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Enzyme-free pretreatment of brewer's spent grain for xylose recovery for potential xylitol production

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Abstract
Brewer's spent grains (BSG), a by-product in beer brewing, have historically been relegated to animal fodder. This study delves into the untapped potential of BSG as a valuable source of fermentable sugars, specifically xylose. Proximate analysis confirmed the presence of xylan-rich hemicellulose (21.5 ± 0.32%) in BSG, making it ideal for xylose production. The FTIR spectrum of BSG confirmed peaks at 900–950 cm^{-1} corresponding to hemicellulose xylan. A combined pretreatment of BSG by hydrothermal (110 °C) and acid hydrolysis (100 °C) resulted in 50.11 ± 0.15% of reducing sugar recovery as quantified by Lane and Eyon method again reconfirmed by HPLC with 48.15 ± 0.05 g/L of xylose. GC-MS analysis of pre-treated BSG hydrolysate revealed presence of some inhibitory compounds with mass spectra viz. Levoglucosenone, 9,12-octadecadienoic acid, n-hexadecenoic acid and furfural derivatives. Further the BSG hydrolysate obtained after combined pretreatment was further used as a carbon source in addition to other ingredients of medium. A preliminary fermentation trial with *Pichia fermentans* was carried out at 30° C, at pH 7.0 for 24 h and resulted in 6.13 ± 0.05 g/L xylitol production at 30 °C at 150 RPM. The results validated the effectiveness of the pre-treatment in maximizing fermentable sugar recovery and give conformity for further optimization of xylitol production making it enzyme free and cost-effective approach.

Keywords Brewer's spent grain (BSG), Reducing sugar (RS), Xylose production, Enzyme free pretreatment, Circular bioeconomy, Xylose

Introduction

Brewer's Spent Grains (BSG) are among the most abundant and complex by-products of beer production and accounts for 85% of the overall by-product generated. For every hundred liters of beer produced, 20 kg of BSG is produced traditionally and further utilized as animal fodder [1, 2]. However, increasing environmental concerns associated with its disposal, combined with its lignocellulosic nature, have sparked interest in its valorization as a sustainable feedstock for bioconversion processes [3]. BSG is rich in complex carbohydrates, particularly cellulose, hemicellulose, polysaccharides, lignin, and arabinoxylans [4]. A variety of constituents such as fiber (30–50% w/w) and protein (19–30% w/w) make it a promising candidate for integration into various markets [4, 5]. BSG is one of the several agricultural by-products that have been researched for finding a wide variety of bioactive compounds such as phenolic acids, flavonoids, lignin, and arabinoxylans with antioxidant, anti-inflammatory, and prebiotic properties that could be utilized for food [6–8].

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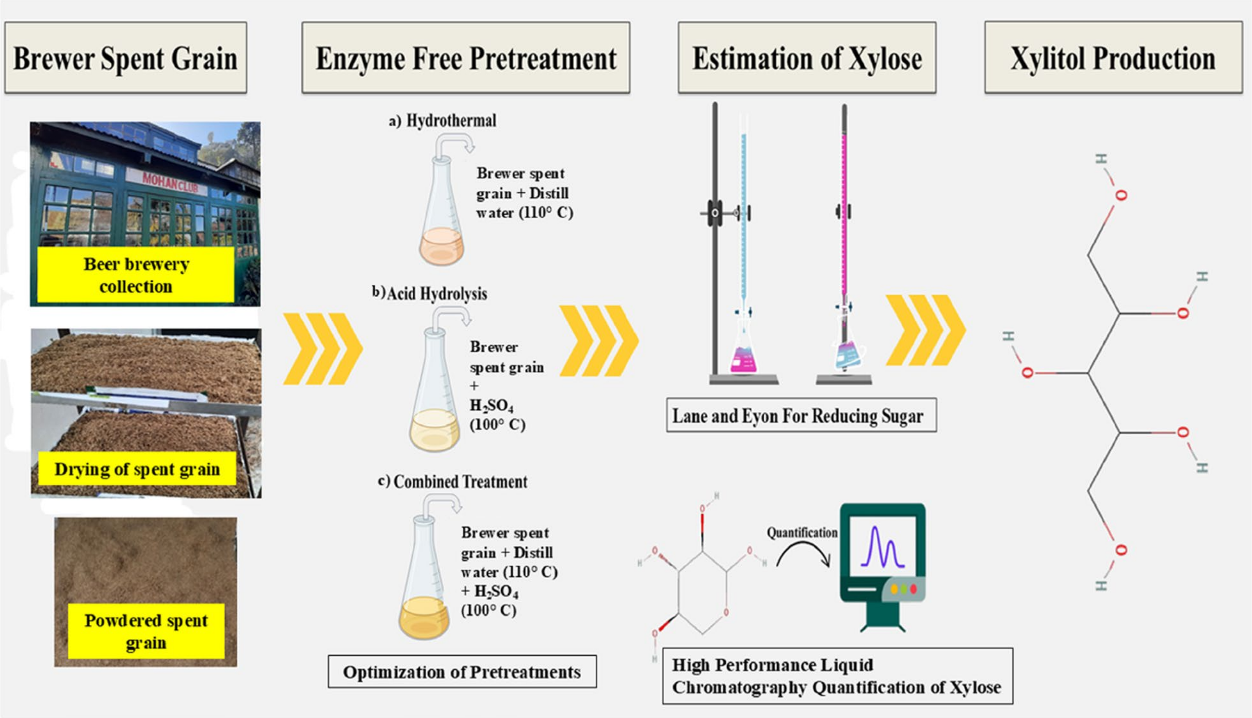
Graphical Abstract



The brewing industry mainly produced 3 types of wastes, which included 0.2–0.4% hot trub, 15% brewer's yeast, and 85% by-products of BSG [4]. Optimization of xylose sugar content in BSG is considered to be very promising research as it opens a number of challenges and areas that need further research. The complexity of the BSG composition further makes its pretreatment a complex process requiring special pretreatment techniques to deal with its intricate matrix [2]. Although research on enzymatic hydrolysis and microbial valorization of lignocellulosic feedstocks is plentiful, the gap still stands for enzyme-free pretreatment methods, particularly for brewer's spent grain (BSG), that are scalable and maximize xylose recovery with minimal inhibitor formation [5]. Another gap that exists is an under-investigation of the potential of BSG-derived hydrolysates for direct fermentation to value-added products like xylitol and using non-conventional yeasts such as *Pichia fermentans* [3, 8]. Closing this gap will improve economic feasibility and sustainability within a circular bioeconomy framework about BSG biorefineries [9]. Analyses indicate that the combination of hydrothermal treatment with dilute acid pre-treatment allows xylose yields from 35 g/L to 40 g/L [10]. Optimization of the xylose release process from BSG has reported as high as 42 g/L xylose under ideal conditions [11].

Hydrothermal pretreatment alone can recover about 70% of the extractable hemicellulose sugars. When used in combination with acid hydrolysis, recovery of xylose can reach beyond 80% and the overall process is an enzyme-free method [11]. This method, theoretically, may offer about 50–100% cost reduction, as it doesn't require costly enzymes. Hydrolysis of enzymes is popular in the recovery of sugars from lignocellulosic; however, it is usually very expensive to operate, takes a long time for reaction, and multistep processing thus limiting scalability for industry. This study investigates an enzyme-free pretreatment method where hydrothermal treatment is followed by dilute acid treatment [2]. In turn, this strategy makes the process less complex and cheaper while effective xylose release can be achieved from hemicellulose-rich BSG. Consequently, it can be economically even more competitive when produced in scale at both laboratory and industrial levels. Today, the commercial price of xylose is reported to be at 1.50-3.00 USD/Kg [12]. For example, for xylanase and other enzymes price falls within 100–500 USD/Kg (Sigma-Aldrich) [13]. This study explores the potential of using brewing spent grains, an available and underutilized by-product, as a valuable source of fermentable sugars, especially xylose, by using enzyme free cost-effective pretreatment method.

Another major challenge in the utilization of BSG for bioconversion is the formation of inhibitory compounds during pretreatment. Phenolic compounds including ferulic acid, p-coumaric acid, and gallic acid are present in BSG and can be released through acid hydrolysis. These compounds can affect yeast activity negatively during subsequent fermentation processes [14]. At present the adsorption methods or biological detoxification approach has been applied to mitigate the level of such inhibitory compounds and consequently improve the fermentation process [15]. Moreover, the BSG pretreatment processes are highly energy and resource-intensive thus making it a focused area of research for its economic viability. Therefore, there is a pressing need to assess the BSG use for environmental sustainability, evaluating its ecological footprint including potential impacts on biodiversity, water usage, and greenhouse gas emissions [16, 17].

Optimizing pretreatment techniques to elevate the degree of xylose sugar in BSG is important to make it a potential source of xylose sugars for xylitol production [18]. The current research focuses on optimizing an inexpensive and environmentally friendly pretreatment methodology for optimum xylose recovery from BSG for use in xylitol production by *Pichia fermentans* for its sustainability and biorefinery [19].

Materials and methodology

All chemicals and reagents used in this study were of analytical grade and purchased from HiMedia Laboratories Pvt. Ltd. (India) and Sigma-Aldrich (USA), unless otherwise stated.

BSG collection and drying

In the present study, the BSG was procured from the Mohan Meakin brewery in Kasauli, Himachal Pradesh, India. The BSG was dried in a cabinet dryer (Model: MAC-700; Macro Scientific Works Pvt. Ltd., India.) at 60 °C for 7 h per day for 7 days until complete desiccation was achieved. After drying, the BSG was pulverized into a fine powder, sieved through a 0.5 mm mesh, and stored in an airtight container.

Proximate analysis of BSG

A comprehensive proximate analysis of BSG was carried out using the Association of Official Analytical Collaboration (AOAC) method. (2005) [20] to determine parameters viz. moisture content, pH, crude fat, crude protein, carbohydrates, crude fiber, and ash content. Whereas, the assessment of hemicellulose, cellulose, and lignin content in BSG was done as per the methodology of [21]. All experiments were conducted in triplicates. The data obtained from these triplicate experiments were subjected to statistical analysis, and standard deviation (SD) was calculated.

FTIR analysis of BSG

Understanding the chemical composition of BSG is important for unlocking its potential in various applications. For this the BSG was studied with Fourier-Transform Infrared (FTIR) Spectroscopy (Model: FTIR-4100; Thermo Scientific Nicolet iS5) to characterize the relative abundances of major components like cellulose, lignin, hemicellulose, and hydroxyl groups, offering a rapid and non-destructive approach. For FTIR analysis, 1 g of dry BSG powder (collected from the brewery and dried as described in the methodology) was directly placed onto the FTIR crystal and gently pressed to ensure good contact. Each BSG sample was scanned in triplicate with a spectral range of 4000–650 cm^{-1}, resolution of 16 cm^{-1} and 60 scans per spectrum to get a qualitative comparison. To evaluate the structural changes induced by the pretreatment process, FTIR analysis was performed on both untreated BSG and the BSG hydrolysate obtained after combined hydrothermal and acid pretreatment mentioned below.

BSG pretreatment optimization for reducing sugar release

To investigate the reducing sugar content availability in BSG, it was further pretreated to optimize its inherent abundance in hemicellulose to potentially yield xylose for further use in xylitol production. Enzymes were not used in this study to specifically evaluate the efficiency of a cost-effective, chemical-based pretreatment method suitable for scalable sugar recovery. The following pretreatment methodologies were executed in a sequential manner:

Hydrothermal treatment

The optimization started firstly with hydrothermal pretreatment where the concentration of BSG were systematically altered within the range of 1:10 to 5:10 mass ratios with distilled water (i.e., 10 g BSG powder in 100 ml distilled water). The hydrothermal pretreatment was carried out in a water bath, precisely maintained at 110 °C for 15 min first to select the optimized BSG ratio and subsequently, the chosen ratio was transferred into a 250 ml Erlenmeyer flask and subjected to treatment time optimization from 15 to 75-minute duration. The resulting optimized mixture was then filtered using a vacuum filtration apparatus equipped with a 0.45 μm pore size filter membrane. The filtrate was subsequently subjected to reducing sugar estimation by Lane and Eyon method [22].

Acid hydrolysis treatment

In the second experimental set up, the BSG in the above selected ratio was subjected to acid hydrolysis by varying H_2SO_4 concentrations between 0.5 and 1.5% (v/v) for 15 min for optimizing acid concentration. H_2SO_4 was selected for pretreatment based on its established effectiveness in catalyzing the hydrolysis of lignocellulosic substrates, including brewer's spent grain (BSG) [23]. It promotes efficient depolymerization of the hemicellulose part as well as partial hydrolysis of cellulose to liberate fermentable sugars with relatively low formation of inhibitory compounds. Its wide application in biomass processing is due to cheap cost, easy scalability, and downstream action towards fermentation processes [24]. After the acid ratio was optimized the treatment time was also optimized by varying pretreatment duration from 15 to 75-minute in two steps i.e., (i) room temperature acid hydrolysis (25 °C) and other (ii) with heating acid hydrolysis at 100 °C in 250 ml Erlenmeyer flasks using a thermostatically controlled water bath (REMI, India) to refine the hydrolysis process.

Combined treatment

In the third optimization method, both the hydrothermal process and acid hydrolysis were combined in sequence for the treatment. The BSG was subjected to hydrothermal treatment under the previously optimized conditions, followed by acid hydrolysis with H_2SO_4 for the durations determined as optimal in the individual methods. The total reducing sugar released were determined using Lane and Eynon Method, the titration method [22]. The xylose quantification was done by McCleary and McGeough method (2015) from obtained reducing sugar [25]. The xylose standard curve was prepared in concentrations ranging from 0.1 mg/ml to 1 mg/ml. For each sample, 1 ml of the hydrolyzed BSG filtrate was mixed with 1 mL of DNS reagent in a test tube. The mixture was vortexed thoroughly to ensure uniform mixing. The test tubes were then placed in a water bath maintained at 100 °C for 10 min to allow the color to develop. Now the tubes were cooled to room temperature in a cold-water bath to stabilize the reaction. The absorbance of each sample was measured at 540 nm using a spectrophotometer. The xylose content in the samples was quantified by comparing the absorbance values against the prepared xylose standard curve [25].

Quantification of xylose in reducing sugar release via HPLC

The pretreated BSG obtained after combined pretreatment was also evaluated by HPLC method to reconfirm the reducing sugar released. The HPLC system (Make: Agilent 1260 Infinity) a reversed-phase C18 column (particle size: 5 µm, column dimensions: 250 mm x 4.6 mm) was used with a mobile phase consisting of 10% acetonitrile and 90% water. The flow rate was maintained at 1.0 mL/min, and the analysis was conducted at a temperature of 30 °C. A calibration curve was drawn using standard xylose solution under the similar conditions. The retention periods and peak areas of the standards were determined to identify and quantify the xylose found in the pretreated BSG hydrolysate. All experimental procedures were conducted in triplicate, and the standard deviation was computed. Additionally, the statistical evaluation was performed using one-way analysis of variance (ANOVA). Both the methods as proposed by McCleary and McGeough (2015) and HPLC were used to quantify xylose so that results comparison could take place. The McCleary method gives a quick, inexpensive way to screen reducing sugars [25]. High specificity for accurate quantification of individual sugars in the hydrolysate can be obtained through HPLC. The two methods, together, ensure analytical accuracy and reliability.

Analysis of inhibitory compounds in BSG hydrolysate using GC-MS

BSG hydrolysate, pretreated using a combination of hydrothermal and acid hydrolysis, was analyzed for the presence of inhibitory compounds such as furfural and5-hydroxymethylfurfural [26] via GC-MS (Thermo Fisher Scientific, USA) to assess its suitability for further valorization processes. Samples were centrifuged, diluted to a concentration of 1:10, spiked with an internal standard - d3-furfural at a concentration of 50 ppm, and derivatized before injection. Full scan data was acquired from m/z 45 to 450 for 45 min. Compounds were identified by comparing their mass spectra with reference NIST library for identification of the compounds.

Xylitol production using pretreated BSG hydrolysate

To assess the potential of pretreated BSG hydrolysate for xylitol production, a preliminary fermentation experiment was conducted using *Pichia fermentans* obtained from Cranfield University, UK. The culture medium composition and fermentation conditions were adapted from the method reported by [27], focusing on key components like carbon source (xylose or pretreated hydrolysate), nitrogen source, and essential minerals. The carbon source was supplied at a concentration of 20 g/L, while the nitrogen source and essential minerals were added at concentrations of 1 g/L, 0.5 g/L, and 0.1 g/L, respectively [19]. The fermentation was carried out at 30 °C with continuous agitation at 150 RPM for 24 h and monitored every 6 h. Xylitol production in the fermentation broth was quantified using HPLC. It is important to note that this experiment served as a preliminary assessment and did not involve optimization of fermentation conditions for maximizing the xylitol production.

Results and discussion

Substrate collection

After the procurement of Brewer's Spent Grains (BSG) from local brewery in Kasuali, Himachal Pradesh, India, it was subjected to drying and grinding for further use.

The drying techniques, such as sun drying and oven drying used, are vital for reducing the moisture content in BSG.

Low moisture content prevents microbial spoilage and ensures the material's longevity during storage, making it a practical substrate for further utilization [28]. Additionally, grinding dried BSG into a fine powder increases its surface area, facilitating efficient downstream processes like hydrolysis or fermentation. Storing the powdered BSG in air-tight containers further protects it from humidity and contamination, maintaining its quality for analytical or industrial applications [29].

Physico-chemical analysis of BSG

A detailed proximate analysis revealed the comprehensive composition of the dried brewer's spent grain (BSG). The results of this study are summarized in Table 1.

The proximate analysis of brewer's spent grain (BSG) exhibited a variety of compounds present in it. The brewer's spent grain contained a significant amount of crude protein (21.5 ± 0.23%), whereas, the crude fat (10.06 ± 0.02%) and ash (3.1 ± 0.05%) was present in moderate amount. The results also showed the high carbohydrate content (61.22 ± 0.13%), which act as a veritable reservoir for substantial sugar conversion. This is followed by hemicellulose (21.5%), which can serve as potential source for the liberation of xylose. Cellulose forms the major part of total carbohydrates in BSG and it is present at 25.5 ± 0.40%, however, this work focused on hemicellulose which is 21.5 ± 0.32% because it looks xylan-rich and can be directly converted into xylose that is used for xylitol production. While cellulose is quantitatively more abundant, its conversion to fermentable sugars typically requires enzymatic saccharification, which was intentionally avoided in this enzyme-free approach [13]. Therefore, xylan from hemicellulose was targeted as the more accessible and cost-effective source of fermentable sugar under the chosen pretreatment conditions [8].

Table 1 Physico-chemical analysis of BSG obtained in the present study

Parameter	Value (%)
Moisture	4.12 ± 0.06
pH	7.11 ± 0.18
Crude Fat	10.06 ± 0.02
Crude Protein	21.5 ± 0.23
Carbohydrates	61.22 ± 0.13
Crude Fiber	15.0 ± 0.15
Ash	3.1 ± 0.05
Hemicellulose	21.5 ± 0.32
Cellulose	25.5 ± 0.40
Lignin	10.0 ± 0.56
Total Solid	95.88 ± 0.54

Values in table are expressed as mean ± SD (*n*=3)

The presence of sugars like glucose, fructose or xylose not only can be used for fermentative processes, but can also serve as substrate for catalytic reactions to yield important chemicals and building blocks in the context of biorefineries, such as 5-hydroxymethylfurfural and furfural [30]. Additionally, the moderate moisture content (4.12%) presence ensures the good storage stability, minimum microbial spoilage and energy requirements for drying in pretreatment feedstocks.

The relatively low lignin content of 10% further enhances the suitability of BSG for valorization, as lignin can be recalcitrant and hinder the efficient extraction of sugars during pretreatment processes [31]. While lignin can be valorized for its specific applications, such as the production of biofuels and aromatic compounds, but its presence in BSG can complicate the recovery of valuable sugars like xylose and glucose [27]. The lower lignin content in BSG compared to other lignocellulosic biomass sources reduces the challenges associated with lignin removal and improves the overall efficiency of sugar extraction.

FTIR study of BSG

FTIR spectroscopy is a analytical technique that provides valuable insights into the chemical composition of BSG. The FTIR spectrum of BSG powder (Fig. 1) shows distinct peaks of the relative abundance of cellulose, hemicellulose, lignin, and various hydroxyl groups. By measuring the absorption of infrared radiation at specific wavelengths, FTIR can identify the presence of different functional groups. This information is essential for understanding the structural characteristics and potential applications of BSG as a bioresource [32].

Specifically, the high intensity of peaks falling within the 1050–1100 cm^{-1} range is indicative of the stretching vibrations associated with C-O-C and C-O-H bonds [33]. Bands at 2925 cm^{-1} (90.002%) and 2858 cm^{-1} (90.933%) were attributed to C–H stretching and asymmetric stretching vibrations, respectively, with peak intensities of 0.78 ± 0.10 AU and 0.81 ± 0.09 AU. C = O stretching of carboxyl was at 1644 cm^{-1} with 97.915% transmittance and peak height of 1.02 ± 0.09 AU [34]. The of carbohydrate structures namely, C–O–C glycosidic linkages at 1033 cm^{-1} (77.037%, 0.73 ± 0.11 AU), C–O–C asymmetrical stretching at 1050 cm^{-1} (87.910%, 0.82 ± 0.05 AU), and C–O–H stretching vibrations at 1020 cm^{-1} (89.702%, 0.88 ± 0.05 AU) were also determined [35]. Specifically, the glycosidic bonds were verified at 975 cm^{-1} with 85.320% transmittance and a peak height of 0.75 ± 0.09 AU [34]. This spectral signature is commonly ascribed to hemicellulose, particularly xylan. Additionally, the other peak centered around 900–950 cm^{-1} offers insights into the structural characteristics of hemicellulose xylan with glycosidic linkages [36]. These FTIR observations confirm

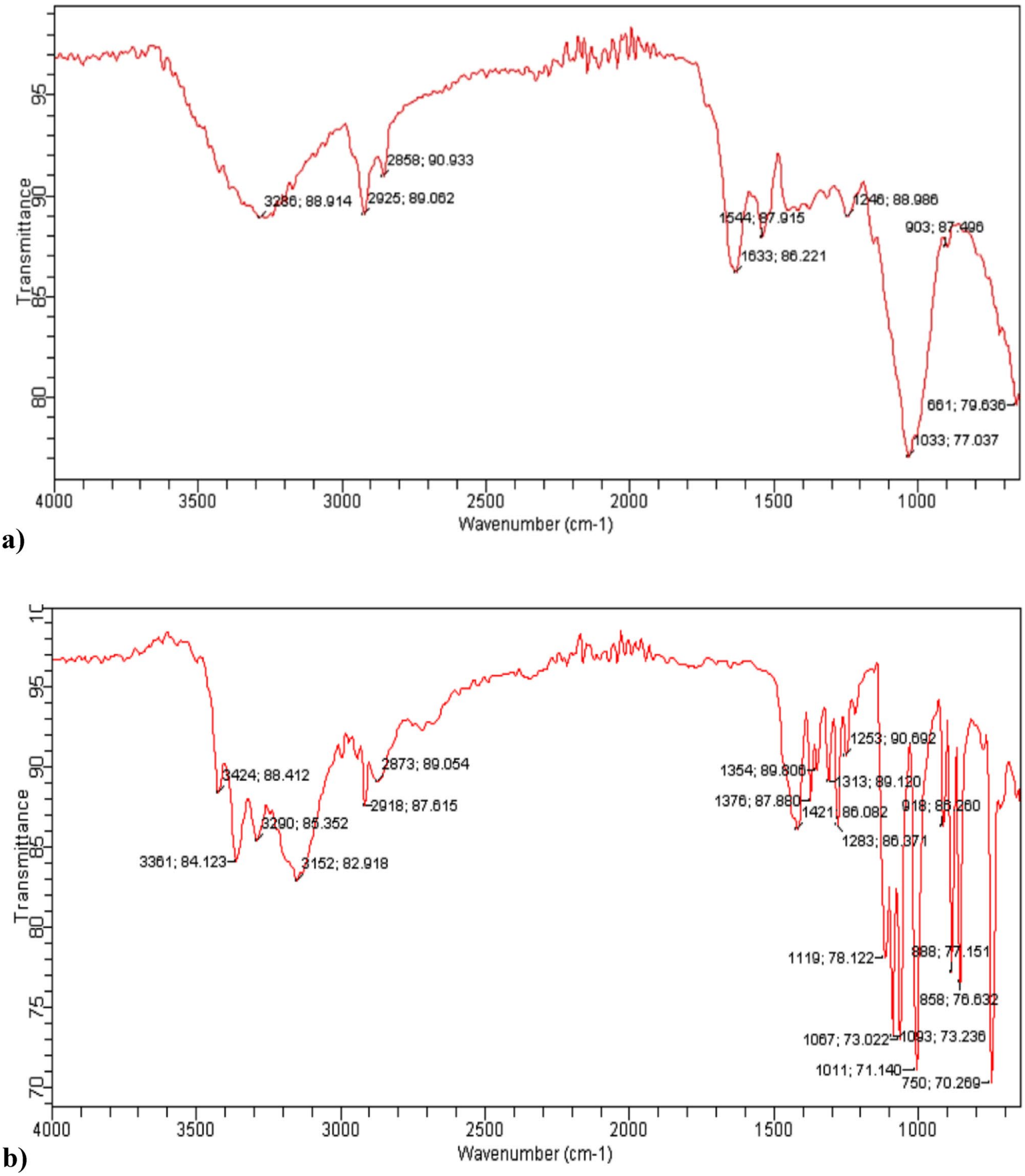


Fig. 1 FTIR spectra of brewer's spent grain (BSG): (**a**) untreated BSG showing characteristic peaks of lignocellulosic components, and (**b**) BSG after combined hydrothermal and dilute acid pretreatment, indicating structural modifications and partial degradation of hemicellulose and lignin within the scan range of 4000–800 cm^{-1}.

the substantial presence of xylose precursors within BSG, supporting the need for targeted pretreatment strategies aimed at optimizing xylose release for downstream xylitol production.

Following combined hydrothermal and acid pretreatment, the FTIR spectrum of BSG showed marked structural modifications. There was a obvious reduction in intensity of the peaks within 1050–1100 cm^{-1} region, indication that xylan's glycosidic bonds were broken and hemicellulose effectively decomposed [34]. Other shifts with much sharper peaks were observed between 900 and 750 cm^{-1} which probably represent newly created vibrational ways related to broken up carbohydrate parts and simple sugars [36]. The C–H and O–H stretching bands around 2900–3300 cm^{-1} are still present however show small changes in intensity suggesting partial retention of cellulose and some lignin [35]. These spectral transitions collectively validate the disruption of the lignocellulosic matrix and support the increased availability of fermentable sugars, aligning with the improved xylose yield observed post-treatment [8, 16].

Optimization of BSG pretreatment for sugar yield

Increasing the xylose (i.e. reducing sugar) yield from BSG is of key importance for optimizing the bioconversion process directed towards the utilization of this abundant by-product in the brewing industry. The present study is focused on pretreatment of waste BSG with the objective to recover maximum reducing sugar using following pretreatment methods by titration method of Lane and Eyon method [22]:

Hydrothermal treatment of BSG

The hydrothermal treatment of BSG, revealed insights into both biomass-to-water ratio and treatment time (Fig. 2a). In the biomass (BSG) to water ratio study a 4:10 ratio resulted in maximum sugar release (10.89 ± 0.22%) at 110 °C emphasizing the role of water availability in effectively converting hemicellulose to reducing sugars while minimizing sugar degradation, at elevated temperatures while minimizing sugar degradation to compounds like humins and others, particularly at 110–140 °C temperature range [37].

On the under hand, when the time of hydrothermal treatment was varied, the best yield of reducing sugars (20.23 ± 0.33%) was observed at a treatment time of 30 min at 110 °C (Fig. 1b) using Lane and Eyon method [22]. Whereas, the amount of reducing sugars were low at 15-minute pretreatment and when the pretreatment time was increased beyond 30 min up to 75 min. One-way analysis of variance (ANOVA) confirmed the statistical significance of these findings ($p < 0.05$). The increased pretreatment durations may be outweighed by the increased energy consumption and potential for degradation of sugars during prolonged exposure to high temperatures and pressures [26] as seen beyond 30 min to 75 min [24]. investigated the effects of pretreatment time and acid concentration on sugar release from corn stover and found that excessive pretreatment can lead to sugar degradation.

Acid hydrolysis of BSG

In this study the acid hydrolysis of BSG was carried out in a range of 0.5–2.5% H_2SO_4 acid and time of treatment was also optimized. The results of this study are shown in (Fig. 2a). In this study 1% H_2SO_4 concentration with optimized BSG ratio (4:10 g/ml) resulted in maximum sugar release of 14.23 ± 0.15% after 15 min at 100 °C (Fig. 3a).

After the optimization of acid concentration required for BSG pretreatment, the time of acid pretreatment was optimized and (Fig. 3b) 30-minute duration treatment resulted in effective hydrolysis of hemicellulose into reducing sugar release (35.97 ± 0.19%). Whereas, the decreased and prolonged acid treatment time does not yield optimal results of sugar release from BSG in the present study [38]. reported a reducing sugar yield of 30.2% using 1% sulfuric acid for 60 min. This suggests that the optimal conditions for acid hydrolysis of BSG

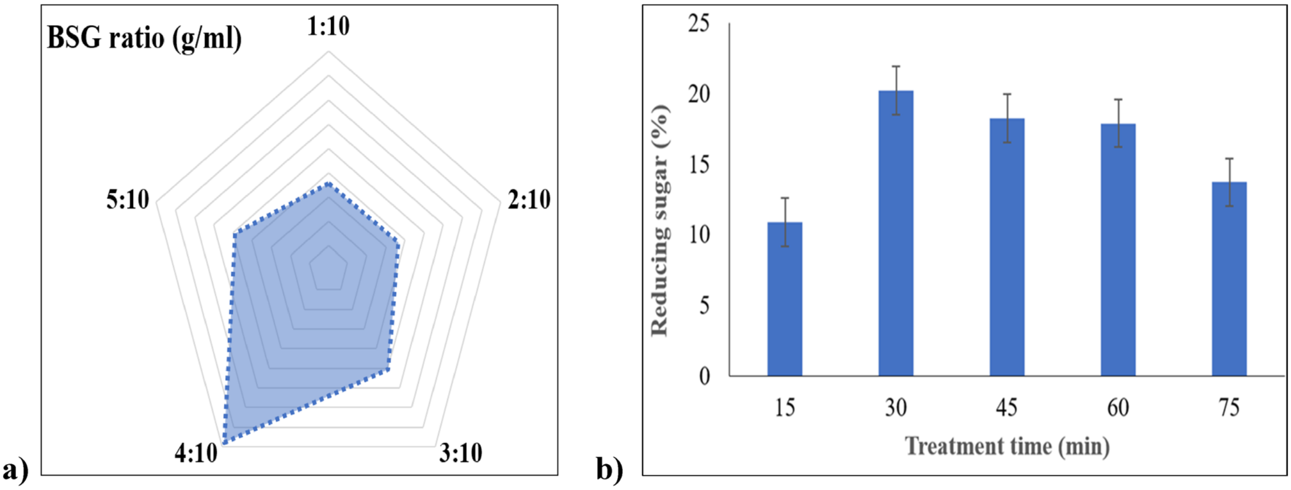


Fig. 2 Optimizing of reducing sugar release from BSG under hydrothermal treatment at 110 °C. (a) using 1:10 to 5:10 BSG to water ratio for 15 min treatment and (b) variation of time of treatment. Results were analyzed using ANOVA (p < 0.05) with standard deviation (SD)

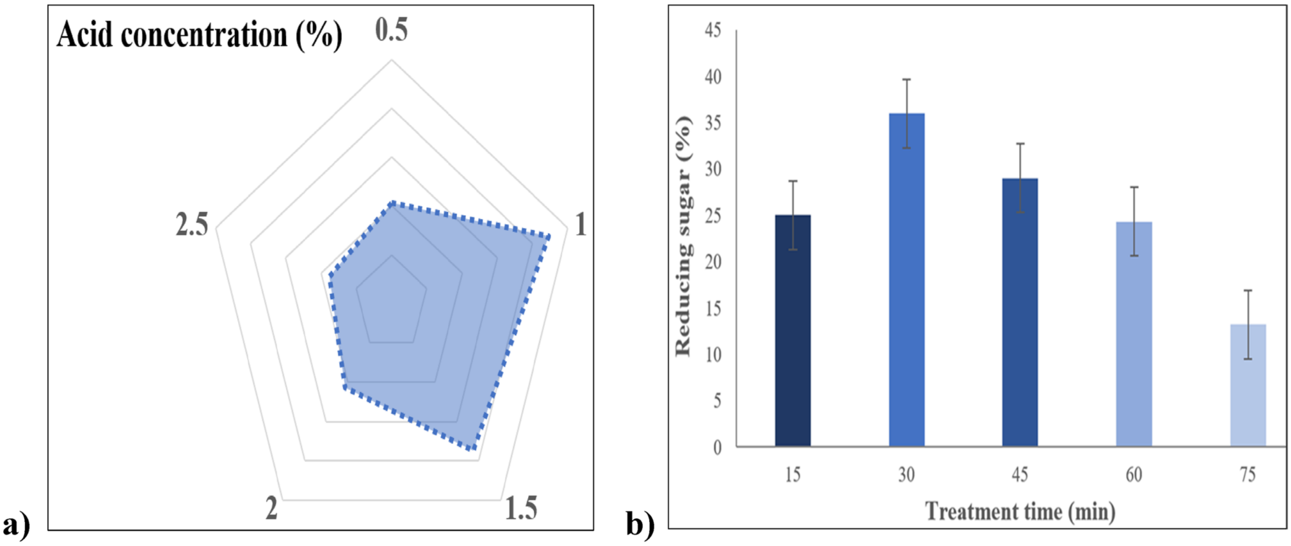


Fig. 3 Optimization of reducing sugar release from BSG under acid hydrolysis treatment at 100 °C (**a**) using 0.5–2.5% H_2SO_4 concentration for 15 min and (**b**) variation of time of treatment. Results were analyzed using ANOVA ($p < 0.05$) with standard deviation (SD)

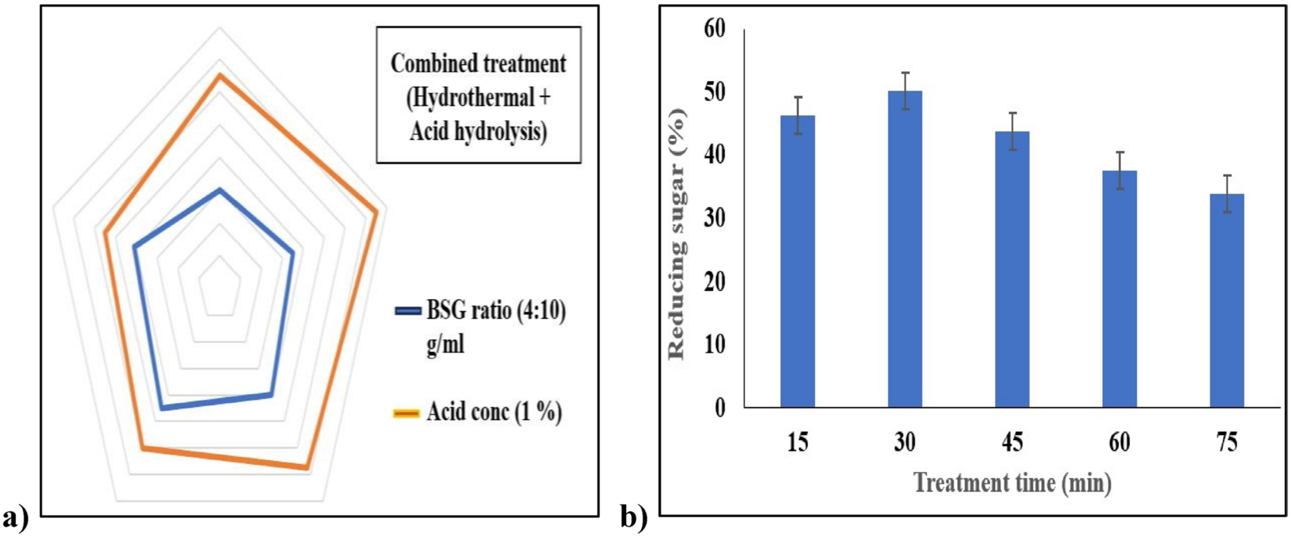


Fig. 4 Optimization of reducing sugar release from BSG after combined pretreatment (a) i.e., hydrothermal at 110 °C for 30 min using 4:10 g/ml BSG followed by acid (1%) hydrolysis at 100 °C for 30 min and (b) variation of the time of treatment. Results were analyzed using ANOVA ($p < 0.05$) with standard deviation (SD)

may vary depending on specific process requirements and feedstock characteristics.

Combined pretreatment of BSG

In this study the BSG samples were pretreated in sequential manner i.e., first with hydrothermal approach consisting of biomass to water ratio of 4:10 g/mL at 110 °C followed by acid hydrolysis step (1% H_2SO_4 and then heated at 100 °C) (Fig. 4a) under above optimized conditions.

The combined pretreatment of BSG (Fig. 3b) yielded highest total reducing sugar (50.11 ± 0.15%) at 30-minute treatment time and others yielded low content (46.21 ± 0.09%, 43.81 ± 0.22%, 37.61 ± 0.09%, 33.88 ± 0.11% for 15, 45, 60 and 75 min), respectively, shown in (Fig. 4). The overall pretreatment results indicates that the combined pretreatment not only outperforms the individual hydrothermal and acid pretreatments but also attained the highest yield of reducing sugar from the BSG. The one-way analysis of variance (ANOVA) revealed a highly significant difference among three pretreatment groups.

The xylose content specifically in the reducing sugar recovered after combined pretreatment via Lane and Eyon titration method [22] was further quantified by McCleary and McGeough (2015) [25] method using following xylose standard (Fig. 5).

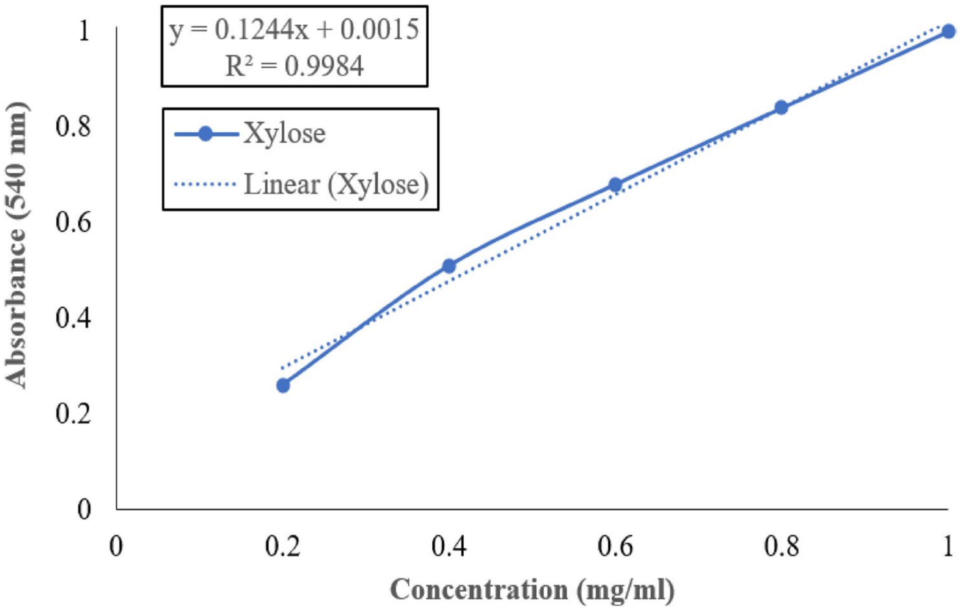


Fig. 5 Standard xylose graph used for reducing sugar (xylose) quantification (McCleary and McGeough, 2015)

Based on the standard calibration curve obtained the calculations performed were as following:

$y = mx + c$,

According to standard xylose curve equation obtained was: $y = 0.1244x + 0.0015$.

Where, y: absorbance (OD) and x: concentration of xylose.

Using the standard curve equation:

$$x = y - 0.0015 \div 0.1244$$

Where:

"y" = 0.561 (measured OD of the diluted sample),

"x" is the xylose concentration in mg/mL.

Substitute $y = 0.561$.

$x = 0.561 - 0.00150 \div 1244 = 4.49$ mg/ml.

Since the sample was diluted by 10 times: 1 ml (sample) + 9 ml (DW) = 10 ml.

So, the dilution factor (DF) is 10.

4.49 × 10 = 44.95 mg/mlffig

The results showed a recovery of 44.95 mg/ml xylose in the reducing sugar in BSG obtained after optimized combined pretreatment (Fig. 4). The results demonstrated that shorter pretreatment durations were generally more effective in releasing reducing sugars, with a 30-minute pretreatment yielding the highest sugar in all cases. This is in line with previous studies on BSG pretreatment using enzymatic hydrolysis, which also reported optimum xylose recovery at shorter incubation times [23, 39].

However, the pretreatment methods optimized in this study, without using enzymes, has potential to reduce the production cost which could contribute to its scalability and sustainability. For example, the combined hydrothermal and acid pretreatment method in this study yielded a reducing sugar yield of 50.11% within 30 min, surpassing the yields reported in several studies using enzymatic hydrolysis- [40] reported 16% approx. maximum reducing sugar yield after 30 h of enzymatic hydrolysis (50% (v/w) [24]. reported 42.00% reducing sugar yield after 72 h of enzymatic hydrolysis [41]. demonstrated 41.82% reducing sugar yield after 120 h of enzymatic saccharification. These results highlight the potential of enzyme-free pretreatment for efficient valorization of BSG, offering a promising alternative to traditional enzymatic methods.

To assess the effectiveness of the pretreatment on xylose release, the xylose yield was calculated with respect to the initial BSG biomass used which was 4:10 (w/v) ratio. Total xylose recovered = Concentration × Volume of hydrolysate = 44.95 mg/mL × 10 mL = 449.5 mg. This value was then divided by the initial BSG weight to give a final xylose yield = 112.38 mg/g BSG = 0.1124 g/g. This value reflects the efficiency of the enzyme-free combined pretreatment in solubilizing hemicellulose into fermentable xylose.

Quantification of xylose in released sugar via HPLC

Following the determination of total reducing sugars, HPLC analysis was employed to specifically quantify the xylose in pretreated and untreated BSG samples. The BSG hydrolysate, prepared using a 4:10 biomass-to-water ratio in a 100 mL distilled water solution without any pretreatment (heated at 110 °C for 15 min to extract soluble sugars, further filtered through Whatman No.1 filter paper, and the supernatant was centrifuged and collected for HPLC analysis), yielded 11.13 ± 0.06 g/L xylose, resulting in a yield of 27.83 mg/g BSG (1.113 g ÷ 40 g). Whereas, 48.15 ± 0.05 g/L xylose was present in BSG pretreated with combined method resulted in yield of 120.38 mg/g BSG (4.815 g ÷ 40 g). The more than 4-fold increase in xylose yield clearly demonstrates the enhanced effectiveness of the combined hydrothermal and dilute acid pretreatment in breaking down hemicellulose and releasing fermentable sugars from the BSG matrix. In reported literature the xylose concentrations from various lignocellulosic feedstocks typically range between 18 and 45 g/L under comparable conditions like [42] documented a xylose yield of 18.19 g/L from coffee husk waste using dilute acid hydrolysis. Similarly, in another study [38] the liquid portion of pretreated acid hydrolysis wheat straw waste (124.2 g/L on dry basis) generated 19.5 g/L xylose (total sugars, 28.9 g/L). Overall, the precise quantification of xylose using HPLC, ensures the accuracy of xylose recovery as compared with the colorimetric methods or less precise quantification techniques [43]. To ensure the reliability of xylose quantification, both the McCleary and McGeough method and HPLC analysis were employed. The former enabled rapid screening during pretreatment optimization, while HPLC provided precise sugar profiling. The consistency between the two methods supports the accuracy of the observed results.

Study of inhibitory compounds in pretreated BSG hydrolysate using GC-MS

The BSG hydrolysate after combined pretreatment was further analyzed for inhibitory compounds using GC-MS. The results of this study are shown in Fig. 6; Table 2. The identification of inhibitory compounds in the pretreated brewer's spent grain (BSG) hydrolysate is a critical aspect of optimizing xylitol production.

The chromatogram (Fig. 6) illustrates the compounds detected in the pretreated BSG.

Among the compounds listed in Table 2, furfural derivatives (2-furanethanol, furan), levoglucosenone, phenolic compounds such as 4pyridinol-1-oxide, and long-chain fatty acids (n-hexadecenoic acid, oleic acid) have been identified as inhibitors with substantial effects for downstream fermentation processes [44]. The GC-MS analysis of BSG pretreated by a combination of hydrothermal and acid hydrolysis revealed the presence of several compounds with the potential to impede the downstream fermentation of xylose to xylitol conversion. Notably, furfural derivatives (2-furanethanol, furan) and levoglucosenone are well-established inhibitors of microbial metabolism particularly in the fermentation of xylose to xylitol as they are capable of disrupting cellular integrity [45]. The presence of phenolic compounds viz. 4-pyridinol-1-oxide underscores the release of lignin-derived moieties during pretreatment, which can further hinder fermentation due to their disruptive effects on

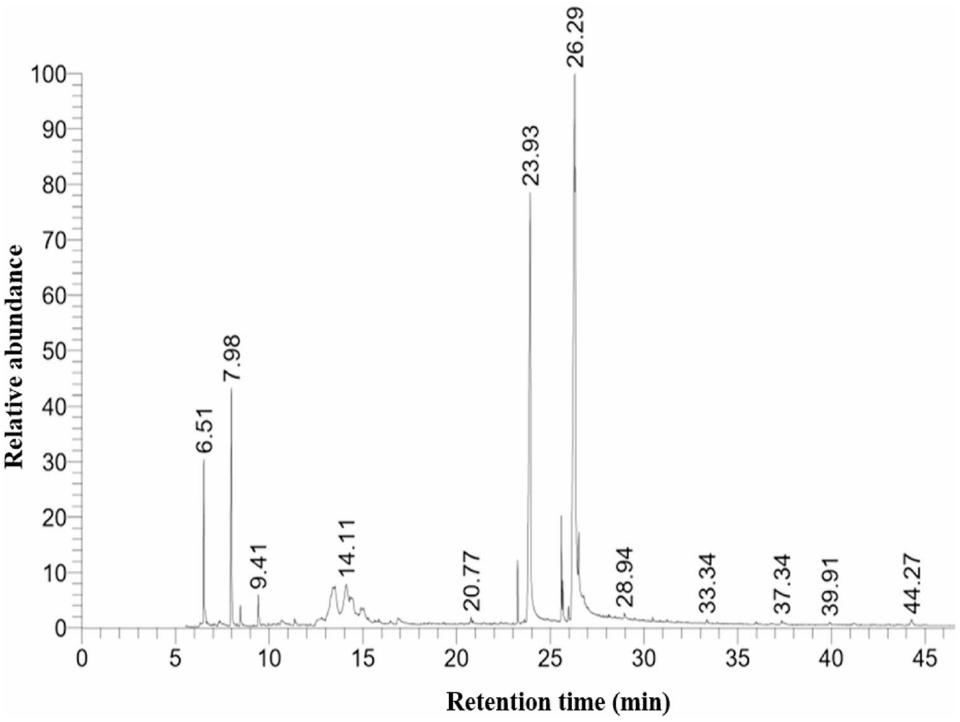


Fig. 6 GC-MS chromatogram of inhibitory compound detected in BSG hydrolysate obtained after combined pretreatment with hydrothermal followed by acid hydrolysis (1% H_2SO_4)

Table 2 GC-MS identification of compounds present in pretreated BSG hydrolysate and their potential relevance to downstream fermentation

Compound Name	RT (min)	Probability (%)	Area (%)	Molecular Formula	Molecular Weight (g/mol)
2-Furanethanol, á-methoxy-(S)−5.76	6.51	27.26	5.76	C7H10O3	142
4-Pyridinol-1-oxide	6.51	13.24	5.76	C5H5NO2	111
3-Aminopyrazine 1-oxide	6.51	12.21	5.76	C_4H_5N3O	111
Dihydro-3-methylene-5methyl-2 furanone	7.98	63.86	9.91	C6H8O2	112
Furan	7.98	17.06	9.91	C_4H_4O	68
Levoglucosenone	9.41	95.30	1.29	C6H6O3	126
2-Furanmethanol	9.41	2.55	1.29	C5H6O2	98
á-D-Ribopyranoside, methyl	13.40	46.43	2.68	C6H12O5	164
à-d-Ribopyranoside, methyl	13.51	29.93	1.80	C6H12O5	164
à-d-Ribopyranoside, methyl	14.11	43.29	2.09	C6H12O5	164
Hexadecenoic acid, methyl ester	23.26	60.05	2.13	C17H34O2	270
n-Hexadecenoic acid	23.93	69.74	24.94	C16H32O2	256
Methyl 9-cis,11-trans-octadecadienoate	25.59	12.42	3.14	C19H34O2	294
9,12-Octadecadienoic acid (Z, Z)	26.29	18.34	40.67	C18H32O2	280
Oleic Acid	28.94	26.34	1.67	C18H34O2	282

cell membranes [46, 47]. Furthermore, the detection of long-chain fatty acids (n-hexadecenoic acid, oleic acid) suggests a potential for antimicrobial activity, posing an additional challenge for efficient xylitol production. This analysis highlights the crucial considerations for optimizing pretreatment strategies. Additionally, detoxification methods and the selection of appropriate xylitol producing microorganisms with higher tolerance to the identified inhibitors could prove effective in overcoming these fermentation obstacles [48].

Use of pretreated BSG in xylitol production by Pichia fermentans

In this study, the shake flask fermentation of pretreated BSG was carried out by supplementing the BSG in place of carbon source in the xylitol production medium used by [19] using seed culture of *Pichia fermentans*. The fermentation was carried out at a pH of 5.5 and a temperature of 30 °C with continuous agitation at 150 RPM for 24 h. No additional carbon source was provided, as the pretreated BSG hydrolysate itself served as the substrate. Xylitol production in the fermentation broth was quantified using HPLC and 6.13 ± 0.05 g/L xylitol production was observed after 24 h fermentation.

However, the xylitol yield from the untreated BSG hydrolysate was recorded as 1.13 ± 0.05 g/L. This comparative analysis highlights the significance of pretreated BSG over untreated hydrolysate in enhancing xylose release, which subsequently leads to higher xylitol production. The conversion of xylose to xylitol rate was reported to be 12.73% (6.13 xylitol/48.15 xylose (g/L) * 100%) in BSG pretreated by combined method and fermented by *Pichia fermentans* [7]. reported a xylitol concentration of 24.0 g/L using a fed-batch strategy with *Candida tropicalis* after 48 h fermentation. Whereas [49], employed *Candida tropicalis* to obtain a yield of 5.88 g/L in 48 h fermentation and Debnath et al. (2024) further validated the potential of BSG as a substrate by producing 6.2 g/L xylitol using *Candida parapsilosis* 96 h fermentation timeframe. These findings highlight the versatility of different microbial strains in converting BSG into xylitol and suggest that further optimization of fermentation conditions and strain selection could lead to even higher yields and improved process efficiency. While this preliminary study provides valuable insights into the potential of utilizing pretreated BSG for xylitol production, it is evident that further optimization is essential to achieve commercially viable yields. By focusing on the identified research directions, future studies can contribute to the development of a more efficient and sustainable process for xylitol production from BSG.

Conclusion

The present study demonstrates the potential of enzyme-free pretreatment of BSG for xylose production, highlighting its economic advantages. The proximate analysis and FTIR confirmed BSG's rich xylan content, establishing it as a suitable substrate for xylose production. The hydrothermal method combined with acid hydrolysis, devoid of expensive enzymes, successfully released a significant xylose concentration of 48.15 ± 0.05 g/L, providing an economically viable solution for industrial applications. *Pichia fermentans* was identified as a promising yeast strain capable of converting xylose to xylitol in the BSG hydrolysate during fermentation and resulted in 6.13 ± 0.05 g/L xylitol after 24 h. This finding provides a basis for further optimization of the fermentation process to enhance xylitol production. The enzyme-free nature of the process significantly reduces costs, making it scalable and sustainable, thus contributing to the broader goals of a circular bioeconomy by valorizing BSG as a resource.

Authors' contributions

Conceptualization, S.M., D.K. and V.K; validation, V.K., W.A. and D.K.; formal analysis, D.K., V.K., W.A. and R.V, data curation, S.M.; writing-original draft preparation, S.M; writing— review and editing, S.M.; visualization, S.M.;

supervision, W.A., V.K., D.K., and R.K. All authors have read and agreed to the published version of the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

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