained. Then, 10wt % of distilled water was added to ease the homogenization of the mixture.

2.2 Pretreatment of Rice Husks

Raw rice husks were collected from a rice processing mill, Dibuk Sdn Bhd, Perlis, Malaysia. The husks were dried at 80° C overnight to remove any moisture in the raw material. The dried rice husks were then grounded and sieved using a 250 μm sieve to obtain consistent particle size. Subsequently, the grounded rice husks particles were pretreated using 3% w/v sodium hydroxide (NaOH) solution. Then, the alkaline treated rice husks were bleached using 1% w/v sodium chlorite ($NaClO_2$) at 70 °C for 1 hour.

2.3 Dissolution of Treated Rice Husks in NADES

The isolation of CNCs were performed by dissolution of the treated rice husks in NADES at 1:40 solid to liquid ratio. The rice husks/NADES mixture were subjected to heating at different temperatures between 60 °C to 120 °C and reaction time between 4 to 8 hours. One-factor-at-a-time (OFAT) method was used to investigate the effect of these parameters on the solubility of the CNCs. After pre-determined reaction time, cold distilled water was added to stop the reaction. The mixture was stirred for another 20 minutes at room temperature, centrifuged at 8000 rpm for 10 minutes to collect the CNCs suspension, followed by drying at 40 °C to obtain the CNCs powder. The solubility percentage of CNCs was determined using Equation 1:

$$Solubility\ (\%) = \left(\frac{\text{weight of dry CNCs (g)}}{\text{initial weight of treated rice husks (g)}}\right) \times 100 \quad (1)$$

2.4 Morphological Analysis

Microscope analysis was conducted to observe the apparent shape and morphological appearance of raw rice husks, alkaline-treated rice husks, bleached rice husks, and CNCs produced. The visual images were recorded and analyzed using an optical microscope (B-150 Series, Optika).

2.5 Antibacterial Testing

The antibacterial activity of the CNCs isolated from rice husks were determined using disc diffusion method. Paper disc containing 5 μL of the CNCs suspension were placed onto an agar plate which was inoculated with bacteria prior testing. The Gram-negative *Escherichia coli* and Gram-positive *Bacillus subtilis* were used for this antibacterial test. The plate were incubated at 37 °C for 24 and 48 hours. The inhibition zones were measured and recorded.

3. RESULTS AND DISCUSSION

3.1 Effects of Reaction Time on CNC solubility

This study investigated the effect of reaction time on the solubility of CNCs from rice husks in NADES (Figure 1).

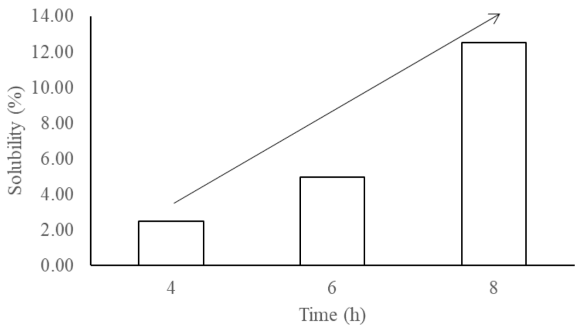


Figure 1: Solubility of rice husks in NADES based on reaction time.

The reaction times were set at 4, 6, and 8 hours, with the reaction temperature maintained at 60 °C. The results indicated that CNC solubility in NADES increased with reaction time: 2.50% at 4 hours, 5.01% at 6 hours, and 12.53% at 8 hours. This trend suggests that longer reaction time enhance CNC dissolution in NADES due to the prolonged resistance of highly crystalline cellulose to hydrolysis. The increased contact time of the hydrolyzing agent with the substrate results in swelling and loosening of the intra- and intercellular cellulose network, allowing the agent to penetrate the cellulose structure gradually and release nanoscale cellulose (Rana et al., 2021). Specifically, a longer reaction time in NADES, containing HBD and HBA components, led to greater disruption of the cellulose hydrogen bond network (Dhali et al., 2021).

3.2 Effects of Different Reaction Temperature on solubility of rice husks in NADES

Figure 2 illustrates the solubility of rice husks in NADES as a function of reaction temperature. The reaction temperature was varied at 60 °C, 90 °C, and 120 °C, while the reaction time was fixed at 8 hours. The results showed that the solubility of rice husks in NADES increased with rising reaction temperatures: 7.95% at 60 °C, 10.00% at 90 °C, and 12.50% at 120 °C. This increase is attributed to the progressive reduction of the amorphous regions of cellulose, leading to a higher yield of crystalline cellulose (Rana et al., 2021). The combination of high temperature and extended reaction

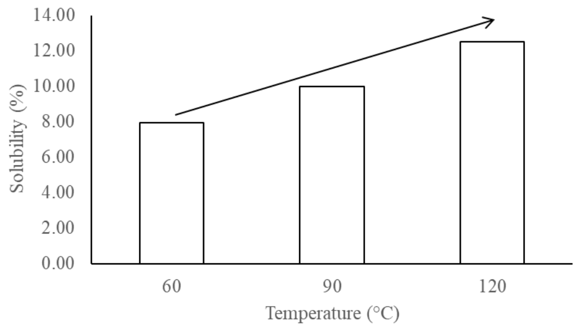
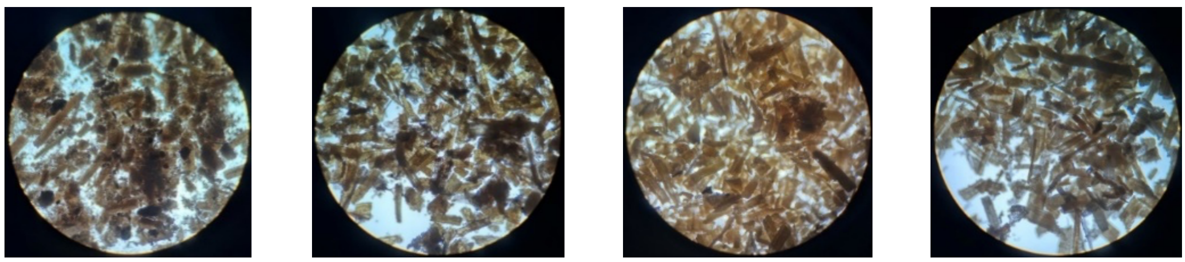


Figure 2: Solubility of rice husks in NADES based on reaction temperature.

time resulted in more efficient hydrolysis, which facilitated the depolymerization of cellulose, increasing its solubility and resulting in higher crystallinity and a more homogeneous structure (Bondancia et al., 2020). Based on the results above, at 120 °C, the solubility of rice husks in NADES is higher than its solubility in sulfuric acid, a conventional hydrolyzing agent. CNCs have polar hydroxyl groups on their surface, which are attracted to the polar molecules in NADES. This attraction facilitates the dissolution of CNCs in NADES. Conversely, sulfuric acid, being a non-polar solvent, does not interact effectively with the polar groups on the surfaces of CNCs (Mahmud et al., 2019). Therefore, NADES might be a more efficient solvent for dissolving rice husks compared to sulfuric acid.

3.3 Morphological Analysis

A microscope was used to observe the apparent shape and morphological appearance of raw rice husks, alkaline-treated, bleached, and rice husks dissolved in NADES. The raw rice husks was ground to a fine particle size of 250 µm. The surface of the raw rice husks appeared rough and solid with a brown coloration. The cell walls were thick and lignified, with individual cells easily visible. This is because the cell walls of raw rice husks have not been subjected to chemical treatments. Raw rice husks contains lignin and hemicellulose, which act as cementing materials, firmly binding bundles of individual fibrils (Abu-Thabit et al., 2020). Additionally, the presence of silica particles provides further resistance to the hydrolysis of rice husks into solitary fibers, contributing to the fiber's closely packed fibrillar arrangement (Otoni et al., 2021). Figure 3 shows the microscopic images of raw rice husks, alkaline-treated rice husks, bleached rice husks, and the as-produced CNCs.



(a) (b) (c) (d)

Figure 3: Visual image under microscope of a) raw rice husks, b) alkaline treated rice husks, c) bleached rice husks and d) as-produced CNC.

Raw rice husks were subjected to alkaline treatment to remove silica particles. Alkaline-treated rice husks exhibited a smoother and more porous appearance than raw rice husks. Although the cell walls became thinner and less lignified, individual cells were still visible. This is because alkaline treatment partially degrades lignin and hemicellulose in the rice husks, increasing cell wall porosity. Hemicellulose removal and partial depolymerization of lignin were achieved with alkaline treatment (Xiao et al., 2019).

Further treatment with a bleaching agent was performed to promote further defibrillation and reduce fiber diameter. The dark color of the cellulose indicated that an insufficient amount of lignin was removed during the pre-treatment process (Huang et al., 2021). Bleached rice husks had a very smooth, white, and fluffy appearance. The bleaching procedure completely destroyed the cells, making them indistinguishable as separate entities. This is due to the use of strong chemicals during bleaching to remove all color and contaminants, including cell walls, from the rice husks. Lastly, CNCs appeared as transparent solids. This is because the NADES treatment completely dissolved the rice husks. NADES can dissolve lignin and hemicellulose in rice husks while keeping the cellulose intact, resulting in the transparent appearance of CNCs.

3.4 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR analysis was performed to observe and obtain information on the hydrogen bonding activity during the pretreatment of rice husks and the dissolution of treated rice husks in NADES. The FTIR spectra of raw rice husks, alkaline-treated rice husks, bleached rice husks, and CNCs are presented in Figure 4.

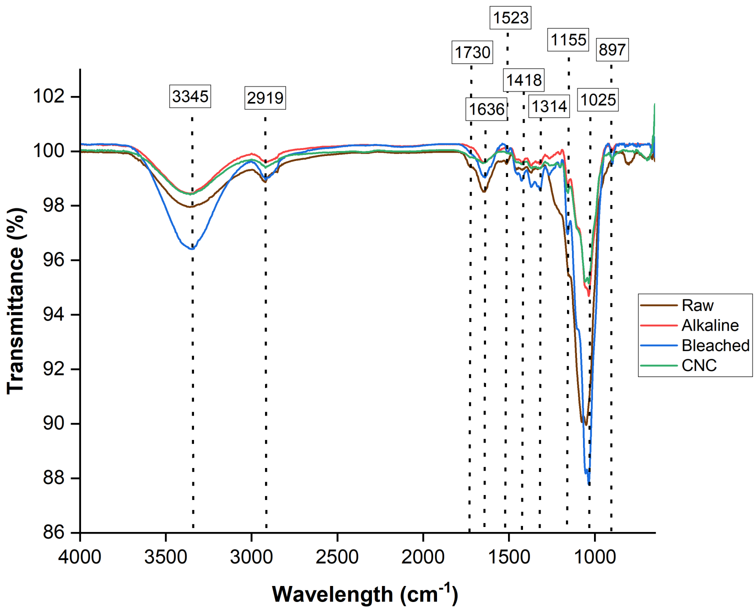


Figure 4: FTIR spectra of raw rice husks, alkaline treated rice husks, bleached rice husks and CNC.

According to the FTIR analysis results, rice husks undergoes modifications in its chemical composition and functional groups during pretreatment and dissolution. The stretching vibration of the free -OH group caused by hydrogen bonding in cellulose molecules appears in all samples (raw, alkaline-treated, bleached, and CNC) as a broad band between 3000-3700 cm^{-1} (Mankar et al., 2021). A peak between 3300 and 2900 cm^{-1} suggests the presence of hydroxyl groups and aliphatic saturated C-H stretching in lignin and cellulose (Sai Prasanna & Mitra, 2020).

A peak at 1636 cm^{-1} in all three samples indicates -OH bending of absorbed water due to interactions between cellulose and water molecules (Maheri et al., 2022). The disappearance of the transmittance peak at 1418 cm^{-1} in CNC, alkaline-treated, and bleached rice husks samples suggests that lignin has been removed. This peak represents the C=C aromatic skeletal vibration of lignin, present only in raw rice husks samples . A prominent peak at 1155 cm^{-1} in raw rice husks spectra is associated with C-O stretching of lignin, also found in CNC and treated rice husks, indicating residual lignin. Hemicellulose in raw rice husks was completely removed after alkaline treatment and bleaching, evidenced by the absence of the peak at 1730 cm^{-1}, which represents the C=O stretching vibration of hemicellulose (Sai Prasanna & Mitra, 2020). This peak, observed in raw rice husks spectra, reappeared in CNC samples due to residual citric acid from the NADES solution used for extraction. The peak at 1165 cm^{-1} in all samples (CNC, alkaline-treated, and bleached rice husks) indicates the presence of C-O-C asymmetric vibration, suggesting that the cellulose structure was not significantly damaged during pretreatment (Yang et al., 2021). The presence of β-glycosidic linkages in cellulose is confirmed by the peak at 897 cm^{-1} in all samples, specific to β-glycosidic linkages, indicating that the cellulose in the rice husks samples remained intact during the pretreatment process.

3.5 Antibacterial Testing

The antibacterial activity of raw rice husks, alkaline-treated rice husks, bleached rice husks, and CNCs was investigated against a gram-negative bacteria, *E. coli*, and a gram-positive bacteria, *B. subtilis* using the disc diffusion method. The inhibition zones were measured to assess the susceptibility of the microorganisms. Table 1 presents the disc inhibition zone of these components against *E. coli* and *B. subtilis* over 24 and 48 hours.

Table 1: Digital images of inhibition zone of raw and treated rice husks as well as CNC against *E. coli* and *B. subtilis* for 24 and 48 hours.

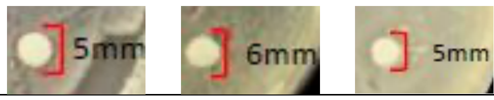
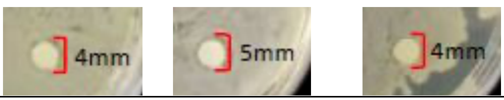
		24 Hours	48 Hours
E. coli	Raw rice husks		
	Alkaline treated rice husks		
	Bleached rice husks		
	CNC		
B. subtilis	Raw rice husks		
	Alkaline treated rice husks		
	Bleached rice huskss		
	CNC		

Table 2 displays the measurements for raw rice husks, alkaline-treated rice husks, bleached rice husks, and CNCs isolated from rice husks. The values in Table 2 were obtained by measuring the diameter from edge to edge across the zone of inhibition for all tested components. Each result represents the mean diameter of the inhibition zone, as the experiment was conducted in replicates. The values enclosed in brackets indicate the acceptable plus-minus range for the inhibition zone.

Table 2: Measurement of raw rice husks, alkaline treated rice husks, bleached rice husks and CNC isolated from rice husks.

Sample	Components	Zone of Inhibition (mm)			
		E. coli		*B. subtilis*	
		24 hours	48 hours	24 hours	48 hours
1	Raw Rice Husks	0	0	0	0
	Alkaline treated rice husks	0	0	0	0
	Bleached rice husks	2 (±1.00)	2 (±1.00)	0	0
	CNC isolated from rice husks	2.5 (±1.00)	2.5 (±1.00)	2 (±1.00)	3 (±1.00)
2	Raw Rice Husks	4.5 (±1.00)	2.5 (±1.00)	0	0
	Alkaline treated rice husks	4 (±1.00)	3 (±1.00)	0	0
	Bleached rice husks	0	0	0	0
	CNC isolated from rice husks	3 (±1.00)	3 (±1.00)	2.5 (±1.00)	2.5 (±1.00)
3	Raw Rice Husks	2.5 (±1.00)	2.5 (±1.00)	0	0
	Alkaline treated rice husks	3.5 (±1.00)	3 (±1.00)	0	0
	Bleached rice husks	0	0	0	0
	CNC isolated from rice husks	2.5 (±1.00)	2.5 (±1.00)	2 (±1.00)	2.5 (±1.00)

The inhibition zone for raw rice husks against *E. coli* was larger at 24 hours compared to the inhibition zone against *B. subtilis* at the same time point. This difference is attributed to the presence of silica in the raw rice husks. Silica can damage the outer membrane of *E. coli*, a gram-negative bacterium with a protective outer membrane that makes it more resistant to environmental factors like heat and chemicals. In contrast, *B. subtilis* lacks this outer membrane, making it less affected by silica. Consequently, raw rice husks is more effective against *E. coli* than against *B. subtilis*.

Furthermore, the inhibition zone for raw rice husks against *E. coli* was larger at 24 hours than at 48 hours. This can be explained by the concentration of antimicrobial compounds in the rice husks. During the initial 24 hours, higher concentrations of antimicrobial substances, such as ferulic acid and p-coumaric acid, are present. These substances are released when the rice husks are wet but decrease as the husks dry out. Additionally, by 48 hours, *E. coli* cells may develop increased resistance to these compounds due to prolonged exposure (Zhao et al., 2023).

Both alkaline-treated and bleached rice husks exhibited antimicrobial activity against *E. coli* at both 24 and 48 hours. This is attributed to the higher content of ferulic acid in these samples, which is known to have antibacterial properties against pathogens like *E. coli* (Zhao et al., 2023). CNCs isolated from rice husks, dissolved in NADES, also demonstrated antimicrobial activity against both *E. coli* and *B. subtilis* at 24 and 48 hours. This effect is due to the presence of antimicrobial compounds such as ferulic acid and p-coumaric acid in the rice husks.

4. CONCLUSION

This study investigated the isolation of cellulose nanocrystals (CNCs) from rice husks using natural deep eutectic solvents (NADES). It was found that CNCs could be effectively extracted from rice husks at lower temperatures and with a shorter reaction time compared to conventional acid hydrolysis. The optimal conditions were determined to be 8 hours at 120 °C, resulting in a CNC solubility of 12.50%. Characterization of the CNCs was performed using microscopy and FTIR analysis. Microscopic imaging revealed that raw rice husks had a rough, brown surface, while alkaline-treated rice husks exhibited a smoother, more porous appearance. Bleached rice husks had a very smooth, white, and fluffy surface. The CNCs isolated from rice husks were observed as transparent solids. The antibacterial activity of the CNCs was evaluated using the disc diffusion method, showing effectiveness against both gram-negative and gram-positive bacteria. Notably, CNCs were more effective against *E. coli*, a gram-negative bacterium with an outer membrane that makes it more resistant to environmental factors. The silica content in rice husks can damage the outer membrane of *E. coli*, increasing its susceptibility to CNCs. The results suggest that NADES is a promising green solvent for CNC extraction from rice husks. The CNCs obtained through this method demonstrate significant antibacterial activity, particularly against *E. coli*, highlighting their potential for applications in food packaging and other fields.

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