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Colorimetric Evaluation of the Efficiency of Alkaline Pretreatment of Date Palm (*Phoenix dactylifera* L.) Lignocellulosic Biomass

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Abstract: The aim of this study was to develop a fast and sensitive method for evaluating lignocellulosic biomass pretreatment efficiency, a key step in producing biofuels and bioproducts. The research focused on applying a colorimetric technique using Anthrone reagent to estimate the accessible structural carbohydrate content released after alkaline pretreatment. The scope included assessing total carbohydrate concentrations in biomass treated with varying potassium hydroxide (KOH) levels (control (0%), 10%, 20%, and 50% w/w). Absorbance measurements at 620 nm were used for quantification, showing a strong correlation with standard glucose concentrations. The results demonstrated significantly increased carbohydrate release with higher KOH concentrations, reaching 539.44 mg/L, 897.22 mg/L, 1171.11 mg/L, and 1276.11 mg/L, respectively ($p = 0.009$). The study concludes that the Anthrone method is an efficient, reliable, and cost-effective tool for quantifying pretreatment effectiveness through carbohydrate accessibility. It is recommended as a practical screening method for optimizing biomass pretreatment processes, especially in resource-limited settings, to support sustainable biofuel and bioproduct development.

Keywords: Alkaline pretreatment, *Phoenix dactylifera* L., Lignocellulosic biomass, Carbohydrate accessibility, Anthrone assay.

1. Introduction

Lignocellulosic biomass comprising agricultural residues, woody materials, and grasses is considered one of the most promising renewable resources for the sustainable production of biofuels and bioproducts. Its abundance, low cost, and carbon-neutral nature make it a strategic alternative to fossil-based feedstocks in many developing countries, including those in Africa [1,2]. However, its complex structure, mainly composed of cellulose, hemicellulose, and lignin, presents a major barrier to efficient conversion into fermentable sugars.

Pretreatment is a crucial step in breaking down this recalcitrant structure and enhancing the enzymatic digestibility of cellulose. Among various methods, alkaline pretreatment with potassium hydroxide (KOH) has proven particularly effective in delignification and improving sugar yields from different lignocellulosic feedstocks [3,4]. Techniques such as steam explosion, acid/alkaline treatment, and enzymatic hydrolysis aim to disrupt lignin and hemicellulose structures to improve cellulose accessibility [5].

However, conventional methods for evaluating pretreatment efficiency, including chromatography and gravimetry, remain inherently time-consuming, expensive, and labor-intensive. In recent years, analytical techniques have emerged as valuable tools for rapid and non-destructive assessment of biomass composition and structural changes

during pretreatment, with promising results for industrial application [6,7]. However, access to advanced analytical instrumentation, particularly chromatographic systems, remains limited in many laboratories in resource-constrained settings. This study aims to develop a rapid, cost-effective, and sensitive method to evaluate pretreatment efficiency by quantifying the release of accessible structural carbohydrates using the Anthrone colorimetric assay [8,9]. The lignocellulosic biomass investigated in this work was derived from date palm (*Phoenix dactylifera* L.) residues, an abundant agricultural by-product in arid regions of Algeria. We hypothesize that higher KOH concentrations will significantly increase the accessibility and release of structural matrix carbohydrates, which can be reliably quantified through single-wavelength spectrophotometric measurement. By comparing different pretreatment levels, we seek to validate this method as a practical alternative for palm biomass evaluation of pretreatment.

2. Materials and Methods

2.1 Study Area and Biomass Source

The study was conducted in Algeria using biomass derived from dry leaves of date palm (*Phoenix dactylifera* L.), a widely available agricultural residue in the arid regions of the country. The biomass was collected locally, air-dried, and ground into fine powder for use in pretreatment experiments.

2.2 Pretreatment Procedure

A total of 25 g of powdered date palm biomass, ground and sieved to a particle size of less than 1 mm, was mixed with 250 mL of sterile distilled water in Erlenmeyer flasks. Potassium hydroxide (KOH) was added to achieve concentrations of 10% (2.5 g KOH), 20% (5.0 g KOH), and 50% (12.5 g KOH) (w/w, calculated based on the dry biomass weight), alongside a 0% control. The KOH pellets were added slowly with gentle manual swirling to prevent excessive heat generation caused by the exothermic dissolution of the alkali salt. The mixtures were then incubated under static conditions at 20°C for 24 hours following the room-temperature alkaline extraction protocol described by Liu et al. [4]. After incubation, the slurry was filtered through an organza fabric filter, and the pretreated biomass residue was recovered in a sterile beaker. To remove residual alkali, the biomass was washed on the filter three times with 100 mL of sterile distilled water. After each wash, the organza cloth was thoroughly pressed to remove excess water. The recovered pretreated biomass was then transferred into sterile polypropylene containers and stored at 4°C for a maximum of 48 hours prior to downstream carbohydrate analysis. All pretreatment experiments were performed using three independent biological replicates (n=3).

2.3 Measurement of Accessible Structural Carbohydrate Release

The quantification of released accessible carbohydrates (expressed as glucose equivalents) was performed using the Anthrone colorimetric method. For each treatment, 1 g of pretreated biomass was mixed with 9 mL of distilled water. From each mixture, 1 mL of solution was added to 5 mL of Anthrone reagent [9]. The reaction mixture was incubated at 80°C for 20 minutes, then cooled, and absorbance was measured at 620 nm using a UV-visible spectrophotometer. A green color indicated the presence of released sugars.

2.4 Empirical Model

A linear regression model was applied to analyze the relationship between absorbance values and the carbohydrate concentration (expressed as glucose equivalents):

$$Y = \beta_0 + \beta_1 X + \epsilon$$

Where:

Y = Absorbance at 620 nm

X = Carbohydrate concentration (mg/L, glucose equivalents)

ϵ = Error term

β_0, β_1 = Model parameters

This model was used to confirm the linearity and reliability of the Anthrone method for estimating accessible structural carbohydrate content.

2.5 Statistical Analysis

One-way analysis of variance (ANOVA) was conducted to compare the effect of different KOH concentrations on the release of accessible structural carbohydrates. Significant differences between treatment means were assessed using Tukey's HSD test at a 5% significance level. All statistical analyses were conducted using SPSS software.

3. Results

Linear regression analysis was applied to examine the relationship between carbohydrate concentration (expressed as cellulose equivalents) and absorbance at 620 nm measured using the Anthrone method (Figure. 1). The calibration curve was constructed using 6 distinct calibration points of standard cellulose ranging from 100 mg/L to 1000 mg/L.

The resulting linear regression equation was determined as follows:

$$Y = 0.000922 \times X - 0.01103$$

Where Y represents the absorbance at 620 nm, X is the carbohydrate concentration (mg/L, cellulose equivalents), the slope is 0.000922 (with a standard error of ± 0.000052), and the y-intercept is -0.01103 (with a standard error of ± 0.0289). The model demonstrated a highly significant and strong linear correlation, with a coefficient of determination of $R^2 = 0.988$ and a residual standard error of 0.0378, indicating that 98.8% of the absorbance variation was explained by changes in the concentration of released carbohydrates ($df = 5$; $p < 0.05$).

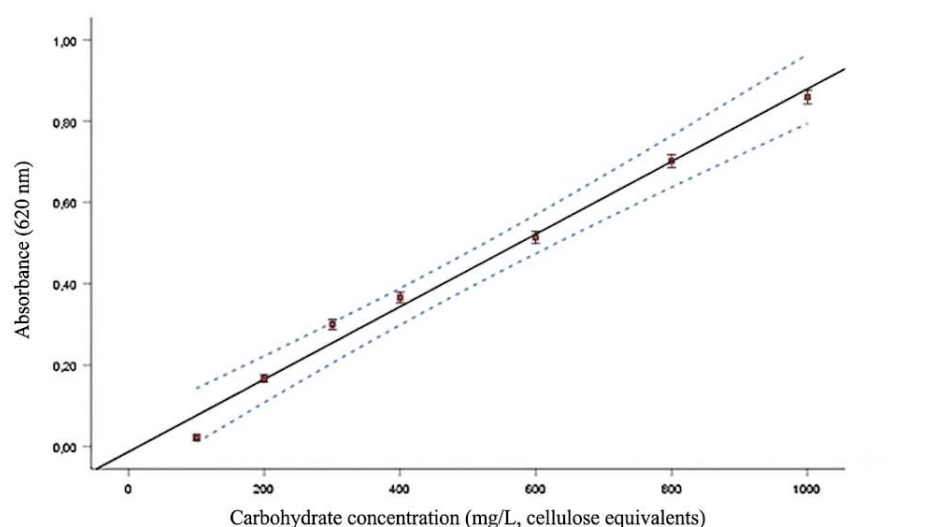


Figure 1. Linear regression and its 95 % confidence interval for the relationship between cellulose equivalents and absorbance ($R^2 = 0.988$; $df = 5$; $P < 0.05$)

The effect of alkaline pretreatment intensity on accessible carbohydrate release was also evaluated (Figure 2). A significant increase in carbohydrate concentration was observed with increasing KOH concentrations, reaching 539.44 mg/L for the control (0%), 897.22 mg/L for 10% KOH, 1171.11 mg/L for 20% KOH, and 1276.11 mg/L for 50% KOH. This dose-dependent response highlights the effectiveness of KOH as an alkaline pretreatment

agent for disrupting the biomass matrix and increasing structural polysaccharide accessibility. Statistical analysis revealed that while the carbohydrate release was significantly higher in the 20% and 50% KOH treatments compared to the untreated control ($p < 0.05$), the difference between the 20% and 50% concentrations was not statistically significant, indicating a threshold effect in matrix solubilization. Spectrophotometric analysis further confirmed these results, revealing statistically significant differences in carbohydrate-related absorbance values with increasing KOH concentrations ($p = 0.009$), indicating enhanced structural carbohydrate availability following alkaline pretreatment.

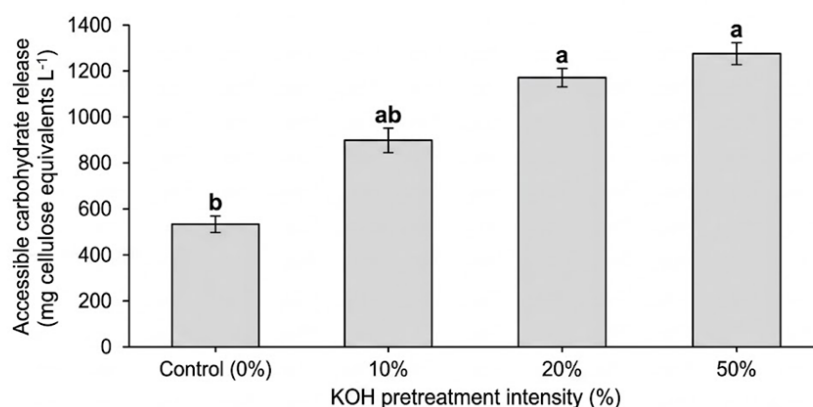


Figure 2. Effect of pretreatment intensity on accessible carbohydrate release (mean $\pm$ SE, expressed as mg/L of cellulose equivalents) from pretreated date palm biomass. Bars bearing different letters differ significantly according to Tukey's HSD test ($p < 0.05$), ($n = 3$)

4. Discussion

The high coefficient of determination ($R^2 = 0.988$) obtained in this study demonstrates the reliability and sensitivity of the Anthrone colorimetric assay for structural carbohydrate estimation under the applied experimental conditions. Although strong linear correlations have previously been reported, slightly lower or comparable R^2 values have been observed depending on biomass type and analytical protocols—for instance, $R^2 = 0.995$ for an optimized Updegraff-Anthrone method on plant biomass [10] and high correlations for saccharide analysis in berries [11] whereas standard protocols such as those described by Moretti *et al.* [12] confirm sensitivity for cellulosic glucose without specifying exact R^2 values. These variations underscore the influence of factors such as reagent freshness, biomass matrix, and hydrolysis steps, reinforcing that $R^2 > 0.97$ is typical for Anthrone assays with peaks exceeding 0.99 under optimal conditions.

It must be acknowledged, however, that the Anthrone method is a non-specific colorimetric assay that quantifies total soluble hexoses and pentoses rather than specifically measuring cellulose alone. Nevertheless, in the context of this study, the rigorous three-step washing of the solid residue after the KOH treatment successfully removed free sugars and alkali-soluble hemicelluloses. Therefore, the observed increase in carbohydrate concentration with rising KOH concentrations directly reflects the release of easily accessible structural polysaccharides (predominantly cellulose-derived glucose units) that were destabilized from the lignocellulosic matrix.

This concentration-dependent response confirms the effectiveness of alkaline pretreatment in disrupting the lignin-carbohydrate complex, thereby enhancing structural polysaccharide accessibility. These findings are consistent with earlier reports indicating that alkaline agents such as NaOH and KOH improve carbohydrate availability by breaking lignin-cellulose linkages, facilitating subsequent enzymatic hydrolysis [3, 13].

At higher KOH concentrations, the compositional changes observed in this study align with those reported by Liu et al. [4], who demonstrated improved carbohydrate availability and enzymatic digestibility in KOH-pretreated wheat straw. The spectrophotometric data obtained in the present work further support these structural modifications by indicating increased matrix polysaccharide exposure.

On a molecular level, the observed increase in accessible carbohydrates from 10% to 20% KOH is driven by the saponification of intermolecular ester bonds cross-linking hemicelluloses and lignin, alongside the partial cleavage of labile ether linkages within the lignin matrix [3,4]. This targeted chemical disruption induces a significant swelling of the crystalline cellulose structure, effectively increasing the specific surface area and pore volume, which facilitates higher structural carbohydrate accessibility [13]. However, when the alkali concentration is increased to 50% KOH, the carbohydrate yield reaches a statistical threshold (1276.11 mg/L), showing no significant difference compared to the 20% KOH treatment ($p > 0.05$). This plateau effect is highly indicative of competitive degradation reactions occurring under extreme alkaline conditions. Specifically, severe alkali exposure triggers the 'alkaline peeling reaction', a mechanism where monosaccharide units are sequentially cleaved from the reducing ends of cellulose and hemicellulose chains converting them into organic acids (such as isosaccharinic acids) that do not react with the Anthrone reagent [4]. Concurrently, the extensive removal of the protective lignin-hemicellulose sheath at 50% KOH may promote partial structural collapse or local recrystallization of the remaining cellulose microfibrils, thus limiting any further increase in accessible carbohydrate release.

The relatively high carbohydrate concentration observed in the control treatment (539.44 mg/L) indicates that water extraction combined with mechanical processing can release a notable baseline of carbohydrate compounds. This phenomenon is primarily explained by the intensive grinding ($< 1\text{ mm}$), which mechanically sheared the lignocellulosic matrix, thereby reducing cellulose crystallinity and increasing the exposure of surface structural polysaccharides [14]. Furthermore, despite the rigorous triple washing steps performed to eliminate free soluble sugars, the hydrothermal conditions of the Anthrone assay (80°C) likely facilitated the co-extraction and subsequent hydrolysis of loosely bound, water-soluble hemicellulosic fractions (such as arabinoxylans and galactomannans) that are naturally abundant in date palm biomass tissues [15, 16]. This underscores that mechanical grinding acts as a vital primary physical pretreatment, establishing a baseline accessibility that chemical KOH action further enhances in a dose-dependent manner.

Similar trends have been reported for other lignocellulosic substrates. Memon and Memon [5] observed that high-concentration KOH pretreatment of sugarcane bagasse resulted in accessible carbohydrate solubilization exceeding 1,000 mg/L, detectable using spectroscopic techniques. More recently, Wang et al. [2] reported a significant increase in sugar yield following KOH pretreatment of sweet sorghum bagasse, while Zhang et al. [1] demonstrated enhanced cellulose accessibility in *Miscanthus* using FTIR analysis. When compared quantitatively to this previous literature, the maximum accessible carbohydrate yield achieved here (1276.11 mg/L, cellulose equivalents) aligns favorably with optimal pretreatment benchmarks. For instance, Wang et al. [2] and Memon & Memon [5] reported equivalent structural carbohydrate solubilization thresholds ranging between 1000 and 1350 mg/L under similar optimized alkaline regimes [2, 5]. The fact that date palm residues typically considered highly recalcitrant due to their dense, heavily lignified vascular bundles yielded comparable accessibility underscores the exceptional efficacy of the proposed KOH extraction method for this specific hard biomass matrix [13]. While advanced techniques like FTIR or HPLC offer detailed structural and monomeric profiles [1], the Anthrone method validated here provides a practical, low-cost, and high-throughput screening alternative, making it an excellent tool for preliminary biomass assessment in resource-limited research laboratories.

5. Conclusions

These results emphasize the dual value of alkaline pretreatment and spectrophotometric quantification. Not only do higher KOH concentrations enhance structural carbohydrate availability through delignification and hemicellulose removal, but colorimetric methods also serve as robust, rapid tools for tracking these chemical changes. The strong correlation between absorbance data and accessible carbohydrate content underlines the potential of such techniques for cost-effective, rapid, and real-time process monitoring and optimization in lignocellulosic biomass valorization.

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