Enhanced Enzymatic Hydrolysis of Pine Wood Chips by Two-Step Pretreatment Combining Steam Explosion and Urea

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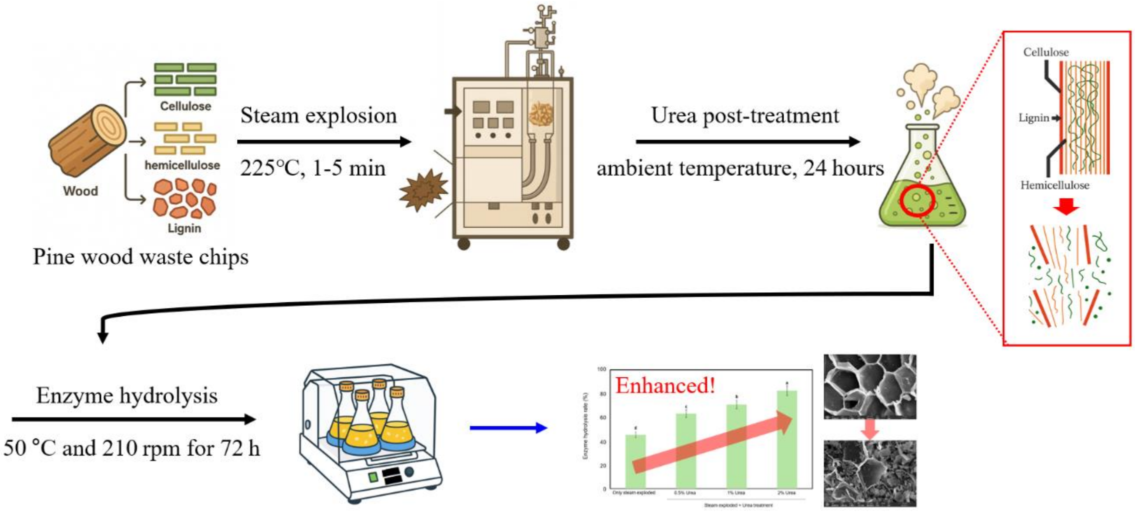
GRAPHICAL ABSTRACT



Enhanced Enzymatic Hydrolysis of Pine Wood Chips by Two-Step Pretreatment Combining Steam Explosion and Urea

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Lignocellulosic biomass such as pine wood offers a renewable alternative to fossil resources but remains challenging to convert due to its recalcitrant structure. Efficient pretreatment is essential to overcome this limitation and enable enzymatic hydrolysis. This study aimed to enhance enzymatic saccharification of pine wood chips through a two-step pretreatment combining steam explosion and urea treatment. Pine wood chips were first subjected to steam explosion to degrade hemicellulose and modify lignin structure, followed by ambient-temperature urea treatment (0.5 to 2%) to disrupt hydrogen bonding and increase porosity. Comprehensive chemical, structural, and morphological analyses were conducted, including BET surface area measurements and SEM imaging. The integrated pretreatment significantly improved enzymatic digestibility, with a maximum hydrolysis yield of 82% achieved at 1% urea concentration. Key factors contributing to this enhancement included increased surface area, reduced lignin–enzyme interactions, and improved cellulose accessibility. The combined treatment outperformed either method alone in terms of glucose release. These findings demonstrate the potential of a steam explosion–urea strategy as a cost-effective and scalable approach for pine wood bioconversion within an integrated biorefinery framework.

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Keywords: Pine; Steam explosion; Urea; Enzymatic hydrolysis

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INTRODUCTION

The transformation of plentiful lignocellulosic resources into biofuels has been considered to be a promising strategy to enhance energy security and reduce greenhouse gas emissions, particularly carbon dioxide (Nanda *et al*. 2015; El-Araby 2024). Pine trees were reported to exhibit a relatively consistent basic composition across different species (Brennan *et al*. 2012). Although the conversion of lignocellulosic biomass into biofuels has been acknowledged to hold significant economic and technical promise, several inherent challenges have been noted. These include the presence of lignin and hemicellulose, high cellulose crystallinity, limited surface accessibility, and covalent linkages between lignin and hemicelluloses (Borrero-López *et al*. 2022). To overcome these challenges, the implementation of a robust pretreatment process was deemed essential to reduce lignocellulosic recalcitrance (Chandra *et al*. 2007). In recent years, several innovative pretreatment techniques—such as steam explosion, hydrothermal treatment, and organosolv methods—were developed and evaluated (Chiaramonti *et al*. 2012). Among these, steam explosion pretreatment (SEP) was frequently employed. In this process, chipped lignocellulosic biomass was treated with high-pressure steam, followed by a sudden decompression. This abrupt pressure release caused explosive disruption of the biomass structure, making it more accessible to enzymatic hydrolysis

(Ramos 2003; Akhtar *et al*. 2016). This method facilitated delignification and significantly improved the enzymatic digestibility of the pretreated biomass (Kucharska *et al*. 2018; Ziegler-Devin *et al*. 2021). Despite its effectiveness, the SEP was recognized to have certain limitations (Pérez *et al*. 2002; Baksi *et al*. 2023).

Urea treatment also has been widely recognized as an effective pretreatment strategy, particularly for agricultural residues. This approach was applied to disrupt lignocellulosic bonds, improve the nutritional quality of straw, and was valued for its cost-effectiveness (approximately $449.7 per ton) and accessibility (Joy *et al*. 1992). The interaction of urea with lignin or cellulose surfaces occurred via hydrogen bonding, which formed hydration layers that weakened the lignin–cellulase binding through steric hindrance. As a result, non-productive cellulase adsorption on lignin was substantially reduced, thereby increasing the free cellulase concentration in the reaction medium and enhancing hydrolysis efficiency (Lou *et al*. 2018). Recent studies have demonstrated that the combination of steam explosion and urea treatment leads to significant improvements in biomass digestibility. For instance, Zhang *et al*. (2023) reported that a dual-step pretreatment of corn stalk using SE and 4.87% urea yielded up to 350 mg/g of reducing sugars. Recent studies highlight the synergistic effects of combining SE with urea-based alkaline treatments. For instance, the swelling induced choline alkali-urea (SICAU) process has demonstrated effective fractionation of rice straw, leading to enhanced enzymatic digestibility. Such combined pretreatment approaches not only improve sugar yields but also contribute to the development of sustainable biorefinery processes (Jiang *et al*. 2024). Despite these advancements, the application of combined SEP and urea treatments to softwood biomass, such as pine wood, remains underexplored. Given the abundance and high lignin content of pine wood, investigating its response to integrated pretreatment methods could unlock new pathways for efficient biomass conversion (Chen *et al*. 2024; Bhukya and Keshav 2022; Hoang *et al*. 2023). In this study, a two-step pretreatment strategy combining steam explosion and urea treatment was applied to pine wood in order to enhance enzymatic hydrolysis yield. The primary objective of the study was to identify a pretreatment method capable of both removing lignin effectively and improving the substrate's suitability for enzymatic digestion. Various pretreatment strategies were evaluated in terms of their effects on enzymatic hydrolysis efficiency. Furthermore, detailed chemical and structural characterizations were conducted to investigate the underlying mechanisms of the observed improvements.

EXPERIMENTAL

Material

The raw pine wood chips (residue of such processes as forestry and wood products manufacturing), including bark, were initially rinsed with tap water until the runoff became clear. They were then dried in an oven set to 60 °C until a constant weight was achieved and subsequently stored at room temperature within a desiccator. The bark accounted for approximately 10% of the volume of pine wood chips.

Steam Explosion Pretreatment (SEP)

The samples weighing 10 kg based on dry weight were cut into pieces measuring 3 cm × 3 cm. Steam explosion treatment was conducted at a pressure of 25 kg/cm² and a temperature of 225 °C for durations ranging from 1 to 5 minutes, using a 100 L batch reactor with saturated steam (Youlim Company, South Korea).

Urea Post-treatment

Urea treatment (0.5%, 1%, and 2%) was conducted under static conditions at ambient temperature for 24 h. After treatment, the mixture was filtered through Whatman No. 2 filter paper.

Enzymatic Hydrolysis

Samples intended for enzymatic hydrolysis were sterilized at 121 °C for 30 minutes after 1 g was transferred to a 30 mL test tube. The sterilized samples were cooled on a clean bench, followed by the addition of 0.1 M sodium citrate buffer (pH 5.0), 2% sodium azide, TWEEN 80 (polysorbate 80), and Cellic® CTec2 (Novozymes, Denmark) enzyme at 440 FPU/glucan. After incubation at 50 °C and 210 rpm for 72 hours, the reaction mixture was filtered using a 2G3 glass filter, dried for 24 hours in a constant-temperature desiccator at 105 ± 3 °C, and weighed to calculate the enzymatic hydrolysis yield.

Analysis Methods

The chemical compositions of the samples before and after steam explosion treatment were analyzed for glucan, xylan, arabinosyl residues, and lignin content. Analyses were carried out according to the NREL standard method (NREL/TP-510-42618) and TAPPI standard methods for paper and pulp.

The specific surface area of pine wood chips were measured using a MicroPore Physisorption Analyzer (ASAP 2020, Micromeritics Instrument Corporation, USA). To perform this analysis, solid samples were cooled to cryogenic temperatures using liquid nitrogen under vacuum. Nitrogen gas was injected into the steam-explosion-treated pine wood chips, and the weight of adsorbed nitrogen (W) was measured after each injection to calculate the specific surface area using the BET equation.

In the BET equation,

$$B_1/(W((P_o /P-1)) = 1/(W_mC) + ((C-1)/(W_mC))(P/P_o) \tag{1}$$

the constant C is related to the adsorption energy of the first adsorbent layer and indicates the magnitude of the adsorbent–adsorbate interaction. W_m, P, and P_o mean the weight of gas required to form a monolayer on the surface of the solid, equilibrium pressure of adsorbate gas, and saturation pressure of the adsorbate gas at the adsorption temperature, respectively. During measurement, gas pressure was gradually increased until all pores were filled with nitrogen molecules. The pressure was then decreased, and the condensed nitrogen was evaporated from the system. Adsorption and desorption isotherms were evaluated to determine pore volume and size distribution. The total pore volume was calculated from the nitrogen adsorbed at a relative pressure of 0.99.

The micromorphology of steam-explosion-treated pine wood chips was observed using a scanning electron microscope (SEM) (ZEISS Gemini 300, Germany) at an accelerating voltage of 5 kV. Samples were mounted on cover slip glass, fixed to a stub with conductive thermoplastic adhesive, and coated with silver using a Polaron E 5000 sputter coater prior to SEM imaging for surface structure and pore distribution analysis.

RESULTS AND DISCUSSION

Effect of Steam Explosion on Composition of Pine Wood Chips

Figure 1 presents results for the pine wood chip residues that were obtained through steam explosion treatment. As the retention time of the steam explosion increased, the particle size of the pine wood chips was observed to become smaller, and a greater number of fibers was found. It had been reported that samples subjected to longer retention times underwent rapid thermal expansion at the end of the steam explosion process, which contributed to the opening of the grain structure. After steam explosion treatment, the weight including moisture was measured, and the results showed a yield of approximately 100 to 110% at 1 minute, 3 minutes, and 5 minutes. The yield of pine wood chips after steam explosion treatment was approximately 70% based on the total weight, and there was no significant difference depending on the treatment time.

In this study, it was similarly observed that the compact and orderly structure of the pine wood chips was progressively disrupted with increasing steam explosion duration. The structural disruption of the steam-exploded pine wood chips was attributed to the loosening of interlayer lignin, which facilitated the separation of fibers.

Fig. 1. Steam exploded pine wood chips. A: non-treatment; B: 225 °C, 1 min; C: 225 °C, 3 min; D: 225 °C, 5 min

Figure 2 presents the chemical composition results of steam-exploded pine wood chips with bark. A decrease in hemicellulose content and a relative increase in lignin content were identified under higher-intensity steam explosion conditions, consistent with trends reported in previous studies (Börcsök and Pásztory 2021). These changes in chemical composition were considered to have influenced the structural properties of the steam-explosion-treated pine wood chips.

Hemicellulose components (xylan, arabinosyl residues) were degraded following steam explosion treatment in comparison with raw samples. Meanwhile, cellulose—the primary component of bioenergy feedstock—was preserved in the solid residue. The degradation of hemicellulose was found to increase the enzymatically accessible surface area of the substrate.

It was previously reported that a reduction in hemicellulose typically occurred at temperatures above 150 °C (Sun *et al*. 2022), and that hemicellulose, due to its amorphous structure and short-chain polysaccharides, was more susceptible to degradation than crystalline cellulose (Li and Khanal 2017). The release of xylose as a component of the liquid fraction during steam explosion was considered to be an indicator of hemicellulose breakdown.

Furthermore, the hydrolysis of biomass during steam explosion treatment was believed to be enhanced by the formation of acetic acid, which was derived from acetyl group cleavage in hemicellulose. This acid formation likely catalyzed the conversion of hemicellulose to monomeric sugars. The degradation of chemical constituents in steam-exploded pine was considered to result in furfural formation, which may have contributed to improved enzymatic saccharification, particularly through the combined effects of heat and acid-catalyzed reactions that disrupted the amorphous regions of the biomass.

In this study, it was also confirmed that hemicellulose (xylan and arabinosyl residues) content decreased significantly as the severity factor of the steam explosion treatment increased, which aligned with the known mechanism of autohydrolysis.

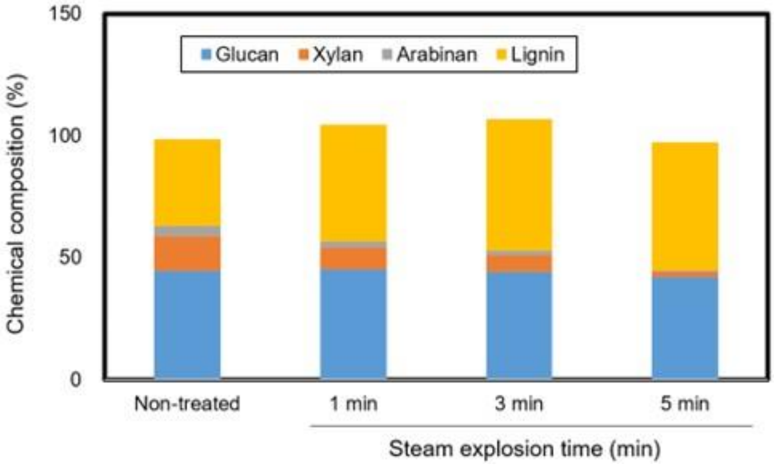


Fig. 2. Chemical compositions of the steam exploded pine wood chips

Effect of Steam Explosion/Urea on Composition of Pine Wood Chips

This paper observed that the dense and regular structure of pine wood chips was gradually destroyed as the steam explosion time increased, so the steam explosion treatment time was fixed at 5 minutes. Most of the lignin in wood was known to be bound to hemicellulose components, which were themselves attached to cellulose in a binder-like fashion, resulting in a complex and inaccessible wood structure. In this study, a two-step pretreatment process combining steam explosion and chemical treatment was developed, and it was expected that this approach would enhance the efficiency of enzymatic hydrolysis due to the reduced hemicellulose and lignin content (Fig. 3). After treating pine wood chips with urea, the yield of the solid fraction obtained was approximately 85% on a dry basis, and there was no significant difference depending on the urea treatment concentration and time. The difficulty associated with delignification and solubilization was attributed in part to the strong bonding between lignin and carbohydrates (Wang *et al*. 2015). Therefore, the removal of most hemicellulose and the complete removal of lignin were considered to promote the solubilization of glucan, albeit to a limited extent. The 24-hour urea treatment was found to be effective in facilitating lignin removal.

While urea is not a conventional organic solvent used in organosolv pulping, this results suggest that certain aspects of its behavior may resemble organosolv-like effects under specific conditions. In this study, urea treatment led to a noticeable reduction in hemicellulose content (as shown in Fig. 3), which may be attributed to partial solubilization or structural disruption of hemicellulose through hydrogen bond cleavage and mild alkaline hydrolysis effects during the treatment. Urea itself does not exhibit the same delignifying power or solvent capabilities as classical organosolv agents (*e.g.*, ethanol, acetic acid), but its degradation products (such as ammonia or cyanate under thermal conditions) may induce mild alkaline conditions. These conditions can contribute to hemicellulose depolymerization and removal, mimicking certain aspects of organosolv delignification. Since this treatment involved aqueous-phase urea without organic solvents, and no substantial lignin removal was observed, this results interpret the urea's role as primarily a hydrogen-bond disruptor and swelling agent, rather than a true organosolv pulping reagent.

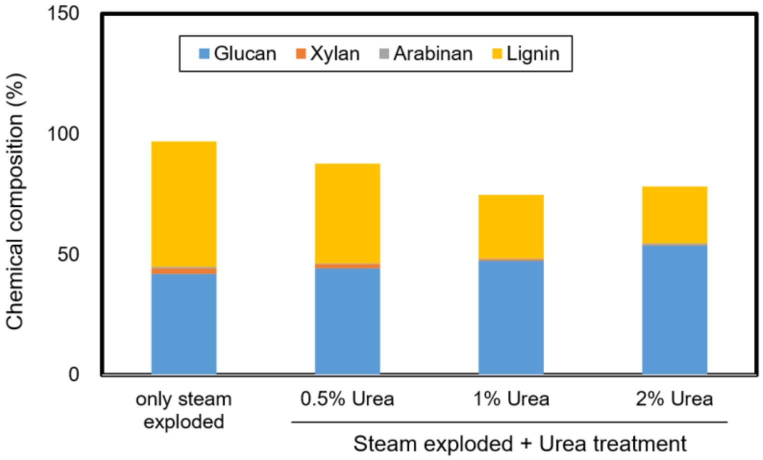


Fig. 3. Chemical compositions as urea treatment of the steam exploded pine wood chips

Crystallinity, Surface Morphology, and BET Surface Area of Substrate

Pine wood chips that were treated with 0.5% to 2% urea at room temperature for 24 h were found to exhibit higher specific surface area values in the samples treated with 1% and 2% urea. Similar results were found for the total pore volume (Figs. 4 and 5). Increases in specific surface area and total pore volume were reported to improve the accessibility of enzymes to cellulose, thereby enhancing the yield of enzymatic hydrolysis.

In this study, the specific surface area after urea treatment was found to be relatively higher compared to that of the pine wood chips treated only by steam explosion. The trend of increased specific surface area and total pore volume following urea treatment was attributed to the partial destruction of the particle microstructure. The formation of a more porous and less compact structure in pine wood chips subjected to steam explosion and urea treatment was believed to allow cellulase broader access to the substrate surface, which was considered to facilitate efficient enzymatic hydrolysis of biomass.

The specific surface area and pore volume of pine wood chips treated with steam explosion followed by urea at room temperature were found to reach their maximum values simultaneously. Therefore, the enhanced specific surface area and total pore volume achieved through the combined steam explosion and urea treatment were speculated to be effective in increasing the enzymatic saccharification yield (Meng and Ragauskas 2014).

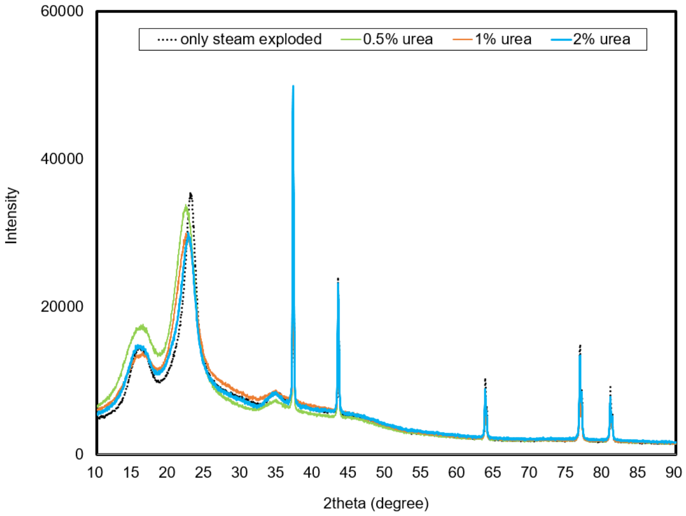


Fig. 4. Crystallization index as urea treatment of the steam exploded pine wood chips

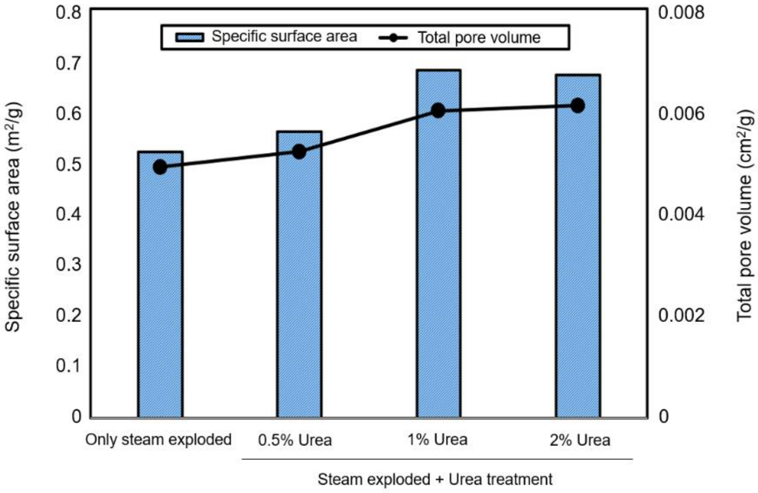
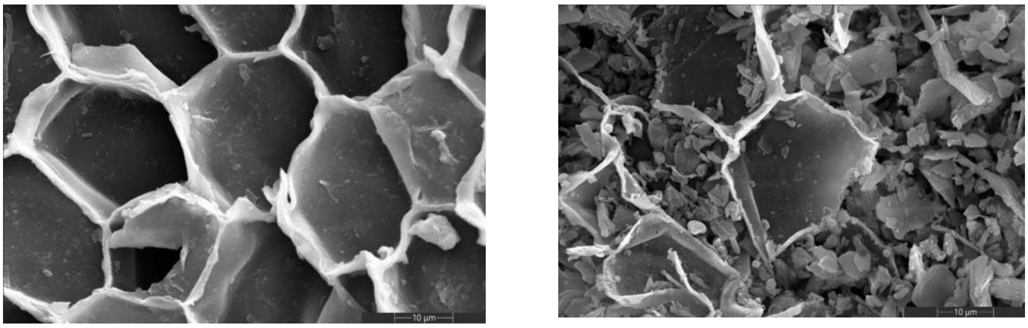


Fig. 5. Specific surface area (m^2/g), total pore volume (cm^3/g) of substrates analyzed by BET method as urea treatment of the steam exploded pine wood chips

Evaluation of Surface Microstructure and Pore Distribution by Steam Expansion/Urea Preparation Treatment Conditions using SEM

Pine wood chips that were subjected only to steam explosion without chemical treatment were found to exhibit a hard and rough surface morphology, which was considered to hinder the accessibility of cellulose to enzymatic action. In contrast, the surfaces of pine wood chips treated with steam explosion followed by urea treatment for 24 h were observed to be disrupted into isolated fibers or fiber bundles, accompanied by visible cracks and small particle-sized debris. The observations represent the entire sample set.

In particular, pine wood chips treated with urea at a given concentration for 24 h after steam explosion were found to exhibit a loosely packed fiber structure due to greater fiber separation, implying that lignin and hemicellulose were effectively removed through the alkaline treatment. It was speculated that pine wood chips treated with urea following steam explosion exhibited an increased enzymatic saccharification yield, as the altered fiber structure created a greater number of reactive sites on the fiber surface, thereby enhancing enzyme accessibility and improving enzymatic performance (Ju *et al*. 2013).



Only steam exploded [1)] Steam explosion + 1% urea

Fig. 6. Scanning electron microscopy (SEM) images of pine wood chips obtained from steam explosion treatment and urea treatment. 1) 225 °C, 5min

Enzymatic Hydrolysis

When steam-explosion-treated pine wood chips was further treated with 1% urea for 24 hours, enzymatic hydrolysis yield of up to 82% were achieved (Fig. 7). These results were found to be consistent with the known effects of 1% urea treatment on pine wood chips, such as the removal of hemicellulose, the elimination and rearrangement of lignin fractions, an increase in surface area, and the loosening of surface morphology. Following 1% urea treatment, the enzymatic hydrolysis yield of pine wood chips pretreated by steam explosion was observed to increase significantly. This enhancement was mainly attributed to the effective removal of hemicellulose and lignin from the substrate by the alkaline treatment. Through hydrogen bonding, urea is known to bind to the surface of lignin or cellulose, leading to the formation of a hydration layer. The concentration of free cellulose in the urea suspension was found to increase, which may have contributed to the improved efficiency of enzymatic hydrolysis of lignocellulosic material. The use of urea for enhancing the enzymatic hydrolysis of lignocellulose was considered suitable as a chemical pretreatment strategy, particularly because urea could serve as a nitrogen source for microbial amino acid biosynthesis during subsequent fermentation. Urea acts as a strong hydrogen bond disruptor, weakening the hydrogen bonds between cellulose chains. As a result, cellulose crystallinity decreases and the amorphous region increases, expanding the surface area available for enzyme binding (Zhang and Lynd 2004). The element induces partial modification or dissolution of lignin under alkaline or heated conditions, thereby reducing nonspecific adsorption of enzymes. Lignin irreversibly adsorbs enzymes, thereby reducing their efficiency. Therefore, structural modification of lignin increases the effectiveness of enzymes (Park *et al*. 2010). The element is a hydrophilic substance that penetrates the cell walls of wood and induces swelling. As a result, the arrangement of cellulose fibers loosens, creating micropores that enzymes can penetrate, greatly increasing surface accessibility (Lou *et al*. 2018). The BET specific surface area increases due to swelling and structural changes, which enhances the contact efficiency of the enzyme. The increase in specific surface area is directly correlated with the hydrolysis reaction yield (Dong *et al*. 2019).

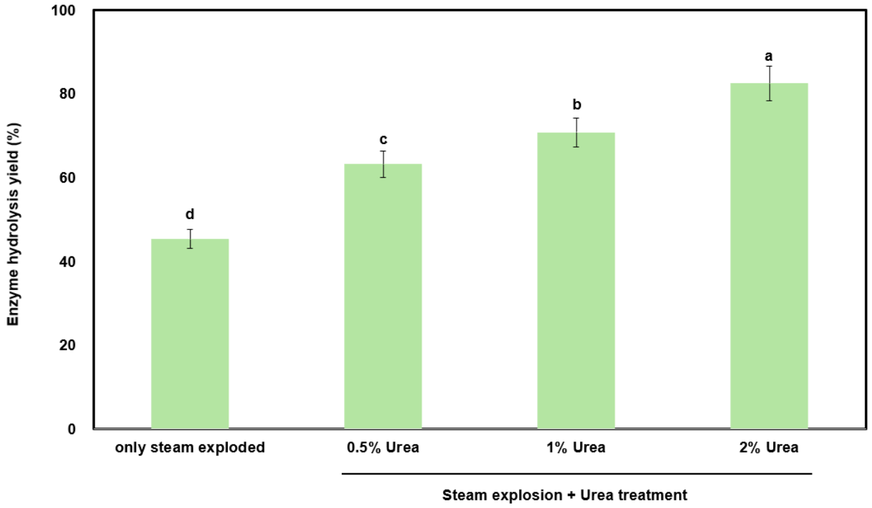


Fig. 7. Enzyme hydrolysis yield of substrates after steam explosion/urea treatment. $p<0.05$

In summary, pine wood chips treated with 1% urea following steam explosion pretreatment was determined to be the most effective condition for increasing enzymatic hydrolysis efficiency. To enhance the economic feasibility of urea-based pretreatment, the possibility of recovering and reusing urea should be considered. Given its water solubility, urea can potentially be recovered through physical separation techniques such as membrane filtration or crystallization, thus improving the sustainability and cost-efficiency of the process. Future research should evaluate the extent and efficiency of such recovery methods in an integrated pretreatment system. Moreover, future work should focus on analyzing the composition of the filtrate following urea treatment to determine the solubilized fractions of lignin and other organics. In addition, evaluating the recovery and reuse of urea through suitable separation technologies will be essential for improving the overall process economics and sustainability.

CONCLUSIONS

1. A dual approach involving steam explosion followed by urea post-treatment was implemented to improve the enzymatic breakdown of pine wood chips.
2. The structure of pine wood chips was disrupted by the steam explosion process, and cellulose surfaces were exposed as the pretreatment pressure and duration were increased, which facilitated the attachment of cellulase enzymes.
3. An enzymatic digestibility yield of 82% for cellulose was achieved using 1% urea for 24 hours at room temperature, following steam explosion and urea application.
4. The combination of steam explosion and urea post-treatment was demonstrated to be a viable strategy for efficiently fractioning and converting the key components of pine wood chips into diverse bio-based products within a biorefinery context.

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