

ABSTRACT

Lignocellulosic biomass, mainly agro-residues such as Cotton Stalk (*Gossypium hirsutum*), represents a sustainable, renewable and abundant feedstock for the production of 2G bioethanol. Cotton Stalk, the key byproduct of *Gossypium hirsutum* harvesting, constitutes an underutilised residue characterised by a favourable lignocellulosic composition. However, its complex and recalcitrant characteristics present a fundamental barrier towards an effective pretreatment method to overcome biomass resistance and improve hydrolysis and downstream bioconversion. The current study investigated a combinatorial green bioprocessing method for the valorisation of Cotton Stalk biomass, integrating enzyme-mediated simultaneous pretreatment and saccharification (SPS), microwave-assisted natural deep eutectic solvent pretreatment (MWDES), and a statistically optimised simultaneous saccharification and fermentation (SSF) method, to achieve efficient and sustainable bioethanol production.

Characterisation of Cotton Stalk revealed 43.4 ± 0.25 % (w/w) cellulose, 12.7 ± 0.30 % (w/w) hemicellulose, 28.42 ± 0.40 % (w/w) lignin, low ash content of 4.2 % (w/w) and a favourable elemental composition characterised by low nitrogen (0.65% (w/w)) and sulphur (0.21% (w/w)). These compositional profiles together confirm the significant bioenergy generation potential of Cotton Stalk as a feedstock with negligible environmental impact during thermochemical and biochemical conversion.

In the enzyme-mediated SPS approach, laccase from *Pleurotus djamour* and cellulase from *Aspergillus niger* were used individually and in combination to study their synergistic activity on the substrate. Independent laccase treatment attained 61.36% delignification through oxidative depolymerisation of the lignin network. The statistically optimised combinatorial SPS system, employing a laccase to cellulase ratio of 1:4 (v/v), pH 5.0, temperature of 50 °C, solid loading of 15 % (w/v), and incubation duration of 24 h, yielded a maximum reducing sugar concentration of 256.89 ± 0.28 mg/g alongside 52.38% delignification. The central composite design response surface methodology model (CCD-RSM) generated a robust statistical model with an R^2 of 97.89 % and an adjusted R^2 of 94.06 %. Structural characterisation (SEM, FTIR, XRD, and BET) confirmed significant morphological disruption, enhanced surface area following laccase treatment, and effective hydrolysis of lignocellulosic components.

MWDES pretreatment of Cotton Stalk was statistically optimised using a choline chloride and lactic acid system at a 1:2 molar ratio. Under optimised pretreatment conditions of 120 °C, 40

min microwave irradiation, and 15 % (w/v) solid loading, the process achieved 79.23 ± 0.32 % delignification. The Box-Behnken design response surface methodology model (BBD-RSM) demonstrated excellent fit with an R^2 of 99.65 % and adjusted R^2 of 99.0 %. Microwave irradiation showed significantly enhanced delignification efficiency as compared to conventional thermal heating, within considerably less treatment time. Structural characterisation confirmed selective removal of lignin and hemicellulose fractions, formation of a highly porous structure, and successful recovery of high-quality amorphous lignin while preserving native aromatic architecture.

For bioethanol production, separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) were evaluated using *Saccharomyces cerevisiae*. Optimised enzymatic saccharification of MWDES-treated biomass produced 730.89 ± 6.2 mg/g reducing sugars, yielding 42.22 ± 0.84 g/L ethanol (5.3 % (v/v)) under SHF. In contrast, the optimised SSF process achieved ethanol concentration of 6.01 ± 0.24 (v/v), equivalent to 52.26 g/L, representing nearly 1.5-fold enhancement over SHF within a reduced total processing period.

In the present investigation, the integrated green bioprocessing approach showed high hydrolysis efficiency and bioethanol yields from Cotton Stalk biomass, while simultaneously enabling valuable lignin co-production. These findings together advance the development of sustainable, energy-efficient, and environmentally benign lignocellulosic valorisation approaches, contributing to the development of the circular bioeconomy and 2G biofuel production.

Keywords: Cotton Stalk, choline chloride, lactic acid, bioethanol, lignin, fermentable sugar, microwave irradiation