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ORIGINAL ARTICLE

Optimization of sequential alkali/acid pretreatment of corn cob for xylitol production by *Debaryomyces nepalensis*

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Abstract

A two-step sequential dilute alkali/acid pretreatment process was developed to improve the breakdown of the lignocellulosic structure of corn cob. Uniform design was used to optimize the hydrolysis process and multiple regression models were developed. Alkali hydrolysis of corn cob at optimum conditions of 1.81% (w/v) NaOH concentration and 5 (w/v) solid:liquid ratio for 90 min resulted in 82.03% lignin removal. The biomass on further dilute acid pretreatment at the optimized conditions of 6% (w/v) H_2SO_4 and 5 w/v solid:liquid ratio for 15 min resulted in 74% xylose yield. The xylose-rich acid hydrolysate was used as a substrate to produce xylitol by *Debaryomyces nepalensis* NCYC 3413. It resulted in a xylitol yield of 0.467 g/g of xylose and a xylitol titer of 21.15 g/L. The changes in the physical and chemical structure of the corn cob due to the sequential alkali/acid pretreatment were analyzed by SEM, XRD, FTIR, and TGA.

Keywords Lignocellulose pretreatment · Sequential pretreatment · Xylitol · *Debaryomyces nepalensis* · Corn cob · Uniform design optimization

1 Introduction

Xylitol is a 5-carbon sugar alcohol with many applications ranging from food and beverage to nutraceuticals and pharmaceutical industries [1]. With sweetness relative to sucrose and 40% of its calorific value, xylitol is an ideal sugar substitute for health-conscious and diabetic patients [2]. Xylitol is commercially produced via chemical route by hydrogenating pure xylose using Raney nickel/Al_2O_3 as a catalyst, which is an energy-intensive and environmentally unfriendly process [3]. The biological route of xylitol production can be carried out in milder conditions and has a minimal environmental impact [4]. In order to have an economical and eco-friendly process, fermentable sugars from agricultural residues as a substrate for fermentation have become a source of interest.

Lignocellulosic biomass (LCB) has attracted much attention over the last few decades as a cheaper and abundantly available source of fermentable sugars. LCBs are extensively investigated for their bioconversion into fuels, food ingredients, pharmaceuticals, and many other value-added products. LCB comprises of three major components viz., lignin, hemicellulose, and cellulose. Some of the most widely studied and commonly used LCBs are sugarcane bagasse, corn cob, rice straw, wheat straw, sorghum straw, brewer's spent grain, etc. India alone produces over 680 metric tonnes of total LCB per annum, in which corncob accounts for 27,880 tonnes. The major challenge faced in lignocellulosic utility is the complete conversion of LCB into constituent components [5, 6]. Many studies have been reported using sequential dilute acid/alkali pretreatment to recover monosaccharides from various LCBs [7–11].

Most previous studies on sequential pretreatment with acid and alkali used dilute acid hydrolysis as the first step, followed by alkali pretreatment with glucose recovery as the primary target. Acid hydrolysis as a first step breaks hemicellulose effectively but also hydrolyze acid soluble fraction of lignin which may hinder fermentation of the hydrolysate to value-added products without detoxification. It also produces other inhibitory products like acetic acid, furfural, and 5-hydroxymethyl furfural (5-HMF). Alkali pretreatment,

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known to remove 60–70% lignin, is used to delignify the biomass [11].

Using alkali hydrolysis as the first step removes lignin from the lignocellulosic waste reducing the structural integrity of the agrowaste, making it susceptible to breakdown on further pretreatment. Acid hydrolysis of alkali-pretreated biomass results in an acid hydrolysate rich in xylose with minimal inhibitory compounds like acid soluble lignin and acetic acid. However, dilute acid hydrolysis may still produce inhibitors such as furfural and 5-hydroxymethylfurfural (HMF) based on severity of the process, affecting the xylitol fermentation efficiency and yield. In order to maximize lignin removal in alkali pretreatment and maximize xylose yield and minimize furfural yield in acid hydrolysis, statistical optimization of the process is necessary. Uniform design (UD), a fractional factorial design developed by Fang et al. [12]. It is a space-filling design where a complete combination of parameters is replaced with fewer combinations spread uniformly over the parameter range [13]. The ability of *Debaryomyces nepalensis* NCYC 3413 to ferment hemicellulose hydrolysate to produce xylitol has been reported previously. The previously reported work demonstrated xylitol production by *D. nepalensis* from hemicellulose hydrolysate of various agrowastes like wheat straw, rice straw, sugarcane bagasse, and corn cob. Corn cob was chosen as LCB due to the high xylose yield compared to rice straw, wheat straw, and sugarcane bagasse [14].

In this study, the optimal conditions for alkali/acid sequential treatment were determined and acid hydrolysate from the process was successfully used to produce xylitol using *D. nepalensis*.

2 Materials and methods

2.1 Biomass and chemicals

Corncob was procured locally from Ambattur Industrial Estate (Chennai, India), sieved to obtain particles with sizes ranging from 2.0 to 2.8 mm using 6–8 mesh (B.S.S.) and stored in a sealed container at room temperature for further use. Sodium hydroxide and sulfuric acid were procured from VWR International. All other chemicals were procured from SRL Chemicals, India.

2.2 Design of experiments

The tables for DoE, based on the number of parameters and range of each parameter, are given on the website (https://www.math.hkbu.edu.hk/UniformDesign/). In this study, we used UD ($U_{12}(6^3)$) to construct a factorial experiment design with 3 independent variables, 6 levels, and 12 runs to optimize the conditions for alkali and acid hydrolysis.

2.3 Alkali pretreatment

Alkali pretreatment was done using different concentrations of NaOH (1–6% w/v), solid to liquid ratio of corn cob to NaOH solution (5–10 w/v), and reaction time (15–90 min). Alkali pretreatment was carried out in glass bottles. Reaction mixture was autoclaved at 121 ℃ and 15 psi [15]. After completion of alkali hydrolysis, the hydrolysate was filtered through Whatman filter paper 1. The filter cake was washed twice with half the filtrate volume with 0.2% acid before subjecting to acid hydrolysis. One set of unwashed biomass samples was also used for acid hydrolysis to study the effect of biomass washing on the process. All experiments were performed in triplicates.

2.4 Acid pretreatment

Acid pretreatment was done by subjecting the alkali-pretreated biomass to different acid concentrations (1–6% w/v), solid:liquid ratio of pretreated corn cob to H_2SO_4 (5–10 w/v), and time (15–90 min). UD ($UD_{12}(6^3)$) with the same six-level factorial experiment design with 12 runs was used. After pretreatment at 121 ℃ and 15 psi [15] in a glass bottle in an autoclave, the samples were filtered through a Whatman filter paper 1. The biomass was washed and dried overnight and the filtrate was neutralized using NaOH pellets. After neutralization, the hydrolysate was centrifuged to remove precipitates and stored at 4 ℃ until further use. The hydrolysate obtained from optimized conditions (solid:liquid ratio, acid concentration, and reaction time) was concentrated at 100 ℃ to increase the xylose concentration 5 folds using a heating mantle. The concentrated hydrolysate was then cooled and centrifuged to remove precipitates before fermentation. The hydrolysate was used as obtained and after fivefold concentration to study the effect of concentrating the hydrolysate on fermentation All experiments were performed in triplicates.

2.5 Microorganism, maintenance, and inoculum preparation

Debaryomyces nepalensis NCYC 3413 was maintained on a solid YPP medium (pH 7.0) containing 10 g/L yeast extract, 20 g/L peptone, and 5 g/L pectin. Cultures were incubated for 24 h at 30 °C. One loopful of culture from the YPP agar plate was transferred into 50 mL YPD seed media in a 250 mL Erlenmeyer flask containing 10 g/L yeast extract, 20 g/L

peptone, and 20 g/L glucose. The seed culture was incubated for 12 h at 30 °C and 180 rpm in a rotary shaker incubator.

2.6 Medium and fermentation conditions

The concentrated hemicellulose hydrolysates were analyzed for xylose content using HPLC. The xylose content was made up to 50 g/L by adding pure xylose. As mentioned in the previous study, other macronutrients and micronutrients were added to support cell growth [14]. The pH of the production medium was adjusted to 6.0 using 3 N phosphoric acid. Eight percent (v/v) of seed inoculum from 12 h old YPD seed culture was taken. The flasks were incubated at 30 °C and 180 rpm for 120 h in a shaker incubator. Samples were collected at 24 h intervals for 5 days and analyzed for biomass and metabolites. All experiments were performed in triplicates.

2.7 Analytical methods

The samples were centrifuged at 2600 × g for 5 min, washed twice, and resuspended in water for measuring absorbance at 600 nm. The biomass titer in gram cell dry weight per liter (CDW) was determined using the correlation CDW = 0.33 × A_{600nm} × dilution factor [14]. The concentrations of glucose, xylose, arabinose, xylitol, glycerol, acetic acid, HMF, and furfural were estimated by HPLC (Jasco Inc., Japan) equipped with Aminex HPX-87H column (Bio-Rad, Richmond, USA) using refractive index (RI) detector. The column was maintained at 45 °C. 0.01 N H_2SO_4 at a flow rate of 0.6 mL/min was used as the mobile phase. The samples were filtered through a 0.2 µm filter before injection. The xylose yield and furfural yields were calculated by the following formulae.

$$\text{Xylose yield (g/g of corn cob)} = \frac{\text{Amount of xylose in acid hydrolysate (g)}}{\text{Amount of corn cob (g)}}$$

$$\text{Furfural yield (g/g of corn cob)} = \frac{\text{Amount of furfural in acid hydrolysate (g)}}{\text{Amount of corn cob (g)}}$$

The biomass composition in terms of lignin content and sugars at each step of pretreatment was determined by a two-step acid hydrolysis protocol established by the National Renewable Energy Laboratory (NREL) [16]. The lignin content in the alkali hydrolysate was estimated by measuring absorbance at 280 nm using kraft lignin as standard for calibration curve [14]. The lignin removal on w/w of corn cob was calculated by the following formula

$$\text{Lignin removal (g/g of corn cob)} = \frac{\text{Amount of lignin in hydrolysate (g)}}{\text{amount of corn cob used for hydrolysis (g)}}$$

The lignin removal percent after alkali hydrolysis was estimated by the following formula.

$$\text{Lignin removal (\%)} = \frac{\text{Amount of lignin in alklai hydrolysate (g)}}{\text{Amount of lignin in corn cob (g)}} \times 100$$

Live cell count of the yeast cells in the media was done by using hemocytometer. The cells were diluted to appropriate concentration and 10 µL of sample was loaded onto a hemocytometer for cell counting. 100 µL of sample was mixed with 100 µL of methylene blue stain (0.1 mg/mL stock dissolved in 2% sodium citrate solution). The dye mixed sample were incubated for 10 min before loading onto the hemocytometer for cell counting [17]

The live cell count was calculated by the following formula

$$\text{Live cell count(cells/mL)} = \text{no. of cells} \times 10^4$$

The cell viability percent was calculated by the following formula

$$\text{Cell viability (\%)} = \frac{\text{number of live cells}}{\text{number of live cells} + \text{number of dead cells}} \times 100$$

2.8 Characterization of LCB before and after pretreatment

Scanning electron microscopy (SEM) was used to analyze the microstructural changes and surface characteristics of the pretreated biomass. All samples were sputter-coated with gold for imaging by SEM. Images were captured at 20 kV (FEI-Quanta FEG 200F) at × 500 magnification. The crystalline nature of corncob samples was studied by obtaining XRD patterns on an X-ray diffractometer (Bruker D8 Discover AXS Powder X-ray Diffractometer) with Cu kα ($\lambda = 1.5406$ Å) at 2.2 kW, 40 kV, and 40 mA. The scanning range, 2θ value, ranged from 8° to 60°. The crystallinity index (CrI) of the biomass was measured by the formula mentioned below

$$CrI = \frac{I_{002} - I_{101}}{I_{002}} \times 100$$

The I_{002} and I_{101} are the intensities of the XRD spectrum at 2θ values of 22.53° and 16.5°, respectively [18, 19].

The thermogravimetric analysis of raw, alkali-pretreated, and sequential alkali/acid–pretreated corncob was carried out in a TGA analyzer with 50 mg samples of corn cob in a

nitrogen environment. Mass loss was plotted as a function of temperature from 25 to 800 °C with a heating rate of 10 °C /min.

To investigate the changes in functional groups in corncob, the Fourier transform infrared spectroscopy (FTIR) spectra of raw, alkali-pretreated, and sequential alkali/acid–pretreated corncobs were recorded on Bruker Alpha Platinum ATR IR. The scans were done from a wavenumber of 4000 to 498 cm^{-1} in absorbance mode. The instrument had a resolution of 2 cm^{-1}.

2.9 Statistical analysis

UD analysis and optimum parameter determination were done using MINITAB® v.2020.1.0 commercial software (Minitab Inc., USA). Analysis of variance (ANOVA) was performed at a confidence level of 95% (p-value < 0.05). The experimental data were fitted into the following second-order quadratic equation to evaluate the effect of each independent variable on the response(s).

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{23} x_2 x_3 + \beta_{31} x_3 x_1 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 \quad (1)$$

where Y is the predicted response; β_0 is model constant; x_1, x_2, and x_3 are independent variables; β_1, β_2, and β_3 are linear coefficients; β_{12}, β_{31}, and β_{23} are cross-product coefficients; and β_{11}, β_{22}, and β_{33} are the quadratic coefficients.

2.10 Xylitol yield and purity measurement

The cells after fermentation were separated from supernatant by vacuum filtration through 0.22-micron filter membrane. The supernatant was treated with 15 g/L activated carbon for 1h at 30 °C and 180 rpm. The activated carbon was removed by vacuum filtration through filter membrane. The clarified broth was concentrated in a rotary vacuum evaporator @ 60 °C to increase the concentration of xylitol in the broth. The concentrated broth was taken in a 15 mL centrifuge tube, seeded with 1 g/L of pure xylitol for nucleation, and incubated in ice for 15 min. The centrifuge tubes were incubated at 0 °C for crystallization for 3 days. The crystals were separated by filtration after 3 days. The crystals were washed with 99% ethanol and dried. 50 mg of this crystal sample was dissolved and analyzed for xylitol content in HPLC. The same procedure was followed for both fermentations using pure xylose and hemicellulose hydrolysate. The yield and degree of purity of xylitol in crystal were calculated as per the following formulae.

$$\text{Xylitol yield } (\frac{g}{g}) = \frac{\text{Amount of xylitol in crystal (g)} - \text{amount of pure xylitol crystal seeded (g)}}{\text{Amount of xylitol in broth (g)}}$$

$$\text{Degree of purity } (\%) = \frac{(\text{Mass of xylitol in crystal} - \text{mass of pure xylitol crystal added})}{\text{Total mass of crystals}} \times 100$$

Table 1 Independent variables and responses for alkali pretreatment optimization using uniform design ($U_{12}(6^3)$)

Runs	X_1 (NaOH concentration) (%)	X_2 (solid:liquid) (w/v)	X_3 (time) (min)	Y_1 (lignin removed) (g/g corncob)	
				Actual	Predicted
1	−1 (≡1)	−0.6 (≡6)	−0.2 (≡45)	0.2233 ± 0.0116	0.2215
2	0.6 (≡5)	0.2 (≡8)	1 (≡90)	0.1754 ± 0.0170	0.1746
3	0.6 (≡5)	−0.2 (≡7)	−1 (≡15)	0.2270 ± 0.0279	0.2260
4	0.2 (≡4)	1 (≡10)	0.6 (≡75)	0.2121 ± 0.0111	0.2123
5	−0.6 (≡2)	0.2 (≡8)	−1 (≡15)	0.1942 ± 0.0088	0.1961
6	−0.2 (≡3)	1 (≡10)	−0.6 (≡30)	0.2275 ± 0.0287	0.2256
7	−0.2 (≡3)	−1 (≡5)	0.6 (≡75)	0.2877 ± 0.0149	0.2888
8	−1 (≡1)	0.6 (≡9)	0.2 (≡60)	0.2354 ± 0.0022	0.2362
9	1 (≡6)	−0.6 (≡6)	0.2 (≡60)	0.2067 ± 0.0022	0.2061
10	1 (≡6)	0.6 (≡9)	−0.2 (≡45)	0.1561 ± 0.0022	0.1576
11	0.2 (≡4)	−1 (≡5)	−0.6 (≡30)	0.2779 ± 0.0021	0.2784
12	−0.6 (≡2)	−0.2 (≡7)	1 (≡90)	0.2631 ± 0.0248	0.2630

*The number within brackets indicate uncoded values while those outside indicate the coded values of the variables. $U_{12}(6^3)$ indicate uniform design with 12 runs, 6 levels, and 3 independent variables

3 Results and discussions

3.1 Optimization of alkali pretreatment

Although dilute acid hydrolysis can yield xylose, it also produces toxic by-products like acid-soluble lignin [20] and acetic acid. Performing alkali pretreatment as the first step reduces acid soluble lignin from the biomass resulting in higher xylose yields and lesser inhibitors from acid hydrolysis. As shown in Table 1, the UD experiments were conducted to optimize the reaction parameters of alkali pretreatment. Lignin removal varied from 0.1561 g/g corn cob to 0.2877 g/g corn cob for the alkali pretreatment. The lignin removal was maximum (0.2877 g/g corn cob) with 3% NaOH concentration, 5 w/v solid:liquid ratio, and 75-min reaction time (run 7). Lignin removal decreased to 0.2121 g/g corn cob at 4% NaOH concentration, 10 w/v solid:liquid ratio, and 75-min reaction time (run 14). This indicated that a lower solid:liquid ratio gave higher lignin removal (g/g corn cob). This is due to the fact that lower solid loading resulted in a higher surface area for reaction [21]. Moodley et al. reported similar findings where 80.5% and 73% lignin removal were observed in sugarcane leaves waste by steam-assisted salt alkali pretreatment and microwave-assisted salt- alkali pretreatment. In that study, they found that increasing solid loading beyond 10% did not increase reducing sugar yields [21]. Similarly, comparing run 5 and run 12 showed that a longer reaction time resulted in higher lignin removal. Higher concentrations of NaOH did not improve lignin removal; a low concentration of NaOH was sufficient for effective lignin removal. Similar findings were reported by Gabhane et al., where they found that increasing NaOH concentration beyond 1% did not significantly increase the reducing sugar yields [22].

For alkali pretreatment, the experimental data was fitted to a second-order polynomial as shown in Eq. (2) for the response of lignin removal (Y_1), which was employed for regression analysis:

$$Y_1 = 0.22422 - 0.023729X_1 - 0.027479X_2 + 0.006810X_3 - 0.02406X_1 \times X_2 - 0.00191X_2 \times X_3 - 0.04010X_1 \times X_3 - 0.02900X_1^2 + 0.02823X_2^2 \quad (2)$$

where independent variables X_1, X_2, and X_3 represent the concentration of NaOH, solid:liquid ratio, and reaction time, respectively.

The ANOVA table indicated that the model was significant ($p < 0.001$). The value of the coefficient of determination (R^2) of model lignin removal ($R^2 = 0.99$) showed good agreement between experimental data and the model, indicating that the model could be used to optimize alkali pretreatment corncob (Table 2).

Figure 1a, b, and c show the response surface plots for alkali pretreatment optimization. It can be observed from the pairwise response surface plot of solid:liquid ratio versus alkali concentration (Fig. 1a) that as the solid:liquid ratio increases, the lignin removal decreases at a constant alkali concentration. As alkali concentration increases beyond 2%, the lignin removal goes down. In the pairwise plot of time versus solid:liquid ratio (Fig. 1b), a drop in the lignin removal can be observed with a decrease in time. Similarly, a drop in lignin removal can be seen with an increase in the solid:liquid ratio. In the plot of alkali concentration versus time (Fig. 1c), the lignin removal increased with increase in time at constant alkali concentration. At constant time, lignin removal increased up to 2% alkali concentration after which it decreased. The optimal conditions for lignin removal were modeled using the MINITAB software, which predicted about 30 g lignin removal per 100 g corncob. The optimal conditions of 1.81% NaOH with a 5 w/v solid:liquid ratio of biomass treated for 90 min at 121 ℃ resulted in maximum delignification of 0.299 g of lignin/g corncob. The lignin in the residue reduced from 37.9% (w/w) to 18.99% (w/w) corresponding to an 82.03% lignin removal. Yang et al. reported 88% lignin removal from sequential acid/alkali-treated corncob with 0.075 g of NaOH/g corncob [23]. Lee and coworkers applied sequential acid and alkali treatment on corn stover, resulting in about 86% delignification [11] and these results are in comparison with our results with one-step alkali hydrolysis. Kaur and Kuhad achieved 55% delignification of sequential acid/alkali-treated rice straw with 4% (w/v) NaOH concentration at 121℃ for 30 min [24]. 70% delignification of empty palm fruit bunch fiber was reported upon being treated with 10 M NaOH at room temperature for 4 h [8]. On comparing the reports by these

Table 2 Analysis of variance of lignin removal during alkali pretreatment

Source	DF	Adj SS	Adj MS	*F*-value	*p*-value
Regression	8	0.05077	0.006346	27.57	0
X_1	1	0.00923	0.00923	40.1	0
X_2	1	0.012372	0.012372	53.76	0
X_3	1	0.000756	0.000756	3.28	0.081
$X_1 \times X_2$	1	0.002813	0.002813	12.22	0.002
$X_2 \times X_3$	1	0.000018	0.000018	0.08	0.784
$X_1 \times X_3$	1	0.007804	0.007804	33.91	0
X_1^2	1	0.003622	0.003622	15.74	0
X_2^2	1	0.00343	0.00343	14.9	0.001
Error	0.00343	0.00343	14.9		
Lack-of-fit	3	0.000049	0.000016	0.06	0.978
Pure error	24	0.006165	0.000257		
Total	35	0.056984			

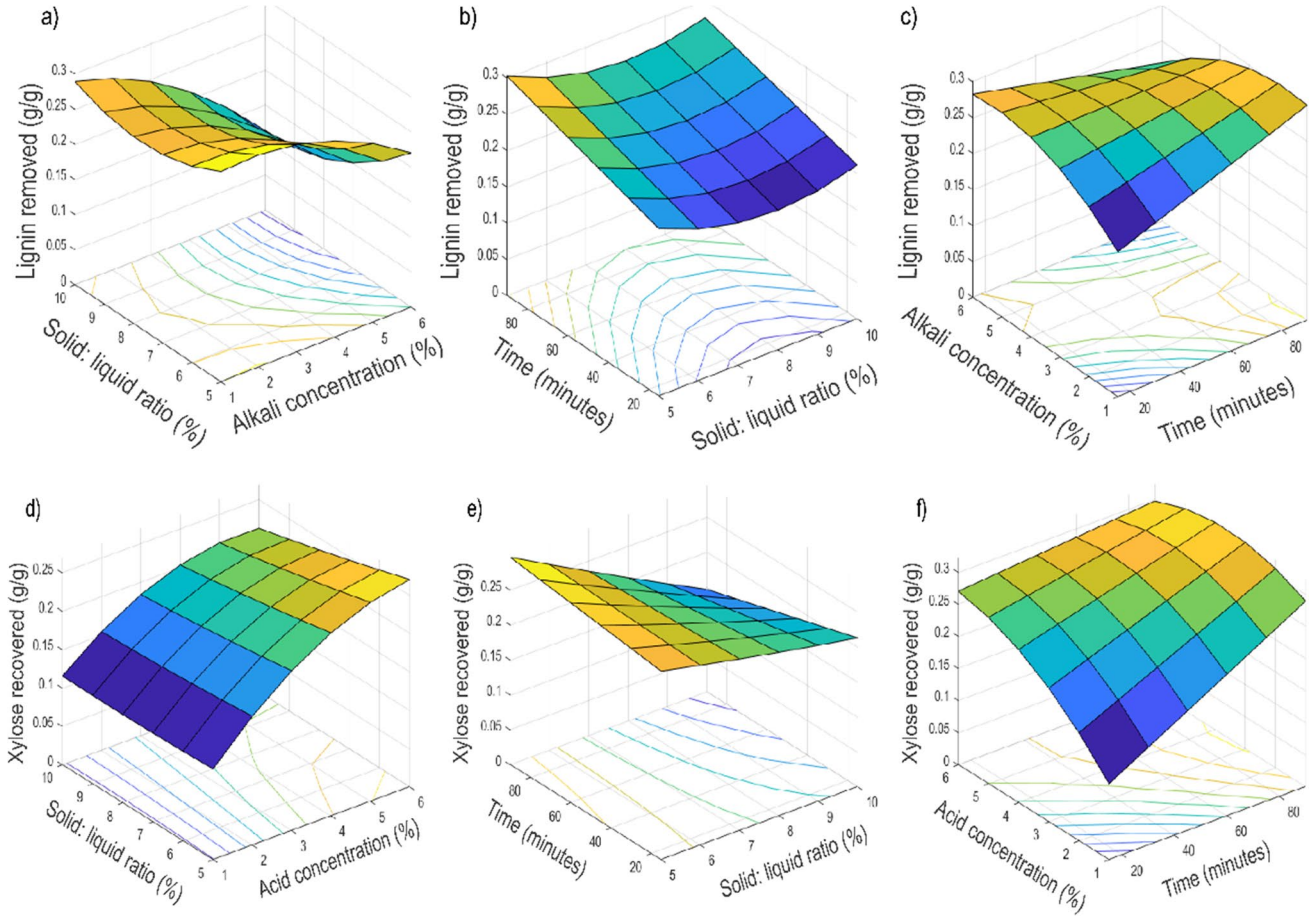


Fig. 1 Response surface plots for alkali pretreatment of corncob and acid pretreatment of alkali-pretreated corncob. **a** NaOH concentration versus solid:liquid ratio. **b** Solid:liquid ratio versus time. **c** Time versus NaOH concentration. **d** H_2SO_4 concentration versus solid:liquid ratio. **e** Solid:liquid ratio versus time. **f** Time versus H_2SO_4 concentration

Table 3 Independent variables, xylose and furfural yield responses of sequential alkali/acid pretreatment of corncob using uniform design ($U_{12}(6^3)$)

Runs	X_1 (acid concentration) (%)	X_2 (solid:liquid) (%)	X_3 (time) (min)	Y_2 (xylose yield) (g/g corncob)		Y_3 (furfural yield) (g/g corncob)	
				Actual	Predicted	Actual	Predicted
1	−1 (≡1)	−0.6 (≡6)	−0.2 (≡45)	0.179 ± 0.010	0.1840	0.0012 ± 0.0003	0.0011
2	0.6 (≡5)	0.2 (≡8)	1 (≡90)	0.226 ± 0.011	0.2362	0.0284 ± 0.0024	0.0264
3	0.6 (≡5)	−0.2 (≡7)	−1 (≡15)	0.233 ± 0.005	0.2456	0.0026 ± 0.0015	0.0044
4	0.2 (≡4)	1 (≡10)	0.6 (≡75)	0.197 ± 0.010	0.2027	0.0016 ± 0.0002	0.0049
5	−0.6 (≡2)	0.2 (≡8)	−1 (≡15)	0.159 ± 0.002	0.1646	0.0006 ± 0.0000	0.0027
6	−0.2 (≡3)	1 (≡10)	−0.6 (≡30)	0.192 ± 0.009	0.1940	0.0026 ± 0.0004	0.0004
7	−0.2 (≡3)	−1 (≡5)	0.6 (≡75)	0.291 ± 0.012	0.3055	0.0188 ± 0.0052	0.0213
8	−1 (≡1)	0.6 (≡9)	0.2 (≡60)	0.166 ± 0.010	0.1791	0.0007 ± 0.0001	0.0009
9	1 (≡6)	−0.6 (≡6)	0.2 (≡60)	0.268 ± 0.011	0.2692	0.0253 ± 0.0016	0.0267
10	1 (≡6)	0.6 (≡9)	−0.2 (≡45)	0.205 ± 0.022	0.2108	0.0073 ± 0.0056	0.0069
11	0.2 (≡4)	−1 (≡5)	−0.6 (≡30)	0.260 ± 0.004	0.2620	0.0094 ± 0.0012	0.0070
12	−0.6 (≡2)	−0.2 (≡7)	1 (≡90)	0.275 ± 0.007	0.2725	0.0171 ± 0.0019	0.0163

*The number within brackets indicate uncoded values while those outside indicate the coded values of the variables. $U_{12}(6^3)$ indicate uniform design with 12 runs, 6 levels, and 3 independent variables

Table 4 Analysis of variance of xylose and furfural yields during acid hydrolysis

Source	DF		Adj SS		Adj MS		*F*-value		*p*-value	
	Y_2	Y_3	Y_2	Y_3	Y_2	Y_3	Y_2	Y_3	Y_2	Y_3
Regression	8	8	0.064984	0.003293	0.008123	0.000412	60.82	40.75	0	0
X_1	1	1	0.009931	0.000121	0.009931	0.000121	74.36	11.96	0	0.002
X_2	1	1	0.000001	0.000181	0.000001	0.000181	0.01	17.87	0.929	0
X_3	1	1	0.009276	0.000189	0.009276	0.000189	69.45	18.75	0	0
X_1^2	1	1	0.003981	0.000037	0.003981	0.000037	29.81	3.71	0	0.065
X_{22}	1	1	0.000012	0.000116	0.000012	0.000116	0.09	11.52	0.765	0.002
$X_1{*}X_2$	1	1	0.000893	0.000121	0.000893	0.000121	6.68	11.98	0.015	0.002
$X_2 \times X_3$	1	1	0.006315	0.000157	0.006315	0.000157	47.28	15.53	0	0.001
$X_1 \times X_3$	1	1	0.002638	0.000204	0.002638	0.000204	19.76	20.15	0	0
Error	27	27	0.003606	0.000273	0.000134	0.00001				
Lack-of-fit	3	3	0.000916	0.000125	0.000305	0.000042	2.72	6.8	0.067	0.002
Pure error	24	24	0.002691	0.000147	0.000112	0.000006				
Total	35	35	0.06859	0.003566						

authors, it can be interpreted that the delignification efficiency also depends on the composition of the lignocellulosic biomass.

3.2 Optimization of acid hydrolysis of alkali-pretreated corncob

Acid pretreatment of lignocellulose results in an almost complete breakdown of hemicellulose to its constituent monomeric sugars and partial digestion of cellulose and lignin [25]. The acid hydrolysis was optimized to recover maximum xylose with minimum furfural in the acid hydrolysate. Acid hydrolysis experiments were carried out at various conditions as given in the UD (Table 3).

The xylose yield varied from 0.159 g/g corn cob to 0.291 g/g corn cob while the furfural yield varied from 0.0006 g/g corn cob to 0.0284 g/g corn cob. The xylose yield was maximum (0.291 g/g corn cob) at 3% acid concentration, 5 (w/v) solid:liquid ratio, and 75-min reaction time at run 7 but the corresponding furfural yield (0.0188 g/g corn cob) was also higher. In run 4, with a reaction time of 75 min, solid:liquid ratio of 10 (w/v), and 4% acid concentration, both xylose yield (0.197 g/g corn cob) and furfural yield (0.0016 g/g corn cob) reduced drastically. This indicated that the furfural yield reduced significantly with an increase in the solid:liquid ratio. Similarly, by comparing runs 2 and 3, we find a significant increase in furfural yield with an increase in treatment time without a significant xylose yield increase.

This result is in agreement with results obtained by Marzialetti et al., where acid hydrolysis of loblolly pine with dilute acid at 150 °C over a range of 15 to 120 min yielded higher furfural and 5-HMF with an increase in reaction time. They concluded that the reaction time resulted in an increase in furfural and 5-HMF and lesser monosaccharides [26]. Sun and Lin, in their research, demonstrated an increase in glucose yields from bamboo pulp with an increase in reaction time up to 3.5 h. This was because the temperature of that study was in the range of 55–75 °C. An increase in temperature accelerates the degradation of monosaccharides to furfural and 5-HMF [27]. On the same lines, it can be interpreted from the data that an increase in acid concentration results in an increase in xylose and furfural yield (runs 1 and 9). Jiang et al. also found an increase in xylose and furfural recovery on pretreatment of corn cob with acid concentration varying from 1 to 10% [28]. Rafiqul et al., reported a maximum yield of 90.6% xylose under optimized conditions of 124 ℃, 3.26% acid, 8 g/g solid: acid, and 80-min reaction time from Meranti wood sawdust [29]. Similarly, 90% hemicellulose recovery was achieved from rice straw under 121 ℃, 3% v/v acid, 10 w/v biomass, and 30 min of reaction parameters [24]. Zhang et al. observed a maximal xylose yield of 91.3% from an empty palm fruit bunch using a combination of 0.5% (w/v) of H_2SO_4 and 0.2% (w/v) of H_3PO_4 at 160 ℃ with a solid loading of 20 ml/g for 10 min [5]. The variation in the xylose recovery is indicative of the difference in the lignocellulosic composition of the biomass and the pretreatment conditions.

The second-order polynomial Eqs. (3) and (4) were generated for xylose and furfural yields, respectively, using the model experiments of acid hydrolysis. The equations which were employed for regression analysis are as follows:

$$Y_2 = 0.23429 + 0.02645X_1 - 0.03839X_2 + 0.02624X_3 - 0.01357X_1 \times X_2 - 0.02333X_2 \times X_3 - 0.03609X_3 \times X_1 - 0.03043X_1^2 + 0.00168X_2^2 \quad (3)$$

$$Y_3 = 0.01344 + 0.00710X_1 - 0.00644X_2 + 0.00950X_3 - 0.00499X_1 \times X_2 - 0.00648X_2 \times X_3 + 0.00569X_3 \times X_1 - 0.00295X_1^2 - 000520X_2^2 \quad (4)$$

where Y_2 and Y_3 represent xylose and furfurals yield (g/g corn cob) respectively and the independent variable X_1, X_2,

Table 5 Comparison of present study with recent studies on sequential pretreatment of lignocellulose

Authors	Salient features	Substrates	Products	Reaction conditions	Comparison with present study	References
Si et al	A comparative study of acid, alkali, sequential acid/alkali and sequential alkali acid pretreatment was made	*Miscanthus*	Pentoses and hexoses	Acid and alkali concentration varied from 0.25 to 8% Alkali hydrolysis: 150 rpm; 50 °C; 2 h Acid hydrolysis: 121 °C; 20 min; 150 rpm; 50 °C; 2 h	73.14% lignin removal with 2% NaOH and 1% H_2SO_4 compared to 82.03% in this study	[15]
Li et al	Sequential alkali/acid hydrolysis with focus on ferulic acid and p-coumaric acid	Sugarcane bagasse	p-coumaric acid and ferulic acid	Alkali hydrolysis:4% NaOH; 2% solid:liquid ratio; 80 °C; and 25 °C; 24 h Acid hydrolysis: 7.3% HCl and 1,4-dioxane mixture; 2% solid liquid ratio; 90 °C; 4 h	54.146% lignin removal compared to 82.03% of present study with higher NaOH concentration and reaction time at lower temperature	[31]
Das et al	Comparison of alkali, sequential alkali/acid and sequential alkali/steam explosion pretreatment on structure of pine lignin	Pine sawdust	pine lignin	Alkali hydrolysis: 5% NaOH; 10% solid liquid ratio; 90 °C; 1 h Acid hydrolysis: 7.5% H_2SO_4; 10% solid:liquid ratio; 150 °C; 30 min	0.99% reduction in klason lignin compared to 64.56% in present study	
Argun et al	Optimization of pretreatment parameters for production of glucose and 5-HMF from wet paper towels	Wet paper towel	Glucose; 5-HMF	Alkali hydrolysis: 16% NaOH; 119 °C; 74 min Acid Hydrolysis: 5% H_2SO_4; 135 °C; 2 h	Very high NaOH concentration compared to 1.81% in this study. Longer acid hydrolysis time since target was 5-HMF	[32]
Qin et al	Different pretreatment strategies were compared for efficient process	Brewer's spent grain	Protein extraction	Alkali hydrolysis: 0.44% NaOH; 50 °C; 2 h; 200 rpm; 5% solid:liquid ratio Acid hydrolysis: 5.63% H_2SO_4; 25 °C; 1 h and 121 °C; 1 h	57.75% lignin and 46.71% lignin and klason lignin removal compared to 82.03% and 64.56 in this study	[33]
Sewsynker-Sukai et al	sequential pretreatment with sodium phosphate dodecahydrate and dilute H2SO4	Corn cob	reducing sugars	Alkali hydrolysis: 12.7% Na_3PO_4,12 H_2O; 14.49% solid:liquid ratio; 121 °C; 30 min Acid hydrolysis: 1.04% H_2SO_4; 121 °C; 30 min		[34]
Sanchez et al	Comparative study of dilute acid, dilute alkali and sequential acid/alkali and alkali/acid pretreatments	Wheat straw	Sugars	Alkali hydrolysis: 3% NaOH; 7.83% solid:liquid ratio; 32.5 min; 121 °C Acid hydrolysis: 0.75% H_2SO_4; 121 °C; 60 min	85.05% lignin removal compared to 82.03% of this study but higher NaOH and lower time	[35]
Hoşgün et al	Comparative study of alkali hydrolysis, acid hydrolysis, liquid hot water pretreatment, and their combinations	Hazelnut shells	Cellulose/glucose	Alkali hydrolysis: 2.25% NaOH; 60 °C; 60 min; 10% solid:liquid ratio Acid hydrolysis: 0.5% H_2SO_4; 120 °C; 60 min; 10% solid:liquid ratio	55.5% lignin removal compared to 82.03% in present study	[36]

Table 6 Composition analysis of hydrolysate from one-step acid hydrolysis and sequential alkali/acid hydrolysis at different concentrations

Components (g/L)	Acid hydrolysate	Sequential alkali/acid hydrolysate		
		1×	2×	5×
Glucose	0.87 ± 0.25	0.39 ± 0.01	0.99 ± 0.01	1.19 ± 0.01
Xylose	12.3 ± 0.64	10.16 ± 0.23	17.35 ± 0.23	41.02 ± 1.35
Acid soluble lignin	1.80 ± 0.03	0.86 ± 0.02	2.4 ± 0.04	2.38 ± 0.15
Acetic acid	1.47 ± 0.31	1.07 ± 0.05	4.44 ± 0.25	3.76 ± 0.23
5-HMF	0.09 ± 0.06	0.09 ± 0.01	0.27 ± 0.05	0.43 ± 0.01
Furfural	0.05 ± 0.02	0.02 ± 0.002	0.03 ± 0.0005	0.04 ± 0.001

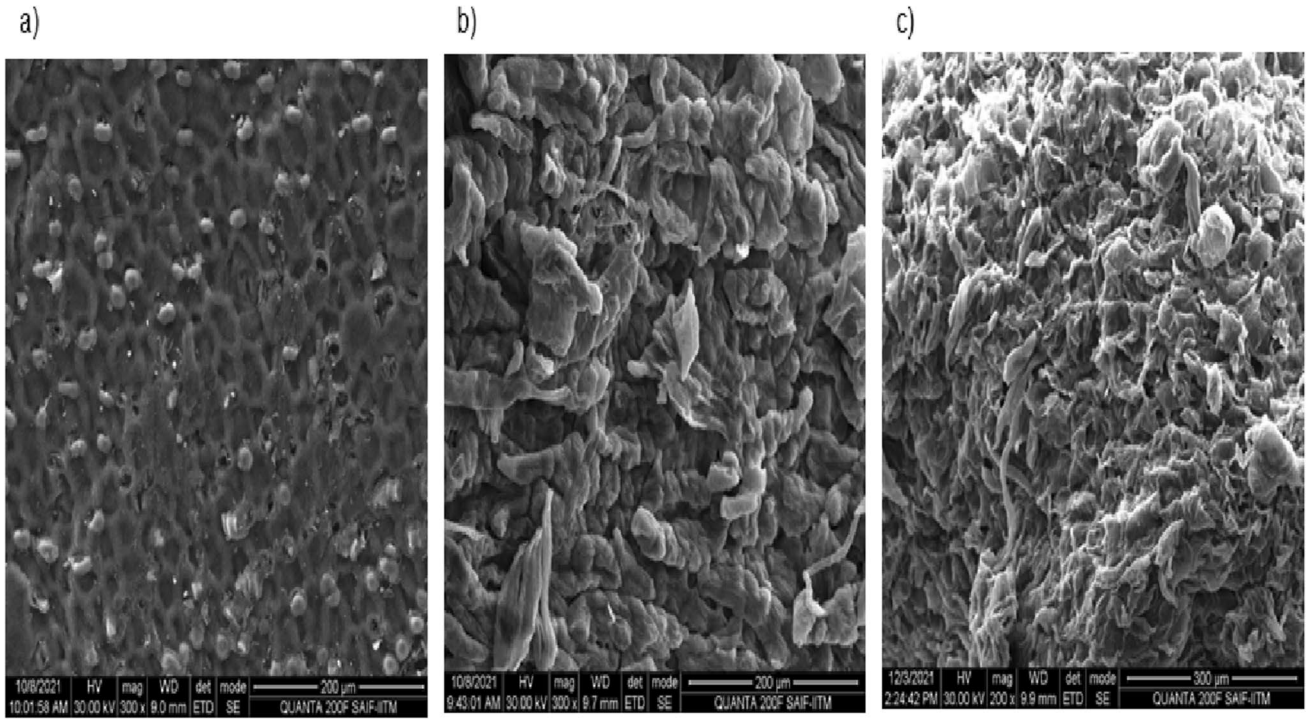


Fig. 2 SEM image of **a** untreated corncob, **b** alkali-pretreated corncob, and **c** sequential alkali/acid–pretreated corncob

and X_3 represent the concentration of H_2SO_4, solid:liquid ratio, and reaction time respectively.

The ANOVA models for xylose yield and furfural yield shown in Table 4 indicated that the models were significant ($p < 0.05$). The values of coefficient of determination (R^2) of the models for xylose yield ($R^2 = 0.98$) and furfural yield ($R^2 = 0.96$) shows good agreement between the experimental data and the model.

The response surface plots for the obtained responses during acid hydrolysate (Fig. 1d, e, f) indicate higher xylose content at higher levels of both H_2SO_4 and time. However, a higher concentration of furfural was observed at reaction times beyond 60 min. Similarly, higher xylose yield was noted at a low solid loading of 5 (w/v). In the pairwise plot of solid:liquid ratio versus acid concentration (Fig. 1d), it can be seen that the optimal point lies towards low solid:liquid ratio and high acid concentration. In the plot of time versus solid:liquid ratio (Fig. 1e), a sharp decline in xylose recovery can be observed as the solid:liquid ratio increases. In the case of time, the decline in comparatively shallower. This indicates a more significant effect of solid:liquid ratio on xylose recovery than treatment time. Similarly, in the acid concentration versus time plot (Fig. 1f), a sharp decline in xylose recovery can be seen with a reduction in acid concentration compared to time. MINITAB software was used to predict optimal conditions for maximum xylose yield with low furfural release in the acid hydrolysate.

The predicted optimized conditions for acid hydrolysis were H_2SO_4 concentration of 6% (v/v), solid:liquid ratio of 5 w/v and reaction time of 15 min. Under optimized conditions, the xylose yield (74%) achieved was 0.201 g/g corncob with unwashed biomass samples compared to

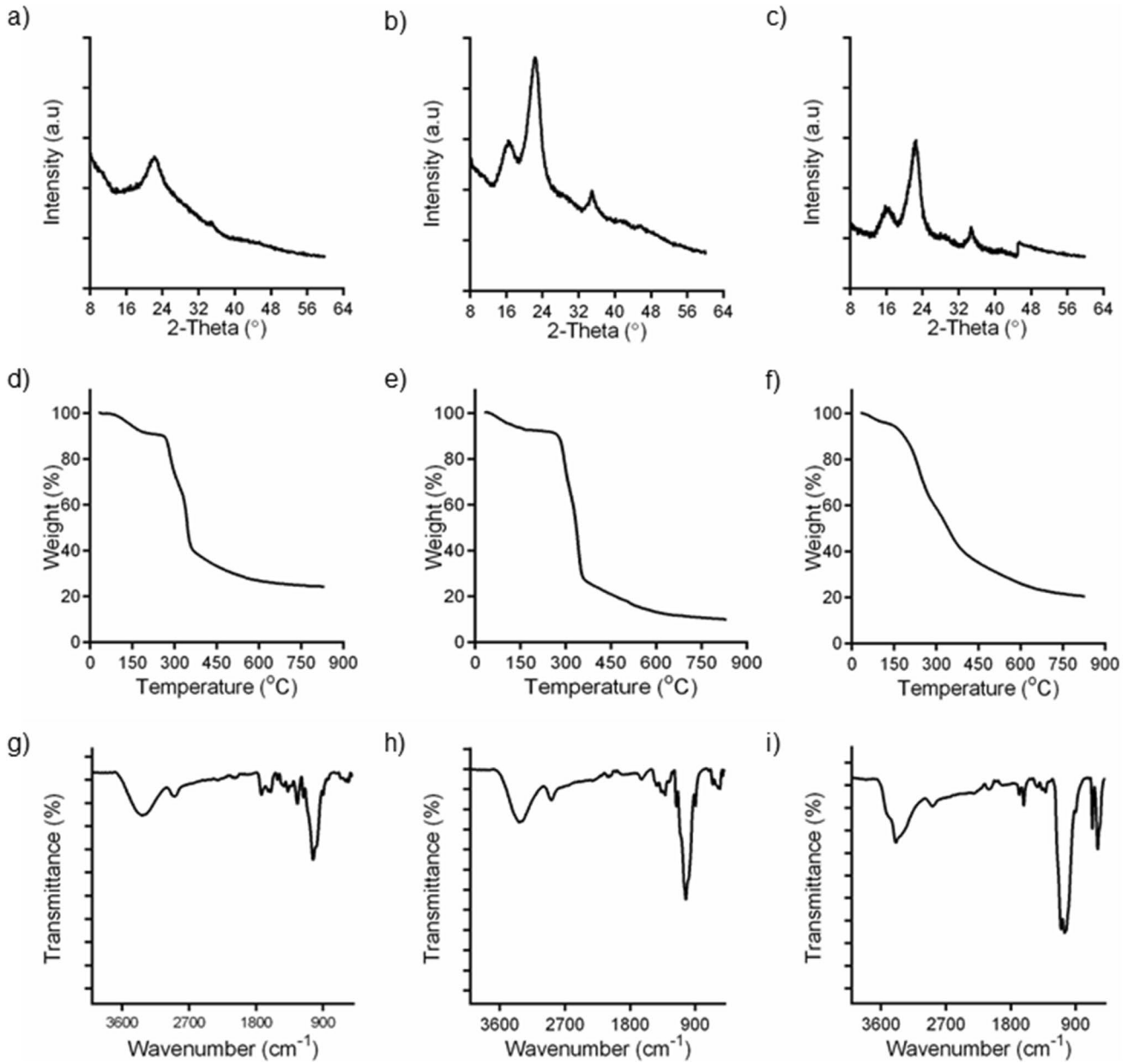


Fig. 3 Characterization of raw corncob, alkali-pretreated corncob, and sequential alkali/acid–pretreated corncob. X-ray diffraction (XRD) spectrum of **a** untreated corncob, **b** alkali-pretreated corncob, and **c** sequential alkali/acid–pretreated corncob; thermogravimetric (TG) analysis of **d** untreated corncob, **e** alkali-pretreated corncob, and **f** sequential alkali/acid–pretreated corncob; and Fourier transform infrared (FTIR) spectrum of **g** untreated corncob, **h** alkali-pretreated corncob, and **i** sequential alkali/acid–pretreated corncob

the predicted outcome of 0.2705 g xylose/g corncob. The formation of the salt due to the presence of NaOH in the unwashed biomass could have reduced effective acid concentration resulting in a lower xylose yield than the predicted value. When washed twice with 0.2% acid, acid hydrolysis of alkali-pretreated corncob yielded 0.2549 g xylose/g corncob. Karuna et al. reported an improvement in the enzymatic hydrolysis of alkali-pretreated rice straw on washing the biomass with water indicating that washing removed residual alkaline lignin solution from the biomass and provided more accessibility for enzymes [30]. For all further experiments, sequential alkali/acid pretreatment was done after washing the alkali-pretreated biomass with 0.2% acid solution.

In an another study with sequential alkali/acid hydrolysis reported 73.14% lignin removal in *Miscanthus* by pretreating the biomass with 2% NaOH at 50 °C temperature for 2 h and 1% H_2SO_4 for 20 min at 121 °C and then 50 °C for 2 h [15]. Table 5 shows comparative analysis of present study with recent studies on sequential pretreatment of lignocellulose. A comparative composition analysis of hydrolysate

from one-step acid hydrolysis from previous study [14] and the sequential alkali/acid hydrolysis is given in Table 6. When compared with one-step acid hydrolysate, the soluble lignin and acetic acid content is less in sequential alkali/acid hydrolysate.

3.3 Characterization of corncob biomass

3.3.1 Scanning electron microscopy

SEM analysis of raw, alkali-pretreated, and sequential alkali/acid–pretreated samples revealed the changes in the structure of corncob due to the pretreatment processes (Fig. 2). The microstructure of raw untreated corncob was compact and rough (Fig. 2a). About 50% of the lignin is present in the middle lamellae, which holds the tissue clusters together [37]. On alkali pretreatment, lignin which forms the outermost layer of lignocellulose, was removed to a great extent loosening the tight structure (Fig. 2b). The SEM image had a lot of porous structure indicative of lignin removal. This is also supported by the fact that the hydrolysate after alkali hydrolysis had only minor quantities of sugars. This is in agreement with SEM images obtained by Su et al., where the biomass was treated with alkaline H_2O_2 [19]. Similar findings were reported on two *Pennisetum* sp. grass pretreatment with 1% NaOH [37] and alkaline pretreatment of corn stover [38]. Sequential alkali/acid pretreatment further broke down the compact structure as the hemicellulose was broken down into simpler sugars (Fig. 2c). The hemicellulose hydrolysis led to further opening of the microstructure. A similar loosening of the structure was observed by Zhu et al. when they pretreated corncob with formic acid [39]. Dilute acid pretreatment of poplar increased the porosity of the lignocellulosic sample [40]. A sequential acid/alkali treatment of poplar wood resulted in a similar breakdown of lignocellulosic structure to form porous microstructures [9]. A rough microsurface with piths and pores was reported in a similar study on sequential acid/alkali pretreatment of sugarcane bagasse [10].

3.3.2 X-ray diffraction studies

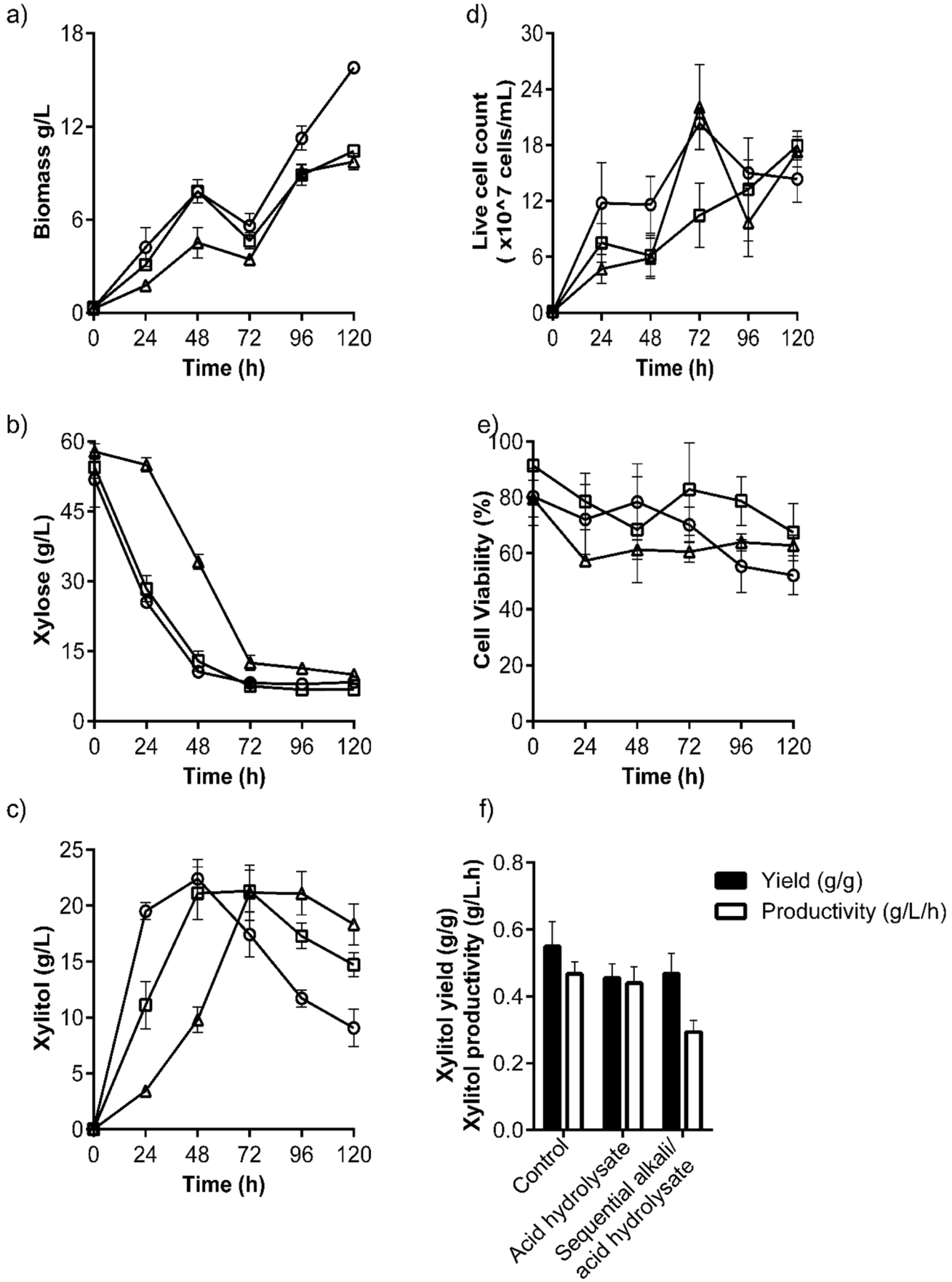
The CrI gives the ratio of crystalline to amorphous fractions in biomass [22]. The crystallinity index of untreated, alkali-pretreated, and sequential alkali/acid–pretreated corncob was calculated based on the intensity obtained from the XRD spectrum. The crystallinity index of untreated, alkali-pretreated, and sequential alkali/acid–pretreated corncob was 22.09%, 35.49%, and 45.10%, respectively. The XRD spectrum of untreated corncob (Fig. 3a), alkali-pretreated corncob (Fig. 3b), and sequential alkali/acid–pretreated corncob (Fig. 3c) are shown in Fig. 3. The crystallinity of the lignocellulose is due to the presence of crystalline cellulose, while lignin and hemicellulose form the amorphous part [22]. The increase in CrI of biomass on pretreatment is due to the removal or degradation of amorphous components such as hemicellulose, lignin, amorphous cellulose, starch, and protein [9]. Alkali pretreatment of corncob resulted in lignin removal, thereby increasing the CrI of the pretreated biomass. Similarly, acid pretreatment of alkali-pretreated corncob resulted in the removal of hemicellulose fraction, further increasing relative crystalline cellulose content and thus the CrI. The relative increase in crystallinity on the removal of lignin and hemicellulose was also reported by Xu et al. [41]. Liu et al. while comparing various pretreatment methods for corn stover also observed a similar increase in crystallinity index due to the removal of noncellulosic fractions [42]. Sequential acid/alkali pretreatment also resulted in an incline in the CrI of pretreated biomass [9].

3.3.3 Thermogravimetric analysis

The thermogravimetric analysis provides the mass loss as a function of temperature. The initial peak from 25 to 100 °C indicates the removal of residual moisture from the biomass. The thermal degradation temperature of hemicellulose was 250–300 °C, cellulose was 300–350 °C, and lignin was 300–500 °C [43]. Figures 3d, e, and f represent the weight % versus temperature profile of untreated, alkali-pretreated, and sequential alkali/acid–pretreated corncob, respectively. The initial degradation between 180 and 300 °C can be observed in untreated, alkali-pretreated, and sequential alkali/acid–pretreated samples. The second degradation between 300 and 350 °C can be attributed to lignin degradation to volatile compounds [37]. This is visible in the TGA curve for untreated samples (Fig. 3d) but not significant in alkali-pretreated (Fig. 3e) and sequential alkali/acid–pretreated samples (Fig. 3f) due to the removal of a majority of the lignin fraction. Thermal gravimetry of corn stem, husk, and leaves with aqueous ammonia [42] and dilute acid–alkali pretreatment of poplar biomass also resulted in similar TGA curves [9].

3.3.4 FTIR spectroscopy

FTIR spectroscopy gives insights into functional groups present in the biomass and the changes in the functional groups within the biomass due to various pretreatment. The peaks at 1245 cm^{-1} and 1726 cm^{-1} are attributed to ether and ester linkages between lignin and carbohydrates [23]. The peak at 1511 cm^{-1} signifies aromatic skeletal stretching [44]. From the FTIR spectrum (Fig. 3g, h, i), after alkali pretreatment of corncob, a decrease in the intensity of these peaks can be found, signifying the removal of lignin. The peak at 1055 cm^{-1} is attributed to C–O–C in cellulose [45]. The intensity of this peak can be seen increasing from untreated



◂**Fig. 4** Fermentation summary of *Debaryomyces nepalensis* NCYC 3413 in unconcentrated acid hydrolysate and sequential alkali/acid hydrolysate. **a** Biomass growth, **b** xylose consumption, **c** xylitol production, **d** live cell count, **e** cell viability, and **f** xylitol yield and productivity. ⊖: control with pure xylose; ⊟: acid hydrolysate; △: sequential alkali/acid hydrolysate

(Fig. 3g) to alkali-pretreated (Fig. 3h) to sequential alkali/acid–pretreated corncob (Fig. 3i) signifying an increase in the proportion of cellulose in the sample. Denanath grass and hybrid napier grass on NaOH pretreatment showed similar changes in the FTIR profile to that of alkali pretreatment in the present study [37]. Sequential acid–alkali pretreatment of corncob [23] had FTIR spectrum similar to sequential alkali/acid pretreatment shown reported in this study. The FTIR results were complementary to the compositional analysis of the biomass done in the present study.

3.4 Xylitol production from acid hydrolysate

The sequential alkali/acid hydrolysate and one-step acid hydrolysate were used for fermentation of xylose to xylitol with the halotolerant yeast *Debaryomyces nepalensis* NCYC 3413. Semisynthetic media with pure xylose as sole carbon source was used as control. The xylose concentration in each case was made up to 50 g/L. The biomass growth (Fig. 4a),

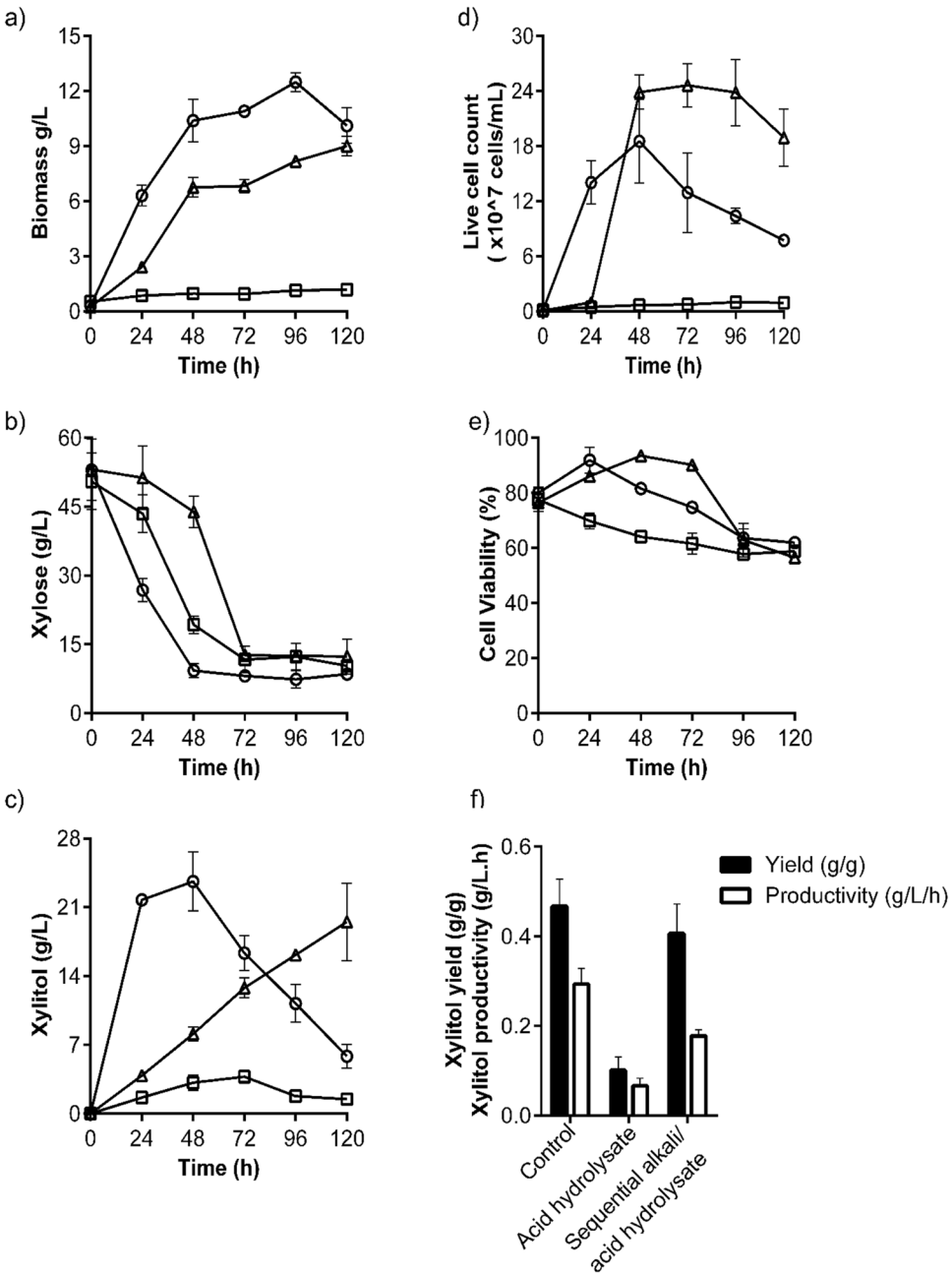


Fig. 5 Fermentation summary of *Debaryomyces nepalensis* NCYC 3413 in fivefold concentrated acid hydrolysate and sequential alkali/acid hydrolysate. **a** Biomass growth, **b** xylose consumption, **c** xylitol production, **d** live cell count, **e** cell viability, and **f** xylitol yield and productivity. ⊖: control with pure xylose; ⊟: acid hydrolysate; △: sequential alkali/acid hydrolysate

Table 7 Comparison of studies on xylitol production from lignocellulose hydrolysate

LCB substrates	Pretreatment methods	Pretreatment conditions	Xylose concentration (g/L)	Acetic acid (g/L)	Furfural (g/L)	5-HMF (g/L)	Organism	Xylose consumption (%)	Xylitol titre (g/L)	Ref
Corn Cob	Acid hydrolysis	0.5% H_2SO_4 + 1.5% H_3PO_4, 128 °C; 1 h; 25 S:L ratio	29.54 ± 0.14*	4.02 ± 0.51	0.19	0.33	*Kluyveromyces marxianus* CICC 1727–5	73	28.03 ± 0.41	[50]
Corn Cob	Sequential Hydrothermal/ ezymatic pretreatment	25 g corn cob/100 g water, 205 °C, 1 h	36.8	-	-	-	*Saccharomyces cerevisiae* PE-2-GRE3	-	72#	[51]
Typha latifolia (aquatic weed)	Sequential alkali/ acid	2% $NaHSO_3$, room temperature, 18 h, 2% H_2SO_4, 121 °C, 1 h	13.80 ± 0.30	-	0.50 ± 0.08 (total furans)		*Candida tropicalis* JFH5	73.6	6.15 ± 0.17#	[49]
Corn cob	Acid hydrolysis	1% H_2SO_4, 125 °C, 1 h, 15 S:L ratio	21.67	2.42	0.54	0.3	*Candida tropicalis* CCTCC M2012462	95	38.8	[48]
Corn cob	Acid hydrolysis	1% H_2SO_4, 121 °C, 40 min, 10 S:L ratio	65*	2.26 ± 0.15	1.29 ± 0.06	0.26 ± 0.01	*Cyberlindnera (Williopsis) saturnus*	83.5	29.1	[52]
Corn cob	Acid hydrolysis	0.5% HNO_3, 121 °C, 30 min, 10 S:L ratio	16.2	1.88	-	-	*Candida tropicalis* ATCC 96,745	-	0.6 g/g xylose#	[53]
Corn Cob	Sequential alkali/ acid hydrolysis	1.81% NaOH, 121 °C, 90 min, 5 S:L ratio, 6% H_2SO_4, 121 °C, 15 min, 5 S:L ratio	10.16 ± 0.23	1.07 ± 0.05	0.02 ± 0.002	0.09 ± 0.01	*Debaryomyces nepalensis* NCYC 3413	81.73 ± 1.94	21.15 ± 2.02	This study

*Concentration after concentrating the hydrolysate; #detoxified hydrolysate media

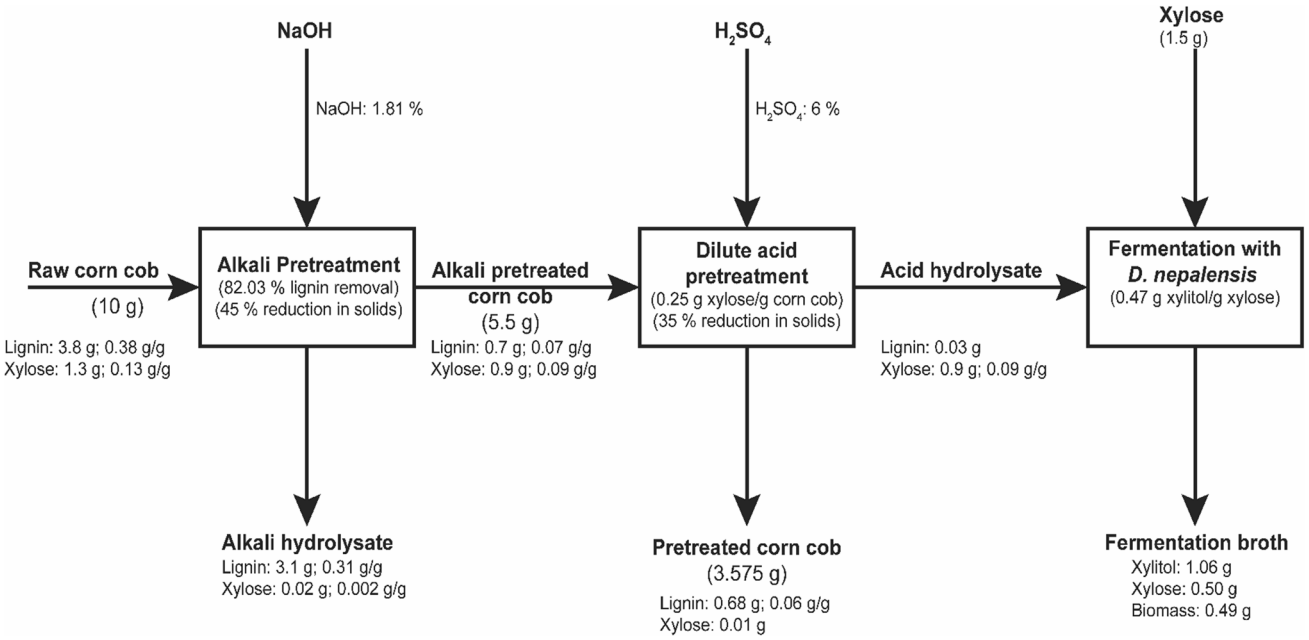


Fig. 6 Mass balance of sequential alkali/acid pretreatment process of corncob and fermentation of hydrolysate to xylitol

xylose consumption (Fig. 4b), xylitol production (Fig. 4c), live cell count (Fig. 4d), cell viability (Fig. 4e), and xylitol yield and productivity (Fig. 4f) are given in Fig. 4.

The acid hydrolysate and sequential alkali/acid hydrolysate gave comparable titers of xylitol viz. 21.11 ± 1.92 g/L and 21.15 ± 2.02 g/L (Fig. 4c). The xylose consumption in sequential alkali/acid hydrolysate was slower compared to control and acid hydrolysate (Fig. 4b). This may be due to presence of salts in the media used for adjusting the pH of the hydrolysate. Since adjusting the pH of hydrolysate causes salt formation, it may lead to osmotic stress causing an increase in lag phase for the yeast to adjust to the media conditions. This is also supported by biomass profile (Fig. 4a) and live cell count (Fig. 4d) which shows slower growth in sequential alkali/acid hydrolysate compared to control and acid hydrolysate. The cell viability profile (Fig. 4e) also decreased initially in sequential alkali/acid hydrolysate compared to control and acid hydrolysate. The xylitol yields in sequential alkali/acid were comparable to acid hydrolysate and lesser than control. The decrease in yield in comparison to control is due to presence of inhibitory compounds. The productivity was higher in acid hydrolysate compared to alkali hydrolysate and this also can be attributed to the longer lag phase in sequential alkali/acid hydrolysate compared to acid hydrolysate. The maximum xylitol yield obtained in sequential alkali/acid hydrolysate was 0.467 ± 0.049 g/g xylose and maximum xylitol productivity of 0.29 ± 0.028 g/L.h (Fig. 4f).

In order to study the effect of concentrating hydrolysate before fermentation, fermentation was done with sequential alkali/acid hydrolysate and acid hydrolysate after concentrating them by fivefold. The biomass growth, xylose consumption, xylitol production, cell viability profile, live cell count, and xylitol yield and productivity are shown in Fig. 5. The biomass growth (Fig. 5a), xylitol yield (Fig. 5f), xylitol titers (Fig. 5c), live cell count (Fig. 5d), and cell viability (Fig. 5e) were lesser in acid hydrolysate after concentrating fivefold compared to sequential alkali/acid hydrolysis. This may be attributed to the increased presence of 5-HMF and furfural and some lignin degradation products in the media [14]. The xylose consumption rates of control with pure xylose and sequential alkali/acid hydrolysate were similar (Fig. 5b), but a significant difference was observed in the xylitol titers (Fig. 5c). This stark difference in xylitol production may be due to higher salt content in the hydrolysate media than in semisynthetic media. *D. nepalensis* is known to thrive in a hyperosmotic environment by producing glycerol and other polyols [14]. This may have reduced the flux of xylose towards xylitol production resulting in reduced xylitol production.

It was found that biomass growth and cell viability was higher in 5 × concentrated hydrolysate (Fig. 5a and e) compared to 1 × concentrated hydrolysate (Fig. 4a and e) but the corresponding xylitol titers were comparatively lower in 5 × concentrated hydrolysate (Fig. 5c) compared to 1 × concentrated hydrolysate (Fig. 4c). The concentration of hydrolysate will result in increase in concentration of salts in the hydrolysate making the media hyperosmotic. This would have caused the cells to produce glycerol and other polyols to thrive in the media reducing the flux of xylose towards

xylitol [14]. In the case of 1 × concentrated hydrolysate, the osmotic shock would be comparatively less resulting in an increase in flux of xylose towards xylitol and decrease in flux towards other metabolites.

Candida sp. is one of the most researched species for xylitol production. Cheng et al. reported 0.32 g/g of xylitol yield from non-detoxified acid-pretreated corncob [46]. This yield was lesser to the one reported in our study. Adapted cultures of *Candida tropicalis* InaCC Y799 were reported to produce a xylitol yield of 0.56 g/g xylose and xylitol titer of 11.2 g/L [47]. The titers reported with unadapted *Debaryomyces nepalensis* NCYC 3413 were slightly higher. The xylitol titre of 21.15 g/L and xylitol yield of 0.467 g/g xylose for *Debaryomyces nepalensis* NCYC 3413 are comparable to wild type organisms studied in literature. The culture used in the experiment was unadapted to the hydrolysate media which significantly affects the biomass growth and xylitol production profiles. Ping et al. [48] in their study used non detoxified acid hydrolysate of corn cob for xylitol production using *C. tropicalis* CCTCCM2012462 obtained xylitol titre of 38.8 g/L. Goli et al. [49] reported 6.15 g/L xylitol titre and 0.65 g/g xylitol yield using *C. tropicalis* JFH5for fermenting sequential alkali/acid hydrolysate of aquatic weed. A comparison table with xylitol production from lignocellulosic biomass has been mention in Table 7. The optimization of the *D. nepalensis* for production of xylitol from sequential alkali/acid hydrolysate for better xylitol yield including growing in higher initial xylose content is still under work.

3.5 Xylitol yield

Xylitol yield and degree of purity for xylitol when fermentation was carried out using pure xylose as carbon source was 0.5 g/g and 83% respectively. However, the xylitol yield was found to be 0.18 g/g and the degree of purity was 11.41% when fermentation was carried out using lignocellulose hydrolysate. The low degree of purity and yield are a result of the presence of salts, other sugars and impurities. The degree of purity and yield can be increased by employing more downstream processing strategies like treatment with ion exchange resins or membrane separation processes. The purification and downstream processing strategy has to be devised and optimized for cost-effective xylitol production.

3.6 Mass balance of sequential alkali/acid pretreatment

Mass balance of any process is vital in understanding process efficiency and scalability. Figure 6 represents the mass balance of the sequential alkali/acid pretreatment process of corncob presented in this article and fermentation of the hydrolysate to xylitol. The first step of the sequential alkali/acid pretreatment viz. alkali pretreatment resulted in a 45% reduction in solids with 82.03% lignin removal. The alkali-pretreated biomass on acid pretreatment had a 35% reduction in the solid fraction. The xylose to xylitol conversion based on the total xylose in the process (including xylose in corn cob and pure xylose added to the media) was found to be 42.28%.

4 Conclusions

In conclusion, the present study demonstrates a two-step sequential pretreatment process with good efficiency for sequential fractionation of corncob into lignin, hemicellulose, and cellulose. This can potentially be integrated into a lignocellulose biorefinery. While the present study indicates an efficient sequential fractionation process for lignocellulose with potential biorefinery applications, the limitation in the process as to recovery/recycle of acid and base for making the process more environment friendly is still under research. The study also demonstrated the use of hemicellulosic fraction in acid hydrolysate for xylitol production by *D. nepalensis*.

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Data availability Data will be available on request.

Declarations

Ethical approval Not applicable to this study.

Competing interests The authors declare no competing interests.

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