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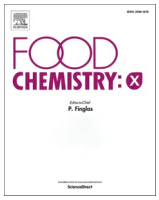

Optimizing the treatment and valorization of spent coffee grounds (SCGs) through the integration of sequential pretreatment steps, enzymatic hydrolysis, and selective fermentation☆

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ABSTRACT

This study aimed to optimize the simultaneous production of D-mannose and bioethanol from spent coffee grounds (SCGs) using an integrated process involving pretreatment, enzymatic hydrolysis, and selective fermentation. Sequential alkali, bleaching, and NaOH-hydrogen peroxide pretreatment steps increased the total sugar content to 74.1 %, with mannose and glucose reaching 45.2 % and 27.2 %, respectively, while effectively removing lignin, as confirmed by colorimetric and solid-state nuclear magnetic resonance analyses. Enzymatic hydrolysis with cellulase and mannanase produced high concentrations of glucose (15.3 mg/mL) and mannose (10.6 mg/mL). To optimize fermentation, a mutant strain of *Pichia stipitis* (MH05) was developed that selectively consumed glucose, leaving 79.9 % of mannose unconsumed after 48 h. This approach resulted in both efficient ethanol production and significant mannose retention, highlighting the potential of SCGs for generating high-value bioproducts and promoting waste valorization and the circular bioeconomy.

1. Introduction

Reducing greenhouse-gas emissions, addressing environmental pollution, mitigating fossil-resource depletion, and optimizing waste management are critical global challenges (Shiferaw et al., 2023; Yang et al., 2023). For the past two centuries, fossil fuel-based energy generation has driven economic growth (Fankhauser & Jotzo, 2018). However, our current reliance on fossil fuels is unsustainable due to excessive CO_2 emissions, which contribute to irreversible environmental damage and accelerate global climate change (Casau et al., 2022). Thus, transitioning to renewable energy sources is imperative (Chen, 2015). Among these alternatives, bioethanol produced from renewable resources, such as biomass, has emerged as a promising option. Waste-to-energy conversion technologies that are both sustainable and environmentally friendly have gained considerable attention in energy research (Mahmood et al., 2016; Mujgaba et al., 2023). Lignocellulosic agricultural waste, such as spent coffee grounds (SCGs), has potential as a renewable biomass feedstock that can partially replace fossil fuels, reduce CO_2 emissions, and circumvent the food-versus-fuel debate (Irmak, 2017).

SCGs, a major byproduct of the coffee brewing process, are an example of lignocellulosic agricultural waste (Saberian et al., 2021). With increasing global coffee consumption, the quantity of SCGs generated is rising. Typically, SCGs are discarded as general waste and sent to landfills (Johnson et al., 2022). This disposal method presents challenges due to SCGs's high oxygen demand during decomposition and the potential release of residual caffeine, tannins, and polyphenols into the environment (Hu et al., 2023; Saratale et al., 2020). Consequently, the effective management and reutilization of SCGs have become increasingly important, prompting research into their valorization (Murthy & Naidu, 2012).

Despite being considered waste, SCGs contain valuable chemical

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components–39.1 % hemicellulose, 12.4 % cellulose, 23.9 % lignin, 17.4 % protein, and 2.3 % fat–which hold significant potential for conversion into useful production (Gebreeyessus, 2022; Lee et al., 2023; Scully et al., 2016; Tapangnoi et al., 2022). Carbohydrates, particularly cellulose and hemicellulose, are abundant in SCGs and serve as valuable carbon sources for bioethanol production and other biotechnological applications (Andrade et al., 2022; Kopp, Willows and Sunna, 2019). However, the complex chemical structure of SCGs, where cellulose, hemicellulose, and lignin are interconnected by cross-linked bonds (Scully et al., 2016), presents challenges for efficient conversion. Therefore, pretreatment is necessary to make these components more accessible to enzymatic hydrolysis and enhance the production of valuable products. Although several pretreatment methods have been developed in previous studies addressing SCGs' structural complexity (Cho et al., 2022; Nguyen et al., 2017; Nguyen et al., 2019; Silva et al., 2023), more effective methods are needed. This study aims to develop an upgraded pretreatment method that enhances enzymatic hydrolysis, thereby improving bioethanol production efficiency and enabling more sustainable biomass utilization.

The sugar D-mannose has recently gained significant attention due to its significant physiological benefits and diverse applications in the food, cosmetic, and pharmaceutical industries (Hu et al., 2016; Nguyen et al., 2017; Nguyen et al., 2019). It has been shown to improve intestinal microecology, prevent obesity caused by high-fat diets, be useful in managing diabetes, and provide various health benefits, including boosting immune function alleviating intestinal diseases, and treating urinary tract infections (Hu et al., 2016; Priya, 2020; Sharma et al., 2018; Wu et al., 2019). Additionally, D-mannose is a key intermediate in the production of vitamins, D-mannitol, and anti-tumor agents, contributing to the growing demand for it (Chen et al., 2007; Mishra & Hwang, 2013; Xu et al., 2016). Despite its increasing market potential, large-scale D-mannose production remains costly and inefficient. Various production methods, including chemical synthesis, microbial fermentation, and biotransformation, are being explored to improve yield and cost-effectiveness (Dhanalakshmi et al., 2023). However, most commercially available mannose is still derived from plant biomass, requiring the extraction and hydrolysis of mannans through enzymatic, acid, or thermal treatments (Wang et al., 2022). These steps are necessary to release D-mannose efficiently from its complex polysaccharide structure. Furthermore, the limited availability of mannan-rich biomass hinders large-scale production.

To address these challenges, SCGs have emerged as a promising alternative feedstock for mannose production. SCGs are a widely available agricultural waste product, making them a cost-effective and sustainable resource. Approximately 66 % of SCGs consist of polysaccharides, with nearly half composed of galactomannan, a key mannose-containing polymer (Batista et al., 2023). This composition makes SCGs an attractive biomass source for large-scale mannose production. Furthermore, utilizing SCGs for mannose extraction contributes to waste reduction and promotes a circular bioeconomy by converting coffee industry by-products into valuable bioproducts.

In addition to mannan, SCGs contain significant amounts of cellulose, which can be enzymatically hydrolyzed into glucose—a key substrate for bioethanol fermentation. This dual valorization potential makes SCGs an ideal candidate for integrated biorefinery applications. Selective fermentation further enhances economic feasibility by selectively converting target compounds while minimizing by-product formation. This process utilizes specialized microorganisms or engineered strains to optimize the conversion of specific sugars into high-value products (Putra et al., 2014; Samtiya et al., 2024). Selective fermentation techniques have been demonstrated as commercially viable for large-scale production (Carvalho et al., 2008).

This study aims to develop an SCG-based biorefinery process that integrates enzymatic hydrolysis and selective fermentation for the co-production of D-mannose and bioethanol (Fig. 1). For fermentation, we employed *Pichia stipites,* a yeast capable of catabolizing various sugars including xylose, glucose, mannose, and galactose (Agbogbo & Coward-Kelly, 2008; Jeffries et al., 2007) and developed a mutant strain through adaptive evolution that selectively ferments glucose while leaving mannose largely unconsumed, facilitating co-production (Kim & Lee, 2019). This dual approach enhances bioethanol production while maximizing the recovery of high-value D-mannose from SCGs, making the overall process both economically feasible and environmentally sustainable. By integrating enzymatic hydrolysis with selective fermentation, this study presents an innovative biorefinery strategy that repurposes agricultural waste into valuable bioproducts. This approach contributes to industrial sustainability, reduces waste, and advances the development of a circular bioeconomy by converting SCGs into high-value resources.

2. Materials and methods

2.1. Materials

The SCGs used in this study were obtained from a Starbucks coffee shop in Kwangju, South Korea; SCGs from the same coffee shop, had been used in a prior study (Cho et al., 2022). Upon collection, the SCG samples were dried in an oven at 70 °C. Sodium hydroxide (NaOH) and hydrogen peroxide (H_2O_2, 30 %) were purchased from Ducksan (Seoul, South Korea). Analytical reagents, including glucose, xylose, galactose, rhamnose, fructose, arabinose, and mannose, were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2. Chemical-composition analysis

The neutral sugar composition of SCGs was determined using gas chromatography (GC) as described by Cho et al. (2022). Samples were initially incubated with 0.25 mL of 72 % (*w*/w) H_2SO_4 at 25 °C for 45

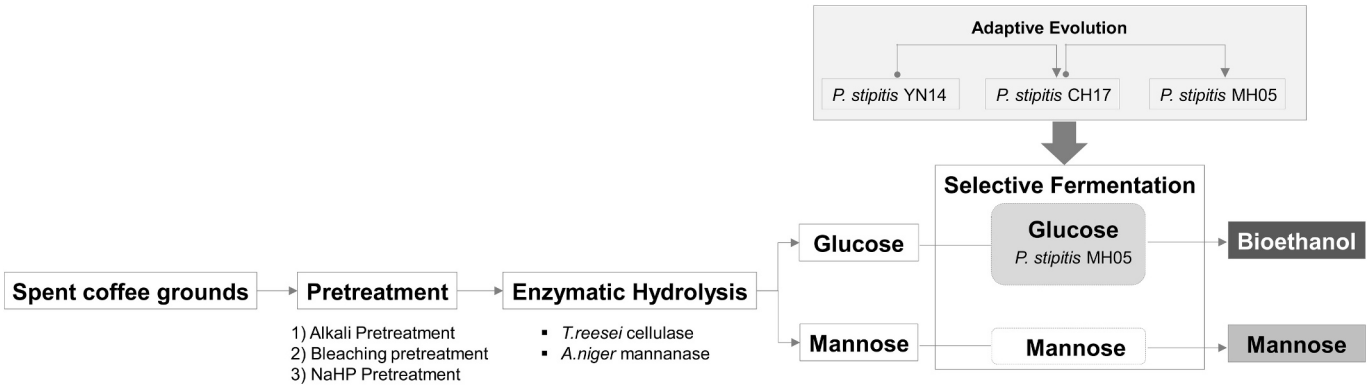

Fig. 1. Production process of D-mannose and bioethanol from SCGs.

min, followed by hydrolysis in an autoclave at 121 °C for 1 h. The hydrolysate, supplemented with 0.1 mL of myo-inositol as an internal standard, was neutralized with 28 % ammonium hydroxide. Sequential additions of 1 mL of 0.02 g/mL $NaBH_4$ in dimethyl sulfoxide, 0.1 mL of 18 M acetic acid, 0.2 mL of 1-methylimidazole, 2 mL of anhydrous acetic acid, and 5 mL of deionized water were performed with vigorous vortexing. The mixture was then extracted with 2 mL of dichloromethane, and any remaining moisture was removed using sodium sulfate. After evaporation under nitrogen gas, 1 μL of the processed sample was injected into the GC column for analysis. The neutral sugar composition of SCGs was determined using gas chromatography (GC) as described by Cho et al. (2022). Samples were initially incubated with 0.25 mL of 72 % (*w*/w) H_2SO_4 at 25 °C for 45 min, followed by hydrolysis in an autoclave at 121 °C for 1 h. The hydrolysate, supplemented with 0.1 mL of myo-inositol as an internal standard, was neutralized with 28 % ammonium hydroxide. Sequential additions of 1 mL of 0.02 g/mL $NaBH_4$ in dimethyl sulfoxide, 0.1 mL of 18 M acetic acid, 0.2 mL of 1-methylimidazole, 2 mL of anhydrous acetic acid, and 5 mL of deionized water were performed with vigorous vortexing. The mixture was then extracted with 2 mL of dichloromethane, and any remaining moisture was removed using sodium sulfate. After evaporation under nitrogen gas, 1 μL of the processed sample was injected into the GC column for analysis.

Klason lignin content was quantified as the acid-insoluble residue remaining after sulfuric acid hydrolysis of extract-free samples. Each sample was first treated with 2.5 mL of 72 % H_2SO_4 at room temperature for 45 min, then diluted to a final concentration of 4 % H_2SO_4 with distilled water. The mixture was subsequently hydrolyzed at 121 °C for 1 h. The resulting insoluble residue was filtered using a Pyrex 1G4 glass filter and thoroughly washed with 500 mL of hot water. The collected Klason lignin was then dried at 105 °C for 12 h before weighing.

Organic solvent extraction was also performed using a Soxhlet apparatus with a 2:1 benzene-to-ethanol mixture. Following extraction, 2.5 g of the residue was treated with 1 g of sodium chlorite and 0.2 mL of glacial acetic acid (18 M), then incubated at 70 °C for 3 h, with homogenization carried out every hour during the treatment.

2.3. Pretreatment

The SCGs underwent a sequential pretreatment process to enhance their enzymatic digestibility. First, alkali pretreatment was conducted by treating SCGs with a 1.0 M NaOH solution at 80 °C for 2 h at a solid-to-liquid ratio of 1 % (*w*/*v*). The treated samples were then filtered, washed with distilled water until a neutral pH of 7 was reached, and freeze-dried. Following this, the alkali-pretreated SCGs were subjected to bleaching using 30 % (*w*/w) H_2O_2 solution at 80 °C for 2 h. After cooling, the samples were filtered, washed with hot and cold water to remove residual H_2O_2, freeze-dried, and stored at room temperature. Finally, NaOH-hydrogen peroxide (NH) pretreatment was performed by treating the bleached SCGs with a 1:1 mixture of 6 wt% NaOH and 0.5 M H_2O_2 under vigorous stirring at 50 °C for 2 h. The pretreated SCGs were filtered and rinsed multiple times with distilled water until reaching a neutral pH. After measuring moisture content, the wet pretreated SCGs were used directly for enzymatic hydrolysis.

2.4. Color measurement

Sample color was measured based on the CIE model using a CR-20 color reader (Konica Minolta, Tokyo, Japan) to determine the L* (lightness), a* (chroma, red/green ratio), and b* (hue, yellow/blue ratio) parameter values. Each measurement was performed in triplicate to estimate average values before and after each pretreatment step. The color difference (ΔE*) between each sample and the standard was determined using the equation

$$\Delta E^{*} = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}} \quad (1)$$

where ΔL* = L*sample – L*standard, Δa* = a*sample – a*standard, and Δb* = b*sample – b*standard. The standard represented the theoretically perfect colorless white using the values L* = 100, a* = 0, b* = 0, and C* = 0.

2.5. Enzymatic hydrolysis

The enzymatic hydrolysis of the SCGs was performed using two in-house-produced enzymes: cellulase derived from *Trichoderma reesei* and mannanase from *Aspergillus niger*. The hydrolysis was conducted at 50 °C for 48 h in 50 mM sodium citrate buffer (pH 5.0). Each enzyme was added at a dosage of 50 mg per gram of dry pretreated SCG, used as the substrate. After enzymatic hydrolysis, the culture broth was centrifuged, and the supernatant was filtered using Whatman No. 1 filter paper to remove insoluble solids. The soluble sugar content was then assessed using high-performance liquid chromatography (HPLC). Enzymatic hydrolysis was performed in triplicate for each pretreatment step and yeast strain (see Section 2.7).

2.6. Microorganisms adaptation and fermentation conditions

Prior to adaptive evolution, the fermentation patterns of *P. stipitis* YN14, a well- studied strain, were investigated for various mixed sugar combinations containing glucose. For pre-cultivation, YP medium (10 g/L yeast extract and 20 g/L Bacto-peptone, pH 6.7) with 20 g/L glucose (YPD20) was used. For the experiment, strain YN14 and the mutant strains derived from it cultivated using YPDX30 (YP medium with 30 g/L glucose and 30 g/L xylose) and YPDM30 (YP medium with 30 g/L glucose and 30 g/L mannose) media. Pre-cultivated yeast cells in the exponential growth phase were harvested and inoculated into 50 mL of YPDX30 in a 250 mL flask at an initial concentration of 0.032 g/L. The flasks were then incubated at 30 °C and 90 rpm under micro-aerobic conditions.

Adaptive evolution was carried out to mitigate the repression of xylose metabolism in the presence of glucose in the YN14 strain. Given that 2-deoxyglucose (2-DOG), a glucose analog, similarly represses the metabolism of other sugars (Kahar et al., 2011), the YN14 strain was serially sub-cultured in YP medium containing 20 g/L xylose and 2 g/L 2-DOG (YPX20-2DG) to induce genetic mutations that alleviate glucose repression of xylose metabolism. After inoculating yeast cells pre-cultured in YPD20 into 50 mL YPX20-2DG in a 250 mL flask at an initial concentration of 0.032 g/L, the flask was incubated at 30 °C and 90 rpm. After 120 h, the yeast cells were harvested and inoculated into fresh YPX20-2DG medium at the same initial concentration. This process was repeated ten times. The tenth culture broth was diluted and spread on a YPD20 agar plate to isolate single colonies. Mixed sugar fermentation was then performed under YPDX20 conditions with 40 randomly picked colonies to screen them for alleviated glucose repression of xylose metabolism.

2.7. Selective fermentation

Pichia stipitis YN14 and the best-performing mutant strain following adaptive evolution, MH05, were used for selective fermentation. To analyze metabolite concentrations during fermentation, the culture broth was centrifuged, and the supernatant was analyzed using an HPLC system. Ethanol yield was determined by dividing the quantity of ethanol produced by the starting total glucose content of the fermentation substrate.

2.8. Analytical techniques

The neutral sugar composition of SCG was determined using **gas chromatography (GC-2010, Shimadzu, Otsu, Japan)**, equipped with a **DB-225 capillary column** (30 m × 0.25 mm i.d., 0.25 μm film

thickness; J&W, Agilent, Folsom, CA, USA). The injector and flame ionization detector (FID) were maintained at 220 °C and 250 °C, respectively. The oven temperature was initially set to 110 °C and increased at a rate of 10 °C/min until reaching 220 °C, where it was held for 35 min. Helium was used as the carrier gas at a constant flow rate of 204 mL/min. The structural and compositional analyses of the samples were conducted using various analytical instruments. In order to characterize the structural properties of the samples, solid-state ^{13}C nuclear magnetic resonance (NMR) spectroscopy with cross polarization-magic angle spinning (CP-MAS) was performed using a Bruker DMX-400 MHz NMR spectrometer equipped with a double resonance H/X CP-MAS 4 mm probe. The powdered samples were spun at 13 kHz, and all experiments were conducted at ambient temperature (294 ± 1 K). A repetition time of 180 s was used for all samples, and up to 2048 scans were accumulated. The soluble sugar content in enzymatic hydrolysates was analyzed using **HPLC** equipped with a Rezex™ RPM-Pb^{2+} ion-exclusion column (4.6 × 300 mm, Phenomenex, CA, USA). Additionally, metabolite concentrations, including glucose, mannose, and ethanol, were measured using **HPLC** (US/e2695, Waters, USA) with a refractive index detector, employing a Rezex ROA-Organic Acid H^+ column (Phenomenex, USA) and 0.01 N sulfuric acid as the mobile phase at a flow rate of 0.6 mL/min.

3. Results and discussion

3.1. Effect of the three-step pretreatment on SCGs

3.1.1. Chemical-composition of raw and pretreated SCGs

Table 1 presents the chemical composition of raw spent coffee grounds (SCGs) and those subjected to sequential pretreatment steps, including alkali treatment (A-SCGs), bleaching (AB-SCGs), and NaOH–hydrogen peroxide (NH) treatment (ABNH-SCGs). The total sugar content of raw SCGs was 37.5 % (dry weight), which increased significantly to 53.5 %, 49.5 %, and 74.1 % after alkali, bleaching, and NH pretreatments, respectively. This progressive increase in sugar content is mainly attributed to the stepwise removal of lignin and extractive compounds, resulting in the relative enrichment of structural carbohydrates in the solid fraction. The removal of non-carbohydrate components not only increases the relative sugar content but also facilitates the accessibility of hydrolytic enzymes to polysaccharide chains in subsequent bioconversion processes.

Among the monosaccharides, mannose and glucose were the dominant sugars in all samples. The mannose content increased from 18.6 % in raw SCGs to 29.9 % after alkali treatment, and further to 45.2 % following NH pretreatment. Similarly, glucose content rose from 8.6 % to 27.2 % across the pretreatment steps. These increases indicate the progressive exposure of mannan- and glucan-rich polysaccharides, particularly from galactomannans and cellulose, as lignin and hemicellulosic barriers were removed. Sodium hydroxide is known to disrupt lignin–carbohydrate complexes by cleaving ester and ether linkages, which facilitates the partial solubilization of lignin and hemicellulose (Campos-Vega et al., 2015; Jomnonkhaow et al., 2024). The subsequent bleaching step, which utilizes hydrogen peroxide, generates hydroxyl and superoxide radicals that promote oxidative degradation of residual lignin and hemicellulose side-chain sugars (Novia et al., 2022; Sun & Cheng, 2002). Although bleaching is traditionally used to target chromophores and lignin, it can also cleave glycosidic bonds and ester linkages in branched hemicellulose structures, leading to the removal of side-chain sugars such as arabinose and galactose. This is supported by our composition analysis, which showed that arabinose content decreased from 1.4 % in raw SCGs to 0.3 % after ABNH treatment, with a significant portion (71.4 %) of the reduction occurring after the bleaching step. Similarly, galactose was reduced from 8.6 % to 1.4 %, with 62.8 % of this reduction also attributed to bleaching. These findings suggest that bleaching plays a more active role in solubilizing hemicellulose-derived sugars than previously assumed, likely through radical-induced degradation mechanisms. Xylose, another hemicellulose-derived sugar, was completely removed after alkali treatment, reflecting its high susceptibility to alkaline hydrolysis. Overall, these losses reflect the high vulnerability of hemicellulose side chains to both alkaline and oxidative pretreatments.

Lignin content showed a substantial reduction from 24.3 % in raw SCGs to just 0.5 % after the final NH pretreatment step. Notably, the majority of lignin removal occurred during the bleaching step (from 17.2 % to 4.5 %), suggesting that oxidative pretreatment plays a critical role in targeting residual lignin structures. This is consistent with previous reports that hydrogen peroxide acts on both condensed and non-condensed lignin structures, promoting delignification (Jomnonkhaow et al., 2024). Similarly, extractive compounds decreased from 8.2 % in raw SCGs to 0.2 % after NH treatment, further confirming the efficient removal of low-molecular-weight phenolics, lipids, and waxes—compounds known to inhibit enzymatic hydrolysis or microbial fermentation.

These compositional changes demonstrate the effectiveness of the sequential pretreatment strategy in selectively enriching fermentable sugars while minimizing inhibitory components. Notably, the final contents of mannose and glucose (45.2 % and 27.2 %, respectively) in ABNH-SCGs exceed values reported in previous studies using similar biomass sources and pretreatment methods (Cho et al., 2022; Kim et al., 2024; Mussatto et al., 2012; Nguyen et al., 2017), indicating the potential of this approach for high-yield sugar recovery. Given the high mannose content, ABNH-SCGs are particularly promising as feedstocks for D-mannose production—a high-value sugar with applications in nutraceuticals and pharmaceuticals—as well as for bioethanol production via glucose fermentation. Therefore, this integrated pretreatment process not only improves the biochemical accessibility of SCGs but also enhances their economic and industrial feasibility as a sustainable bioresource.

Table 1
Chemical composition (% dry weight) of raw and pretreated SCGs.

		SCGs	A-SCGs	AB-SCGs	ABNH-SCGs
Monosugar	Rhamnose	N.D.	N.D.	N.D.	N.D.
	Arabinose	1.4 ± 0.017	1.3 ± 0.10	0.4 ± 0.06	0.3 ± 0.05
	Xylose	0.2 ± 0.18	N.D.	N.D.	N.D.
	Mannose	**18.6 ± 1.68**	**29.9 ± 3.37**	**30.1 ± 1.56**	**45.2 ± 6.25**
	Galactose	8.6 ± 0.73	8.6 ± 0.66	3.2 ± 0.63	1.4 ± 0.24
	Glucose	**8.6 ± 0.82**	**13.8 ± 1.79**	**15.7 ± 1.42**	**27.2 ± 4.18**
	Total sugar	37.5 ± 3.57	53.5 ± 5.89	49.5 ± 3.66	**74.1 ± 10.55**
Lignin		24.3 ± 1.0	17.2 ± 0.5	4.5 ± 0.1	0.5 ± 0.2
Extractive compounds		8.2 ± 0.9	4.3 ± 0.8	1.7 ± 0.2	0.2 ± 0.0

Abbreviation: SCGs, spent coffee grounds; A-SCGs, alkali pretreated SCGs; AB-SCGs, bleached A-SCGs; ABNH-SCGs, NH pretreated AB-SCGs; NH, NaOH-hydrogenperoxide; N.D., not detected.

3.1.2. Color changes

Lignin and phenolic compounds are predominantly responsible for the dark color of SCGs and the color changes depicted in Fig. 2 clearly demonstrate the removal of lignin and phenolic compounds. Though, minimal color change was observed in the SCGs after alkali pretreatment, the subsequent bleaching pretreatment transformed the color to yellow, suggesting an incomplete degradation of lignin and colored phenolic compounds. In contrast, the following NH pretreatment caused a significant color change, with ABNH-SCGs appearing almost white, indicating that the combined pretreatment effectively bleached the SCGs by degrading the lignin and phenolic compounds.

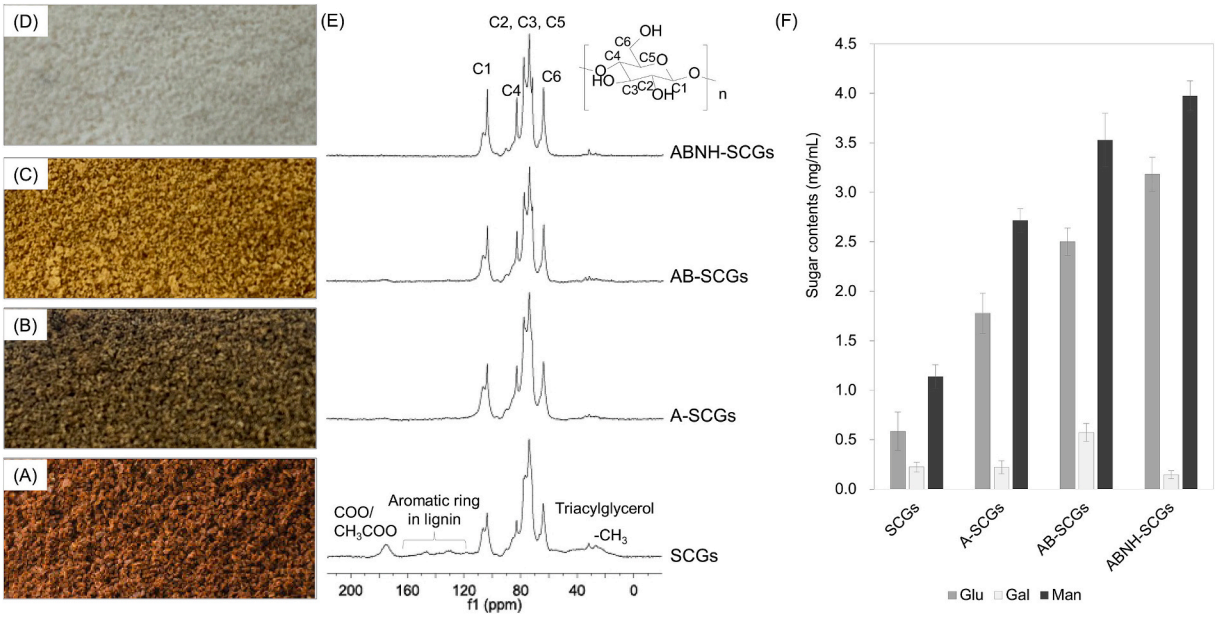

Fig. 2. Visual appearance of (A) raw spent coffee grounds (SCGs), (B) alkali-pretreated SCGs(A-SCGs), (C) bleached A-SCGs (AB-SCGs), and (D) NaOH-hydrogen peroxide-pretreated (ABNH-SCGs). (E) Solid-state ^{13}C-nuclear magnetic resonance (NMR) spectra of untreated and pretreated SCGs. (F) Enzymatic hydrolysis of raw and pretreated SCGs using in house-produced cellulase and mannanase (Glu, glucose; Gal, galactose; and Man, mannose).

The L*, a*, and b* color system closely aligns with the human eye's perception and is superior for color comparisons. Therefore, the effects of pretreatment on the optical properties were evaluated using this system. Table 2 compares the SCG color measurements before and after each pretreatment. Throughout the pretreatment process, SCG lightness (L*) increased significantly from 24.2 to 93.8. The L* value of ABNH-SCGs was closer to the standard value of 100 than the L* values of A-SCGs and AB-SCGs. Furthermore, as the pretreatment process progressed, the total color difference (ΔE) tended to decrease. Between raw SCGs and ABNH-SCGs, the ΔE value remarkably decreased from 77.7 to 13.6, indicating that the ABNH-SCGs were closer to pure white. This phenomenon is attributed to reductions in impurities (such as lignin and hemicellulose) in the SCGs during pretreatment. Therefore, these results corroborate the visual assessment that the combined alkali, bleaching NH pretreatment effectively removes hemicellulose and lignin from SCGs.

3.1.3. *Structural changes assessed by solid-state CP/MAS ^{13}C NMR analysis*

Solid-state NMR is a highly effective non-destructive method for investigating the molecular-level structure of lignocellulosic materials (Kirui et al., 2019). It is especially useful in examining the chemical structure changes that occur during pretreatment processes. Here, a ^{13}C solid-state NMR was used to analyze the chemical composition of SCGs during the three-step pretreatment process (Fig. 2E). In raw SCGs, peaks in the chemical-shift range of 60–110 ppm, corresponding to the cellulose and hemicellulose components, and 110–160 ppm indicating the presence of carbonyl groups and aromatic compounds, which are characteristic of lignin were present. Furthermore, two distinct hemicellulose peaks could be identified at 172 and 22 ppm, corresponding to carboxyl and methyl groups, respectively. After alkali and bleaching pretreatment, the signals corresponding to lignin and hemicellulose decreased but continued exhibiting a weak intensity. After the third NH pretreatment process, the lignin peaks almost completely disappeared, showing that most of the lignin had been removed. However, the ^{13}C solid-state NMR still showed clear peaks for hemicellulose, especially for mannan (C1: 102.2 ppm; C4: 81.5 ppm; C2,3,5: 76.4, 72.7, 70.6 ppm; C6: 62.5 ppm), suggesting that the mannose-rich backbone of mannan remained (Kanai et al., 2024). Mannans, the main type of hemicellulose in SCG, are found in both straight-chain forms (with glucose) and branched forms (with galactose). As also shown in Table 1, the NMR results suggest that side chains like galactose and arabinose were more easily removed during pretreatment, while the central mannose backbone stayed intact. This is likely because the straight-chain mannans are more crystalline and form strong hydrogen bonds, making them harder to break down.

Table 2
Color measurement of raw and pretreated SCGs.

Pretreatments	L*	a*	b*	ΔE
SCGs	24.2 ± 0.1	11.3 ± 0.3	12.8 ± 0.8	77.7 ± 0.2
A-SCGs	30.3 ± 0.2	6.8 ± 0.1	12.9 ± 0.2	71.2 ± 0.2
AB-SCGs	58.9 ± 0.4	16.5 ± 0.1	38.7 ± 0.1	58.8 ± 0.1
ABNH-SCGs	93.8 ± 0.0	−0.8 ± 0.0	12.1 ± 0.1	13.6 ± 0.1

Abbreviation: L*, lightness; a*, the level of greenness and redness; b*, the level of yellowish and bluish; ΔE, color difference.

3.2. *Glucose and D-mannose production by enzymatic hydrolysis*

To evaluate the impact of the combined pretreatment process on SCG hydrolysis, the solid residues obtained after each pretreatment step were enzymatically hydrolyzed. Enzymatic hydrolysis was performed using in house produced cellulase and mannanase (Fig. 2F). The enzymatic-hydrolysis efficiency of SCGs increased after each step of the combined alkali-bleaching-NH pretreatment with the highest reducing-sugar yields obtained from ABNH-SCGs. Specifically, the glucose and D-mannose contents of ABNH-SCGs (3.18 and 3.97 mg/mL, respectively) were 5.44 and 3.49 times higher, respectively, than those of untreated SCGs (0.58 and 1.14 mg/mL). Hence, the full combined pretreatment was necessary to improve enzymatic-catalysis efficiency for optimal glucose and D-mannose production. Moreover, the effective removal of lignin and hemicellulose, which inhibit enzyme activity, likely contributed to efficient glucose and D-mannose production during enzymatic hydrolysis.

3.3. Development of Pichia stipitis mutants unable to ferment mannose

Prior to adaptive evolution, the fermentation patterns of the *P. stipitis* YN14 were investigated using different glucose-containing sugar mixtures. When glucose and xylose were mixed, the YN14 strain began effectively consuming xylose after 18 h of fermentation, by which time the initial glucose content had been depleted (Fig. 3A). Consequently, 22.8 g/L of ethanol was produced from the two sugars, with glucose and xylose consumption rates of 1.59 and 0.65 g/L*h, respectively, over 48 h of fermentation. This pattern is consistent with the fact that most yeast strains preferentially metabolize glucose before other sugars, resulting in glucose repression, in which glucose and its metabolites inhibit the metabolism of other sugars (Kayikci & Nielsen, 2015). Interestingly, the YN14 strain showed roughly simultaneous consumption of glucose and mannose when these sugars were mixed (Fig. 3B). During the initial 24 h of fermentation, glucose and mannose were consumed at rates of 1.23 and 1.24 g/L*h, respectively, resulting in the production of 22.4 g/L of ethanol. This observation suggests that glucose does not repress mannose metabolism in *P. stipitis*, as many proteins involved in glucose metabolism, such as hexose transporters and glycolytic enzymes, are also involved in mannose metabolism (Young et al., 2011).

The results suggested that strain YN14's mannose metabolism could be altered by the presence of glucose, especially if the strain could be selectively bred to effectively metabolize xylose even when glucose is

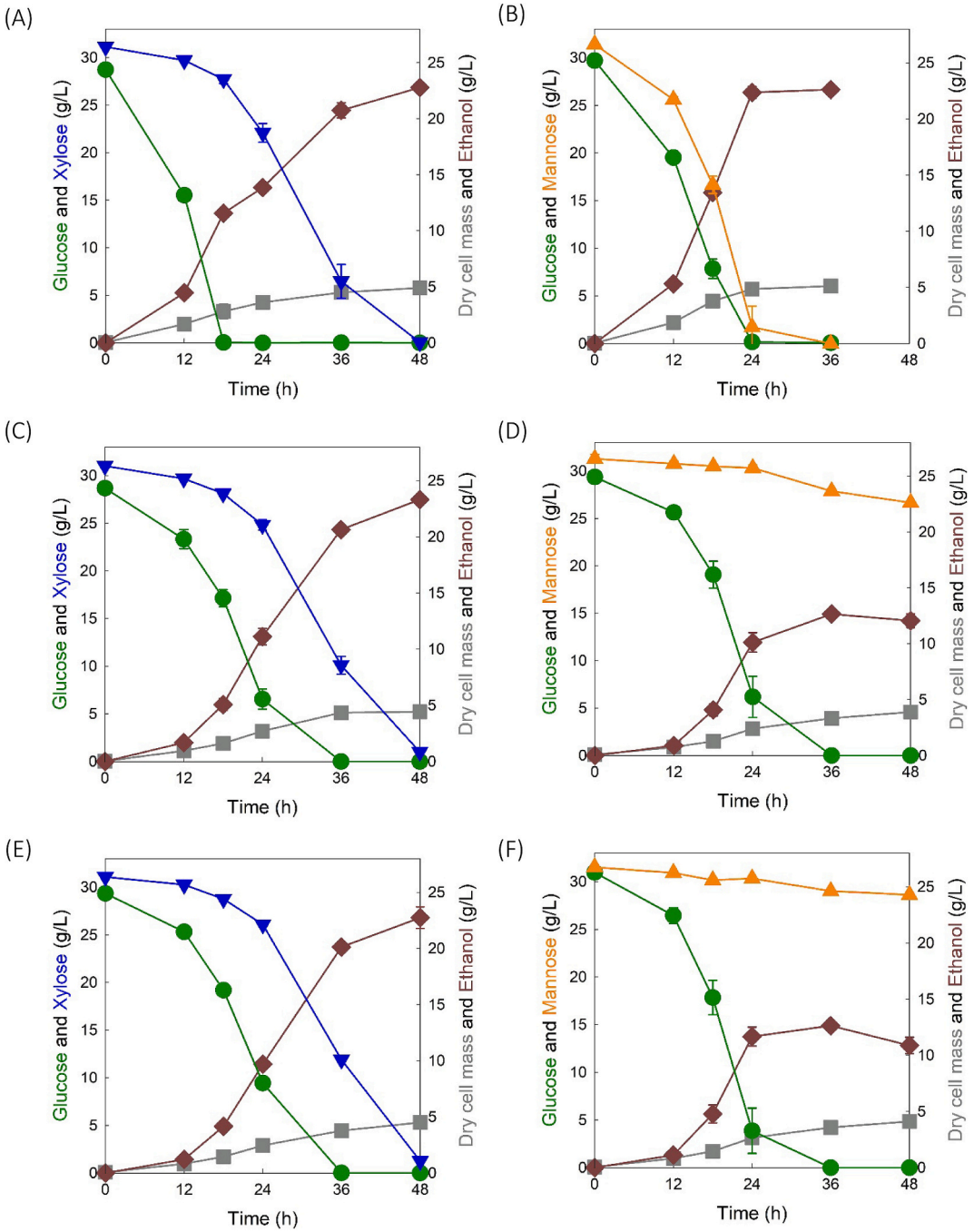

Fig. 3. The fermentation profiles of parental strain *P. stipitis* YN14 (A and B; top) and mutants *P. stipitis* CH17 (C and D; middle) and *P. stipitis* MH05 (E and F, bottom) grown in different glucose-containing sugars mixtures: glucose and xylose (A, C, and E; left) and glucose and mannose (B, D, and F; right). Symbols and colors represent glucose (●), xylose (▼), mannose (▲), galactose (●), dry cell mass (■), and ethanol (◆).

present. Consequently, adaptive evolution was performed to alleviate glucose repression of xylose metabolism in the YN14 strain. Given that 2-DOG, a glucose analog, is known to repress the metabolism of other sugars similarly to glucose (Kahar et al., 2011), it was used for the selective adaptation process. To confirm the inhibitory effects of 2-DOG on growth and fermentation, selected fermentation profiles of the YN14 strain during serial sub-cultures in the YPX20-2DG media were compared to the fermentation profiles in YPX20 (without 2-DOG) (Fig. S1). While strain YN14 consumed 20 g/L of xylose and produced 7.0 g/L of ethanol in 24 h under YPX20 conditions, it neither consumed all the initial xylose nor produced ethanol until 120 h of fermentation in the first sub-culture under YPX20-2DG conditions. This indicates that 2-DOG inhibits xylose metabolism similarly to glucose in the YN14 strain. Compared to the first YPX20-2DG subculture, the YN14 strain showed a considerably reduced xylose consumption rate in the sixth subculture, but in the tenth subculture, a two-fold enhanced xylose consumption rate and the production of 1.9 g/L of ethanol was observed after (72 h of fermentation). These changes suggest that strain YN14 accrued genetic mutations that progressively increased its resistance to 2-DOG, alleviating glucose repression of its xylose metabolism.

To verify the extent to which glucose repression of xylose metabolism was alleviated in the evolved YN14 strain, single colonies were isolated from the tenth sub-culture, and their mixed sugar fermentation capabilities were compared to those of the parental strain under YPDX20 conditions (YP with 20 g/L of glucose and 20 g/L of xylose). Forty isolated colonies from the initial screening showed similar glucose and xylose consumption patterns, so only four were selected for further comparison with the YN14 strain, as shown in Fig. S2. The YN14 strain exhibited sequential consumption of glucose and xylose (with efficient xylose consumption beginning only when glucose was depleted (after 15 h of fermentation), whereas the four mutant colonies showed partial co-consumption of glucose and xylose. Compared to the parental YN14 strain, the mutant colonies had similar xylose consumption rates but more than 40 % reduced glucose consumption rates. The gap between glucose and xylose consumption rates in strain YN14 was about 0.81 g/L*h, while those in the mutants ranged from 0.20 to 0.32 g/L*h (a roughly 60–70 % decrease). This indicates partial alleviation of glucose repression of xylose metabolism in the evolved YN14 strains. It has been shown that yeast mutants exhibiting alleviated glucose repression generally have significantly reduced glucose metabolic capacities compared to their parental strains (Kim et al., 2019; Lane et al., 2018). Among the four mutant colonies, colony number 17, which exhibited the smallest gap between glucose and xylose consumption rates, was selected and dubbed strain CH17 (Fig. S3).

To assess whether allevating glucose repression of xylose metabolism affects the metabolism of other sugars in strain CH17, its fermentation patterns YPDX30 and YPDM30 media were investigated (Fig. 3C and D, respectively). Unlike strain YN14, strain CH17 consumed 6.2 g/L of xylose and 22.1 g/L of glucose over the first 24 h of fermentation in YPDX30 (Fig. 3). Consequently, the strain CH17 produced 23.3 g/L of ethanol from these sugars over 48 h, with a 50 % reduced glucose consumption rate (0.80 g/L*h) but a similar xylose consumption rate (0.63 g/L*h). This pattern suggests that strain CH17 can partially co-consume glucose and xylose, consistent with observations under YPDX20 conditions.

In contrast, in the YPDM30 medium, strain CH17 exhibited a unique fermentation pattern: it failed to consume mannose in the presence of glucose during the initial 24 h and showed significantly slowed mannose consumption after glucose was exhausted. As a result, strain CH17 produced only 12.6 g/L of ethanol from the two sugars, with a 30 % reduction in glucose consumption (0.82 g/L*h) and a 90 % reduction in mannose consumption (0.10 g/L*h) compared to the YN14 strain. This suppression of mannose metabolism might be due to the alleviation of glucose repression, which generally reduces the glucose metabolic capacity of yeast significantly (Kim et al., 2019; Lane et al., 2018; Young et al., 2011).

These results suggest that further alleviating glucose repression might lead to greater suppression of mannose metabolism. To investigate this, additional adaptive evolution was conducted under increased 2-DOG concentrations (YP medium with 20 g/L of xylose and 10 g/L of 2-DOG, YPX20-10DG). Strain CH17 was serially sub-cultured under YPX20-10DG conditions nine times, and single colonies were isolated from the final sub-culture. Mixed sugar fermentation was then performed under YPDM20 (YP with 20 g/L of glucose and 20 g/L of mannose) conditions with 10 randomly chosen colonies.

Selected fermentation profiles strain CH17 in YPX20-10DG medium were compared with those in YPX20 (Fig. S4). Despite a 40 % reduction in the xylose consumption rate compared to YPX20, in YPX20-10DG medium, strain CH17 produced 5.3 g/L of ethanol by consuming 17.9 g/L of xylose over 48 h in the first sub-culture. This indicates that the increased 2-DOG concentration did not significantly inhibit xylose metabolism, as strain CH17 had already developed improved resistance to 2-DOG from its previous evolution. However, no further improvement in xylose consumption was observed during the serial sub-cultures with 10 g/L of 2-DOG, suggesting that significant genetic mutations further alleviating glucose repression may not occur.

To determine if mannose metabolism was further reduced after subcluturing in YPX20-10DG, mixed sugar fermentation was performed in YPDM20 media using single colonies isolated from the ninth subculture. Among the 10 colonies used for initial screening, four colonies were selected, and their fermentation patterns were compared with those of their parent strain, CH17 (Fig. S5). Strain CH17 consumed 20 g/L of glucose over 24 h but only 4.3 g/L of mannose over 48 h under YPDM20 conditions. Compared with strain CH17 strain, the four mutant colonies exhibited similar glucose consumption profiles but greater than 30 % reductions in mannose consumption (1.5–2.6 g/L), indicating further reductions in the mannose metabolism of these strains due to evolution under increased 2-DOG conditions. In terms of consumption rates, strain CH17 showed a mannose consumption rate of 0.09 g/L*h over 48 h, while the four mutant colonies showed rate of 0.03–0.06 g/L*h (Fig. S6). Of the four mutant colonies, colony number 5, which exhibited the lowest mannose consumption rate, was selected and labeled strain MH05.

To assess whether evolution under high 2-DOG concentrations affected the metabolism of other sugars in strain MH05, mixed sugar fermentations were performed with different combinations of sugars containing glucose. Fig. 3E and F show the fermentation profiles of strain MH05 in YPDX30 and YPDM30 media. Under YPDX30 conditions, strain MH05 consumed 19.9 g/L of glucose and 5.0 g/L of xylose over 24 h of fermentation (Fig. 3), amounts similar to those consumed by strain CH17. Over 48 h, strain MH05 produced 22.7 g/L of ethanol exhibited glucose and xylose consumption rates (0.82 g/L*h and 0.62 g/L*h, respectively) similar to those of strain CH17. These results suggest that further evolution under a high 2-DOG concentration did not significantly alleviate glucose repression of xylose metabolism in the MH05 strain.

During fermentation in YPDM30 medium, strain MH05 showed similar glucose consumption when compared to strain CH17, but its mannose consumption rates were lower (Fig. 3F), especially, after glucose was exhausted. Overall, strain MH05 produced 12.5 g/L of ethanol from the two sugars, with a glucose consumption rate of 0.86 g/L*h and mannose consumption rate of 0.06 g/L*h (a 40 % reduction compared to that of strain CH17) over 48 h. Further research, such as genomic and transcriptomic analyses, may be needed to understand why mannose metabolism is significantly reduced without notable changes in xylose metabolism in mutant strains CH17 and MH05.

3.4. Glucose and D-mannose production through the selective fermentation of SCG hydrolysate

After enzymatic hydrolysis, the SCG hydrolysate was fermented into ethanol using the parental strain *P. stipitis* YN14 and mutant strain *Pichia*

stipitis MH05. The SCG hydrolysate initially contained 15.3 mg/mL of glucose and 10.6 mg/mL of mannose. With strain YN14, glucose was entirely consumed after 24 h of fermentation, while mannose was consumed after 48 h. After 48 h of fermentation, the maximum bioethanol production reached 11.6 mg/mL, a yield of 87.9 % (Fig. 4).

In contrast, use of the mutant *P. stipites* strain MH05, resulted in complete glucose consumption after 48 h of fermentation. The highest ethanol yield, 71.7 %, was achieved after 72 h. However, after 48 h of fermentation, a yield of 54.0 % had been obtained, and a significant amount of mannose (79.9 %) remained. Consequently, a fermentation period of 48 h is considered optimal for the simultaneous production of both D-mannose and bioethanol, despite the lower bioethanol production. In the future, further experiments on the separation of mannose and ethanol are warranted.

3.5. Overall mass balance

As illustrated in Fig. 5, the overall mass balance of SCG processing was evaluated through sequential steps, including pretreatment, enzymatic hydrolysis, and selective fermentation using *Pichia stipitis* MH05. Initially, 1 kg of raw SCG contained 86 g of glucose and 186 g of mannose. After alkali pretreatment, these values slightly decreased to 83 g and 179 g, respectively. The bleaching step primarily removed lignin and a portion of hemicellulose, reducing the mannose content to 136 g while glucose remained relatively stable at 82 g. Subsequent NH pretreatment further decreased mannose to 95 g, with glucose still maintained at 82 g. During enzymatic hydrolysis, structural polysaccharides were effectively depolymerized, yielding 76 g of glucose and 95 g of mannose. In the final selective fermentation step, *Pichia stipitis* MH05 converted glucose into 40 g of ethanol with a fermentation efficiency of 71.7 %. While glucose was largely consumed, a substantial portion of mannose (76 g) remained unfermented, indicating the limited ability of *Pichia stipitis* MH05 to utilize mannose under the applied conditions. This stepwise biorefinery process highlights the efficient fractionation of SCG into mannose and bioethanol, demonstrating its potential for value-added bioconversion.

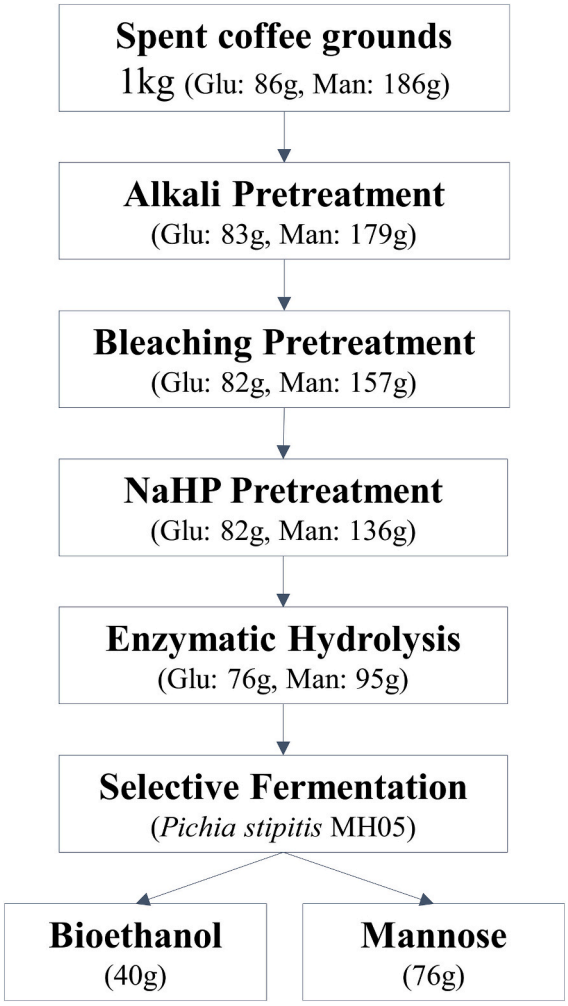

Fig. 5. Overall mass balance for the production of mannose and bioethanol from SCG.

4. Conclusion

This study demonstrates that valuable compounds, such as D-mannose and bioethanol, can be produced simultaneously from SCGs by optimizing pretreatment processes, enhancing enzymatic hydrolysis, and employing selective fermentation techniques. Utilizing SCGs to generate high-value products has the potential to reduce waste from coffee processing, address environmental concerns, and provide economic benefits by converting waste into valuable biorefinery products. Overall, SCGs represent a significant resource for bioethanol and D-mannose production, contributing to the development of a sustainable

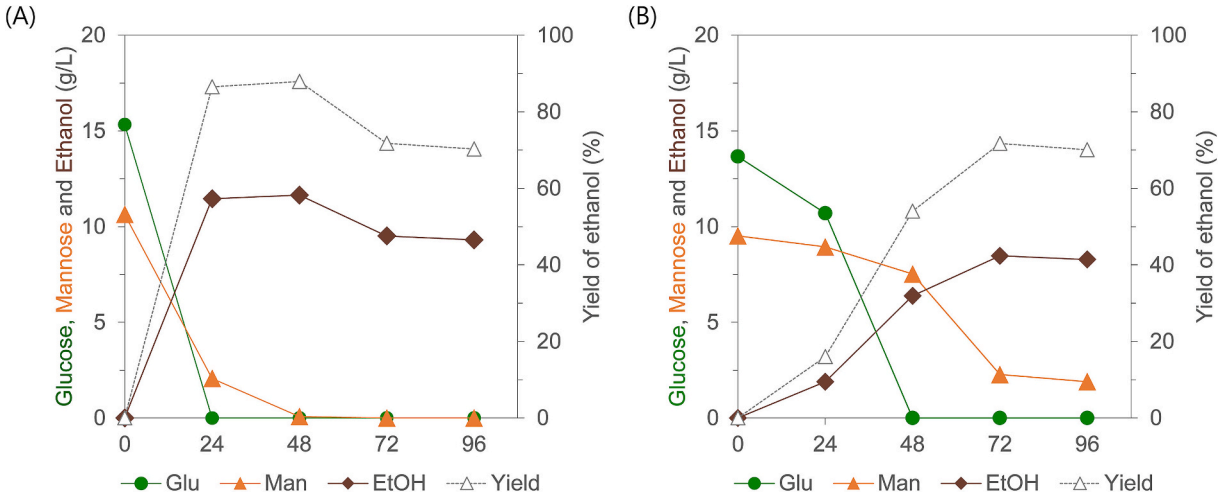

Fig. 4. The fermentation profiles for SCG hydrolysates using (A) parental *P. stipitis* strain YN14 and (B) mutant *P. stipitis* strain MH05 (Glu, Glucose; Man, mannose; EtOH, ethanol).

circular bioeconomy and addressing challenges related to environmental sustainability and energy supply.

CRediT authorship contribution statement

Eun Jin Cho: Writing – original draft, Visualization, Investigation, Funding acquisition, Formal analysis, Data curation. **Won-Heong Lee:** Methodology, Investigation, Data curation. **Yoon Gyo Lee:** Investigation, Formal analysis. **Quang Van Ta:** Investigation. **Ha Yeon Kim:** Investigation, Formal analysis. **Hyeun-Jong Bae:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2025.102606.

Data availability

Data will be made available on request.

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