

ABSTRACT

The intensifying global energy crisis and exhaustion of fossil fuel supplies have generated an urgent demand for sustainable and renewable liquid biofuels with low environmental impact. Biobutanol generated by the acetone-butanol-ethanol fermentation route by solventogenic *Clostridium* sp. is an advanced next-generation biofuel. Biobutanol possesses a calorific value of 29.2 MJ/L, exhibits low hygroscopicity, and is compatible with current internal combustion engines. However, commercialization of second-generation biobutanol from lignocellulosic biomass remains constrained by the structural recalcitrance of biomass, inhibitor generation during pretreatment and multiple downstream fermentation bottlenecks. The present investigation addressed these limitations through an integrated ultrasound-assisted enzymatic biorefinery platform. Corn stalk was chosen as it is an abundant agricultural residue with holocellulosic content exceeding 74 %.

The solid-state fermentation of *Pleurotus djamor* was used to produce laccase in-house. Cellulase and β -glucosidase were obtained from *Trichoderma reesei* RUT C30 and *Aspergillus niger*, respectively. The process established an entirely in-house enzyme supply chain that was both cost-effective and efficient. Free laccase activity was increased by approximately 1.37-fold and cellulase activity by approximately 22 % during a 10 min session at 25 W and 25 kHz of low-intensity ultrasound, without causing enzymatic denaturation. Ultrasound-assisted laccase-mediated delignification optimized via Central Composite Design based Response Surface Methodology across 5 variables achieved 80.56 % delignification at pH 5, 48 °C, 5 h, 12 % (w/v) solid loading, and 400 IU/mL laccase titre. GC-MS analysis confirmed the complete absence of furfural and hydroxymethylfurfural in the resulting hydrolysates, establishing an entirely inhibitor-free pretreatment strategy.

Two saccharification pathways were comparatively evaluated. The sequential ultrasound-assisted delignification followed by a separate saccharification process, referred to as USDC achieved a validated maximum reducing sugar yield of 448.01 ± 5.24 mg/g biomass at 49.58 °C, pH 5.5, and 22.99 h. The integrated single-pot simultaneous pretreatment and saccharification process, referred to as USPS, achieved a superior validated yield of 511.28 ± 3.53 mg/g at 48.58 °C, pH 5.0, 18.18 h, and a cellulase-to-laccase ratio of 5.7:1. The USPS process delivered a 14.1 % higher

reducing sugar than USDC while taking nearly five hours less, demonstrating the synergistic advantage of concurrent enzymatic action within a single reactor. Structural characterization by SEM, FTIR, XRD and BET analysis collectively confirmed selective lignin removal, preservation of the cellulosic backbone and increased mesoporosity, providing mechanistic evidence for the observed saccharification enhancement.

ABE fermentation of corn stalk hydrolysate was conducted using solvent-enriched *Clostridium beijerinckii* ATCC 55025-E604. It demonstrated 39.11 ± 1.5 % residual growth at 16 g/L butanol significantly exceeding wild-type tolerance. Baseline fermentation under optimized conditions of 50 g/L reducing sugar pH 6.5, 37 °C, 72 h, and 10 % (v/v) inoculum produced 9.13 ± 0.33 g/L butanol. Distillers' dried grains with solubles at 15 g/L, sodium sulphate at 2 g/L and bamboo-derived biochar at 6 g/L were evaluated individually as organic nitrogen source, electron carrier and pH buffer, respectively. Multivariate RSM optimization of the three supplements at 15.25 g/L DDGS, 2.08 g/L sodium sulphate, and 5.62 g/L biochar yielded a validated butanol concentration of 14.03 ± 0.53 g/L, representing a 56.3 % improvement over the unsupplemented control. The model demonstrated a R^2 of 99.36 % and less than 1 % deviation between predicted and experimental values. These findings establish that strategic process integration, enzyme synergy and fermentation optimization can collectively overcome the principal barriers to lignocellulosic biobutanol commercialization. The suitability of corn stalk as a viable and abundant feedstock within a circular bioeconomy framework was also established. The platform developed herein offers a scalable, inhibitor-free and cost-effective template for advancing second-generation biobutanol toward industrial relevance in the global transition to sustainable liquid biofuels.

Keywords: Lignocellulosic, Corn stalk, Ultrasound-assisted pretreatment, Laccase delignification, SPS, *Clostridium beijerinckii*, Biobutanol