Xylitol Production From Sugarcane Bagasse Through Ultrasound-Assisted Alkaline Pretreatment and Enzymatic Hydrolysis Followed by Fermentation

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Xylitol production from sugarcane bagasse through ultrasound-assisted alkaline pretreatment and enzymatic hydrolysis followed by fermentation

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Abstract

This study focused on the optimization of xylitol production from sugarcane bagasse by using response surface methodology (RSM). Xylitol was produced through a series of processes, firstly, optimization of ultrasound assisted mild alkaline pretreatment for the xylan extraction from sugarcane bagasse followed by enzymatic hydrolysis of xylan to xylose by enzyme β-1,4-xylanase and finally microbial fermentation of xylose to xylitol using yeast *(Candida guilliermondii)*, bacteria *(Corynebacterium glutamicum)* and their mixed culture for different time periods (0-96 h). Maximum xylan recovery of 12.059% (w/w) was observed at pretreatment; 0.73 M NaOH, 1:38.55 solid to liquid ratio and 34.77 min ultrasonication. The enzyme concentration of 400 U/g xylan at 48 h of incubation showed the highest xylose production (81.51 mg/g bagasse). Yeast *(Candida guilliermondii)* resulted in the highest xylitol yield (Yp/s= 0.43 g/g) after 72 h. This bioprocess route can contribute as a suitable alternative for chemical methods of xylitol production.

Keywords: Xylitol; xylan; response surface methodology; enzymatic hydrolysis; sugarcane bagasse

1. Introduction

Sugarcane *(Saccharum officinarum)* is one of the major tropical crops with the world's annual production of around 1.81 billion tons in the year 2015, which is presumed to reach above 2.21 billion tons by 2024, based on which sugarcane bagasse production is supposed to reach 0.6 billion tons [1, 2]. Sugarcane bagasse is the main byproduct of sugar industry which generates around 280 kg of bagasse per ton of sugarcane processing [3]. This implies the necessity of waste valorization of sugarcane bagasse [3] as lack of proper utilization and irrational disposal of biological waste pose a great environmental threat [4]. Sugarcane bagasse is a lignocellulosic material with high hemicellulose content [5]. The hemicellulose rich agroindustrial wastes are good source of D-xylose [6] which can be hydrolyzed into xylitol by microbial fermentation and enzymatic conversion [7].

Xylitol ($C_5H_{12}O_5$) refers to a polyalcohol having a hydroxyl group connected to each carbon atom in their chain. The relative sweetness of xylitol is equivalent to that of sucrose, but it exerts nearly one third low calorie content [8, 9]. Commercially, xylitol has extensive application in various sectors of pharmaceuticals, nutraceuticals, food and beverage industries due to its distinct pharmacological importance like prevention of dental cavities and ear infection in small children [10]; low glycemic index [11]; higher cooling power [12]; independent of insulin metabolic pathway [13]; and anticariogenic property [8].

The conventional acid hydrolysis method to produce xylitol involves high concentration of acid at high temperature leading to the formation of various toxic compounds hindering the fermentation and purification process [14]. Similarly, the efficiency of solo enzymatic hydrolysis to yield xylose is reduced due to the hindrance of lignin for efficient enzyme penetration into the lignocellulosic biomass [15]. Hemicellulose is strongly bound to cellulose via physical interaction and hydrogen bonding and to lignin via covalent bonding

63 that causes hindrance in the complete recovery of xylan from lignocellulosic biomass [16].
64 Hence, alkali treatment for xylan recovery can be combined with ultrasound treatment [17].
65 Mild pretreatment leading to detaching lignin, lowering cellulose crystallinity, and
66 increasing porosity of the matrix before enzymatic hydrolysis has been reported to increases
67 extraction efficiency [15]. Biotechnological methods such as fermentation and enzymatic
68 hydrolysis for D-xylose to xylitol conversion are frequently studied [4]. The utilization of
69 different strains of yeast, bacteria, and fungi have been reported for the fermentation of D-
70 xylose to xylitol. *Candida guilliermondii* and *C. tropicalis* were reported as efficient xylitol
71 producers [15]. However, bacteria and fungi are found to have lower xylitol production
72 compared to yeast [14].

73 The aim of this study was to optimize the ultrasound assisted alkaline pretreatment and
74 enzymatic hydrolysis of sugarcane bagasse for xylose recovery and to investigate the effect
75 of different starter cultures of yeast (*Candida guilliermondii*), bacteria (*Corynebacterium*
76 *glutamicum*) and their mixed culture on the fermentation of xylose rich sugarcane bagasse
77 hydrolysates to produce xylitol.

78 **2. Materials and methods**

79 **2.1 Materials**

80 Sugarcane bagasse was provided by Mitr Phol Sugar Corporation Limited, Thailand.
81 *Candida guilliermondii* (TISTR 5068) and *Corynebacterium glutamicum* (TISTR 461) were
82 acquired from Thailand Institute of Scientific and Technological Research (TISTR),
83 Thailand. Enzyme, β-1,4-Xylanase refined from *Trichoderma reesei* was obtained from CTi
84 & Science Co. Ltd., Thailand. All other chemicals used were of analytical grade.

85 **2.2 Compositional analysis of sugarcane bagasse powder**

Sugarcane bagasse was dried in an oven at 60 ± 5 °C (Memmert GmbH+Co. KG, D-91126 Schwabach, Germany) and ground with hammer mill (Polymix, PX-MFC 90 D, Kinematica AG, Switzerland) into small fine particles of mesh size 0.5 mm. The compositional analysis (Cellulose, hemicellulose, lignin, ash and extractives) of untreated raw sugarcane bagasse was done by using the gravimetric method as described by Ayeni et al. [18]. The proximate analysis of sugarcane bagasse was performed by the proximate analyzer (LECO – TGA 701, USA).

2.3 Optimization of ultrasound assisted alkaline pretreatment for xylan extraction

The design expert software (Version 10.0.0, Stat-Ease Inc, Minneapolis, MN, USA) was used to optimize the ultrasound assisted alkaline pretreatment condition for the extraction of xylan. Box-Behnken design was used to determine the effect of alkaline concentration (X_1), ultrasonication time (X_2) and solute-to-alkali solution ratio (X_3) on the extraction of xylan from sugarcane bagasse. Xylan recovery % (w/w) was determined as the response variable. The experiments were carried out in a randomized manner and the data was analyzed using a quadratic polynomial regression model as shown in equation 1.

$$Y = \beta_0 + \Sigma \beta_i X_i + \Sigma \beta_{ii} X_i^2 + \Sigma \beta_{ij} X_i X_j \quad (1)$$

Where, Y is the response variable, β_0 is constant, β_i, β_{ii} and β_{ij} are the linear, quadratic, and interactive coefficients determined by the model and X_i and X_j are the independent variables, respectively.

Following the set of experimental design as obtained from Box Behnken design, the sugarcane bagasse sample (2.5 g) was added to NaOH solution (0.0-1.0 M) at different solute to alkali solution ratio (1:10-1:40) and exposed to ultrasonication using an ultrasonic probe

reactor (UP 200S, Hielscher, Teltow, Germany) for different time intervals (10-40 min) at a fixed frequency of 24 kHz.

2.4 Determination of xylan concentration

The xylan from ultrasonicated alkaline pretreated slurry was extracted following Samanta et al. [19] with slight modifications. The slurry obtained after the ultrasonication was filtered through Whatman filter paper No. 1 and the filtrate containing xylan was adjusted to the pH 5.4 with acetic acid (2 M). Precipitate was separated by centrifugation at 6440 ×g for 20 min (Hettich, EBA 8S). Pellets of the precipitate were then oven dried at 60 °C until constant weight. The percentage true recovery of xylan from the bagasse was calculated using the equation 2.

$$True\ Recovery\ \% = \frac{Dry\ weight\ of\ extracted\ xylan\ (g)}{Weight\ of\ the\ sample\ (g)} \times 100 \quad (2)$$

2.5 FTIR analysis

Xylan extracted from the optimized condition and untreated sugarcane bagasse powder were subjected to FTIR analysis for characterization of chemical structure. Spectrum One FT-IR Spectrometer (Perkin Elmer, U.S.) was used to obtain the spectra operating at a spectral range of 4000-500 cm^{-1}.

2.6 Enzymatic hydrolysis of extracted xylan

Xylan extracted from the optimized pretreatment condition was hydrolyzed using the enzyme β-1,4-xylanase following the method as described by Akpinar et al. [20]. Xylan hydrolysis was done by incubating mixture of xylan (2% dissolved in sodium acetate buffer 50 mM, pH 5.4) and xylanase enzyme at different concentrations (10 U, 30 U, 50 U, 100 U,

129 200 U, 400 U and 800 U per g xylan) in an orbital shaker (50 °C at 250 rpm). Sample was
130 taken at regular intervals (0-72 h) for xylose analysis.

131 **2.7 Determination of xylose content**

132 Sample (1 mL) was withdrawn from incubated hydrolysates slurry at different time intervals
133 (0, 1, 2, 4, 8, 16, 24, 48, 60 and 72h), heated (95°C for 15 min) to inactivate the enzyme,
134 followed by cooling to ambient temperature (25 °C). The sample was then centrifuged (4025
135 ×g, 20 min), and filtered through Whatman filter paper no. 1 to get the clear filtrate rich in
136 xylose. Reducing sugar (xylose) concentration in the filtrate was determined by Dinitro
137 salicylic acid (DNS) method by following the method as described by Akpinar et al. [20]
138 with slight modifications. Xylose solution (0.1 mL) was added to DNS reagent (0.1 mL),
139 vortex mixed and incubated in the hot water bath (95 °C for 15 minutes) until the appearance
140 of red brown color. The mixture was cooled (25 °C) and distilled water (8 mL) was added to
141 the mixture and vortex mixed. Absorbance of this solution was noted at 540 nm by using the
142 UV-Vis spectrophotometer (UNICAM, Alva, UK). Xylose concentration (mg/mL) was
143 calculated by using the equation (A_{540nm}=0.6055x-00047, R^2=0.9989) from xylose standard
144 calibration curve.

145 **2.8 Microorganisms and inoculum preparation**

146 The microbial samples of yeast and bacteria were prepared by following the method as
147 described by Hernandez-Perez et al. [21] and Yoshitake et al. [22]. Dried yeast, *C.*
148 *guilliermondii* was rehydrated in yeast malt (YM) extract broth and streaked on the YM agar
149 plate. Similarly, dried bacteria*, C. glutamicum* were rehydrated in nutrient broth and streaked
150 on the nutrient agar (NA) plate. The plates were incubated at 30 °C for 48 h for the growth
151 of desired colonies. The yeast cells were transferred to yeast propagation medium (50 mL)
152 composed of xylose (3%), rice bran extract (4%), $(NH_4)_2SO_4$ (0.2%) and $CaCl_2.2H_2O$

(0.01%). Bacterial cells were transferred to bacterial propagation medium (100 mL, pH 6.5) containing potassium gluconate (2.4%), $(NH_4)_2SO_4$ (0.2%), KH_2PO_4 (0.1%), $MgSO_4$. $7H_2O$ (0.05%), thiamine hydrochloride (20 µg/L) and $FeSO_4.7H_2O$ (5000 µg/L). The propagation cultures were subjected to rotary shaker (150 rpm) at 30 °C for 48 h. Afterwards, the cells were separated by centrifugation at 4025 ×g for 20 min, rinsed twice with normal saline and the cell pellet was resuspended in normal saline and used as an inoculum.

2.9 Fermentation of xylose rich sugarcane bagasse hydrolysates

Sugarcane bagasse hydrolysate rich in xylose obtained from enzymatic hydrolysis was vacuum concentrated at 40 °C to increase the xylose concentration to 0.82 % (w/v). The hydrolysate was subjected to microbial fermentation utilizing *C. guilliermondii*, *C. glutamicum* and their mixed culture.

Fermentation using *C. guilliermondii,* was conducted by following the method as described by Hernandez-Perez et al. [21]. Sugarcane bagasse hydrolysates (30 mL at pH 5.5) was inoculated with initial cell biomass concentration of 1 g/L in 125 mL cotton plugged Erlenmeyer flask and incubated in an orbital shaker at 30 °C at 100 rpm. Fermentation additives used were same as that used in culture propagation medium except xylose. *C. glutamicum* fermentation was done by the method as described by Yoshitake et al. [22]. The bacterial inoculum maintained at cell concentration of 1 g/L was inoculated in gluconate medium (20 mL, pH 6.5) composed of potassium gluconate (9.6%), $(NH_4)_2SO_4$ (0.6%), KH_2PO_4 (0.1%), $MgSO_4.7H_2O$ (0.05%), thiamine hydrochloride (200 µg/L) and $FeSO_4.7H_2O$ (5 ppm) and incubated in the rotary shaker operating at 200 rpm at 30 °C for 2 days. After 2 days, 10 mL of sugarcane bagasse hydrolysates with xylose concentration (24.7 mg/mL) was aseptically transferred into the gluconate medium so that the final xylose concentration in the fermenting substrate was 0.82 % (w/v). For the mixed culture

177 fermentation, sugarcane bagasse hydrolysates (30 mL, pH 6.0) was inoculated with equal
178 proportion of bacterial and yeast inoculum suspension to maintain final gross cell
179 concentration of 1 g/L. All the fermentation additives used previously for *C. guilliermondii*
180 and *C. glutamicum* culture were added. Finally, all the fermentation flasks with three
181 different starter cultures were agitated in an orbital shaker at 30 °C at 100 rpm for a period
182 up to 96 h.

183 **2.10 Determination of xylitol**

184 The xylitol concentration was determined by using D-sorbitol/xylitol assay kit by following
185 the manufacturer's instructions (Megazyme, Ireland). For confirmatory analysis of xylitol
186 concentration, sample from each starter culture condition that yielded the highest
187 concentrations of xylitol was analyzed by high performance liquid chromatography (HPLC,
188 Dionex UltiMate 3000, Illinois, USA) (Data in Supplementary File, Figure S1) following
189 the method as described by Misra et al. [23]. HPLC was equipped with a refractive index
190 detector (RID) and a Sugar Pak I Column (300 mm×6.5 mm). Distilled water was used as a
191 mobile phase with flow rate set as 0.5 mL min^{-1}, temperature at 90 °C and sample injection
192 volume was 20.0 µl.

193 **2.11 Fermentation parameters**

194 For each fermentation, periodic sampling was done at an interval of 24, 48, 72 and 96 h to
195 determine the xylose uptakes and xylitol production. Xylose uptake was calculated by DNS
196 method and xylitol concentration was analyzed by D-xylitol/sorbitol assay kit and later
197 reassured by HPLC for optimum fermentation condition of each culture strains. Yeast cell
198 count was measured by hemocytometer whereas bacterial and mixed culture growth was
199 monitored by measuring optical density at 600 nm. Xylose consumption (%), xylitol yield
200 (Yp/s), and xylitol recovery (%) were calculated using the following equations:

$$Xylose\ consumption\ (\%) = \left(\frac{Initial\ xylose\ concentraion - Final\ xylose\ concentration}{Intial\ xylose\ concentration}\right) \times 100 \quad (3)$$

$$Xylitol\ yield\ \left(\frac{g}{g}\right) = \frac{xylitol\ concentration\ in\ the\ broth\ at\ the\ end\ of\ fermentation}{Initial\ xylose\ concentration\ - Final\ xylose\ concentraion} \quad (4)$$

$$Xylitol\ recovery\ (\%) = \left(\frac{Xylitol\ concentration\ in\ the\ broth\ at\ the\ end\ of\ fermentation}{Mass\ of\ xylan\ incurred\ for\ xylitol\ formation}\right) \times 100 \quad (5)$$

2.12 Partial purification and characterization of xylitol

Xylitol obtained from microbial fermentation was partially purified following the method as described by Misra et al. [23]. Fermentation broth containing xylitol was centrifuged (4025 ×g, 20 min). The sediment containing cell biomass was discarded while the supernatant was treated with the activated charcoal (15 g/L) with a magnetic stirrer (30 °C for 1 h) and filtered through Whatman's filter paper No.1. The filtrate containing xylitol was freeze-dried and was further characterized by Fourier Transform Infrared (FTIR) Spectroscopy (Perkin Elmer, U.S.) by following the method as described by Mukherji et al. [24]. The spectra of pure xylose, xylitol from fermentation and after partially purification were also recorded by FTIR.

2.13 Statistical analysis

Analysis of variance (ANOVA) was used to carry out the statistical analysis of RSM (Response Surface Model) results to show the significance of the model and independent variables. One-way ANOVA was performed by using SPSS statistical software package (SPSS, version 23.0, USA). The significant differences between the mean values of different treatments at 95% confidence level ($p < 0.05$) were calculated by using LSD and Tukey's test.

3. Results and discussion

3.1 Proximate and compositional analysis of sugarcane bagasse powder

The proximate analysis of sugarcane bagasse powder showed 6.2 % moisture, 78.46% volatile matter, 13.16% fixed carbon and 2.18% ash content. Neto [25] reported the proximate composition of bagasse as moisture content (50.2% w/w), volatile matter (79.9% w/w), fixed carbon (18% w/w) and ash (2.2% w/w). The moisture content of the bagasse used in this research was lower as it was oven dried at 60 °C for 24 h. Further, compositional analysis showed that cellulose (49.38%) was the major component of sugarcane bagasse followed by hemicellulose (26.43%), lignin (12.85%), extractives (sucrose, nitrate/nitrite, protein, ash, chlorophyll, waxes) (9.13%). Jackson et al. [26] reported the average composition of sugarcane bagasse as cellulose (42.19%), hemicellulose (27.6%), lignin (21.56%), extractives (5.63%) and ash (2.84%).

3.2 Optimization of ultrasound assisted alkaline pretreatment for xylan recovery

To optimize the ultrasound assisted alkaline pretreatment for xylan recovery from sugarcane bagasse, response surface methodology was utilized. Box-Behnken Design along with the experimental and predicted values is shown in table 1.

A series of three-dimensional (3-D) response surface graphs were generated in which one variable was kept constant and the interactive effects of other two independent variables on xylan recovery was studied (Fig 1). The 3-D graphs clearly indicated that NaOH concentration and solid: liquid ratio had a profound effect on xylan recovery such that xylan recovery increased with the increase in alkali concentration and solid to liquid ratio (Fig 1a). Whereas the effect of combination of alkali concentration-ultrasonication time and solid liquid ratio-ultrasonication time were observed to be non-significant on the response variable (Figure 1b and 1c).

Increase in xylan recovery with the increase in alkali concentration was attributed to the ability of alkali to dissolve hemicellulose and lignin by hydrolysis of uronic and acetic esters and swelling up of cellulose, leading to the reduction in cellulose crystallinity [27]. Jayapal et al. [16] reported that sodium hydroxide concentration when increased from 2 to 12% resulted an increase in true recovery of xylan (sugarcane bagasse) from 2.77 to 19.88% (w/w).

Xylan recovery increased significantly ($p < 0.05$) with the increase in solid: liquid ratio which works based on the principle of mass transfer i.e. xylan concentration gradient was higher in the bagasse than in the solvent (alkali) that acts as a driving force for mass transfer from solid to solvent. With the increase in alkali ratio, concentration gradient also increases accordingly and as the equilibrium concentration is reached, mass transfer stops [28]. Similarly, Sun et al. [29] reported increase in xylan extraction efficiency from corncob with the increase in corncob to alkali solution ratio from 1:10 to 1:30.

Ultrasonication invades and disrupts the cell wall integrity by creating cavitation phenomenon followed by generation of microbubbles and shear forces along with cleaving of ether bonds present in between hemicellulose and lignin resulting in increased accessibility and extractability of xylan [30]. Ultrasonication can accelerate the time to reach the equilibrium concentration and thereby reducing extraction time [31]. Hence, the non-significant effect of ultrasonication time (10-40 min) on the xylan recovery was due to acceleration of time to reach the equilibrium concentration leading to lesser xylan recovery with the extension of ultrasonication time [32].

Based on the desirability function of Design Expert, the experimental xylan recovery value (11.89% w/w) was observed to be similar to predicted maximum xylan recovery (12.059 %) at NaOH concentration (0.73 M), ultrasonication time (34.77) min and solid to liquid ratio

(1:38.55), hence conforming its validity. Jayapal et al. [16] reported that sugarcane bagasse steamed in 1M NaOH solution at a solid: liquid ratio of 1:10 resulted in lower xylan recovery of 6.11% w/w. Therefore, xylan recovery from sugarcane bagasse can be maximized by optimizing alkaline pretreatment condition accompanied with ultrasound treatment.

3.3 FTIR analysis

The effect of combined alkaline-ultrasonication pretreatment on the chemical structure of xylan was studied by the FITR spectroscopy (Fig. 2). The functional groups of the compounds were identified by comparing the obtained FTIR spectrum data with the reference data from Coates [33]. The protruding broad absorption band at 3410.20 and 3404.11 cm^{-1} of bagasse powder and xylan respectively depicted the stretching of hydroxyl (-OH) groups. Development of distinct absorbance bands in xylan spectra at 3410.20, 1414.45, 1043.88 and 897.30 cm^{-1} were associated with the presence of xylan as reported by Jayapal et al. [16]. The spectrum between 1160.45 cm^{-1} and 1043.88 cm^{-1} was also associated with the typical xylan molecule. Absence of peak in xylan at around 1730 cm^{-1} indicated the complete cleavage of ester bonds of xylan molecule due to the ultrasonic and alkaline pretreatment [34].

3.4 Enzymatic hydrolysis of xylan to xylose

The effect of β-1,4 xylanase enzyme at different concentration and hydrolysis time on the production of xylose was studied (Fig. 3). Statistical analysis illustrated that at each enzyme concentration, the xylose production increased significantly ($p < 0.05$) up to 48 h and the increasing trend plateaued thereafter. Hence the time, 48 h was selected as the optimum enzymatic hydrolysis time. Further, at each incubation time, significant increment ($p < 0.05$) in xylose concentration was reported with the increase in enzyme concentration from 10 U/g to 400 U/g, above which (800 U/g), no significant difference ($p < 0.05$) was observed.

293 Therefore, enzyme concentration of 400 U/g xylan was determined as the optimum enzyme
294 dosage. Overall, optimum enzymatic hydrolysis condition was achieved at an enzyme
295 dosage of 400 U/g xylan at 48 h of incubation time with xylose yield of 81.51 ± 1.73 mg/g
296 and 30.84% of total xylose recovery.

297 Increase in xylose conversion with the increase in enzyme concentration and incubation time
298 can be attributed to the proportional relationship of enzymatic reaction rate and
299 corresponding xylose yield with the concentration of enzyme loaded and time of hydrolysis.
300 Damaso et al. [35] reported the enzymatic hydrolysis of alkaline (2 N NaOH) and thermally
301 pretreated sugarcane bagasse using 3000U/g crude xylanase for 24 h which resulted in xylose
302 yield of 25.2 mg/g bagasse (9.52 % of total xylose recovery) which was lower as compared
303 to the present study. Brienzo and Carvalho [36] reported that the optimized xylanase
304 concentration of 120 U/g was found to produce 17.98% of total xylose from sugarcane
305 bagasse. Paiva et al. [37] reported that acid hydrolysis of sugarcane bagasse with sulfuric
306 acid (3.1% v/v) at 126 °C for 18 min of reaction time produced xylose (266.73 mg/g bagasse)
307 which was equivalent to more than 96% of the theoretical yield. However, acid hydrolysates
308 contain various degradation toxic and fermentation inhibitory compounds (furfural, acetic
309 acids, HMFs and LDPs) that requires detoxification step prior to fermentation whereas
310 enzymatic hydrolysates are devoid of these toxic compounds [14].

311 **3.5 Fermentation of xylose into xylitol by different cultures**

312 The sugarcane bagasse hydrolysates rich in xylose obtained by enzymatic hydrolysis were
313 inoculated with three different starter cultures; *C. guilliermondii*, *Corynebacterium*
314 *glutamicum* and their mixed culture to produce xylitol. Figure 4 illustrates the profile of
315 substrate (xylose) consumption and product (xylitol) formation by respective three starter
316 culture strains in a batch culture for a period of 96 h. Initial xylose concentration of 8.25 g/L

was maintained at all culture conditions. At the end of fermentation (72 h), maximum xylose consumption resulted by yeast (67.71%), followed by mixed culture (48.70%) and bacteria (25.76%). Similarly, maximum xylitol concentration was accumulated at 72 h of fermentation by yeast (2.39 ± 0.13 g/L) followed by mixed culture (1.53 ± 0.09 g/L) and bacteria (0.26 ± 0.03 g/L) respectively.

At all culture conditions, xylitol concentration and xylitol yield increased significantly ($p < 0.05$) with the increase in fermentation time until 72 h followed by significant ($p < 0.05$) decrement at 96 h. Whereas xylose concentration in the fermentation broth decreased continuously which indicated xylose conversion into xylitol upon its consumption by the microbial cells for their growth and metabolism. Observance of maximum xylitol accumulation at 72 h followed by decrement in xylitol concentration at 96 h was due to the fact that yeast cells start xylitol utilization for further metabolism when the xylose in the broth is in the exhausting phase [7]. Xylitol (an intermediate metabolite of xylose metabolism) conversion involves two steps, a reduction process followed by an oxidation process. At first, D-Xylose is reduced to D-xylitol by NADPH and then this metabolite is oxidized to D-xylulose by NADP+, resulting these two reactions to be a limiting factor for D-xylose fermentation and D-xylitol production [4]. In case of slight fluctuations in oxygen availability, xylitol formation pathway enters pentose phosphate pathway preventing the xylitol accumulation and similarly, xylitol formation is indispensably associated with the formation of other metabolites like ethanol, carbon dioxide, acetic acid and polysaccharides thereby resulting lower xylitol conversion rate [15]. The reported variations in the values of xylitol are associated with different microbial species and varying growth conditions involved.

3.6 Fermentation parameters

341 The effect of three different starter cultures on xylitol production was evaluated based on
342 xylose consumption, xylitol yield and xylitol recovery (%) (Table 2). Yeast strain *C.*
343 *guilliermondii* showed the highest xylitol yield (Yp/s= 0.43 g/g) and xylitol recovery %
344 (8.97% w/w of total xylan used) compared to bacteria (Yp/s= 0.13 g/g) and mixed culture
345 (Yp/s= 0.38 g/g) that confirmed *C. guilliermondii* as the suitable starter culture for xylitol
346 production. However, the xylitol yield from *C. guilliermondii* was observed to be
347 comparatively lower than those reported previously; Cunha et al. [38] reported the final
348 xylitol yield of 0.49 g/g by using PVA-hydrogel entrapped yeast whereas, Vaz et al. [31]
349 reported the xylitol yield of 0.55 g/g while scaling up to pilot scale. This comparative lower
350 yield can be attributed to difference in fermentation parameters and presence of divergent
351 and inhibitors in the fermenting substrate as fermentation is associated with the microbial
352 tolerances level and growth conditions [5].

353 For the mixed culture, values for these parameters were lower than that of yeast fermentation
354 and higher than that of bacterial fermentation. This implies that in mixed culture, xylitol
355 formation efficiency of bacteria was enhanced whereas that of yeast was reduced. This
356 phenomenon of low product yield can also be attributed to the partial inhibitory effect on
357 each other. Coculture and mixed fermentation exhibits various interactions like competition,
358 commensalism, predation, mutualism, and proto cooperation between microbial
359 communities [39]. Similarly, various process parameters; pH, temperature, oxygen,
360 substrate, and product concentration etc. are responsible for achieving the synergistic effect
361 of mixed and coculture cultivation [40].

362 **3.7 Partial purification of xylitol and characterization by FTIR**

363 Partially purified freeze-dried xylitol obtained from the fermentation was subjected to FTIR
364 analysis which was compared with the spectra obtained for standard xylitol. Pure xylose

spectra were used to explain the peaks obtained in freeze dried xylitol due to the presence of residual sugars. The functional groups were identified by comparing the obtained spectrum data with the reference data from Coates [33]. The spectra of freeze-dried xylitol, pure xylitol and xylose are presented in figure 5.

IR spectra of freeze-dried xylitol showed a broad stretch around 3389.98 cm^{-1} which was similar to the stretch of pure xylitol spectra that ranged from 3427.77- 3190.12 cm^{-1} corresponding to the chemical characteristic of hydroxyl group (-OH) present in five carbon sugar alcohol. A weak bond of C-H stretching band was also observed for both freeze dried and pure xylitol at around 2938.80 cm^{-1} and at a range of 2995.75-2879.71 cm^{-1} respectively. Pure xylitol spectra showed the distinct peak 1420.21 cm^{-1} and in the similar manner, freeze dried xylitol also showed sharp peak at 1412.86 cm^{-1} corresponding to the O-H bending of carboxylic acid. This resembles to a previous study by Mukherji et al. [24] who reported the presence of strong typical peak at around 1410 cm^{-1} and 2931 cm^{-1} during the IR spectral analysis of pure xylitol and xylitol crystal obtained from fermentation broth similar to current study. A very sharp distinct band was observed at 1041.10 cm^{-1} in pure xylose spectra and similar to this peak, a sharp peak at 1046.45 cm^{-1} was observed in freeze dried xylitol which indicated the presence of residual sugars (xylose) in the sample. However, a strong sharp bend of N-H was observed in freeze dried xylitol at 1567.46 cm^{-1} corresponding to secondary amine group which was not observed in pure xylitol spectra. Hence, based on the common spectral peaks between pure xylitol and freeze-dried xylitol and resemblance with xylose structure, the partially purified compound was confirmed to be xylitol with some residual sugars (xylose).

4. Conclusion

The present study demonstrated a biotechnological processing for xylitol production from sugarcane bagasse hydrolysates rich in xylose. It was revealed that sugarcane bagasse can be hydrolyzed by a biobased enzymatic hydrolysis associated with ultrasound assisted mild alkaline pretreatment and consequently, as a substitute to acid hydrolysis, the most common and conventional hydrolysis method of agro-industrial waste. Ultrasound assisted alkaline pretreatment was found to increase the hydrolysis efficiency of xylanase. Furthermore, this study confirmed that yeast (*C. guilliermondii*) was the best starter culture for xylitol production from sugarcane bagasse hydrolysates as compared to bacteria (*Corynebacterium glutamicum*) and the mixed culture (*C. guilliermondii & Corynebacterium glutamicum*). Overall, this study provides a suitable alternative for industrial utilization of hemicellulose rich sugarcane bagasse for xylitol production.

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Consent for publication

Not applicable

Availability of data and material

Data will be available on request from corresponding author.

Code availability

Not applicable

Author contributions

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Declarations

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560 **Figure captions**

561 **Fig. 1.** Response Surface 3D graph (1a, 1b and 1c) showing interactive effects of NaOH
562 concentration, solid to liquid ratio and ultrasonication time on xylan recovery %

563 **Fig. 2.** Comparison of FTIR spectra of xylan and sugarcane bagasse powder

564 **Fig. 3.** Effect of different enzyme concentrations on xylose recovery at different hydrolysis
565 period. Different superscript letters (a-e) indicate significant difference ($p < 0.05$) between
566 different enzyme dosage rate (10-800 U/g) at a particular time.

567 **Fig. 4.** Xylitol formation and xylose consumption (black symbol) at different time periods
568 during fermentation of sugarcane bagasse hydrolysates by *Candida guilliermondii*
569 *Corynebacterium glutamicum* and their mixed culture.

570 **Fig. 5.** FTIR spectra of partially purified freeze-dried xylitol, pure xylitol and xylose
571 standard.

572

573

574 **Table 1** Box-Behnken experimental design with predicted and actual values of xylan
575 recovery

	Independent Variables			Response Variable (Xylan Recovery %)	
Run Order	NaOH Concentration (M) (X_1)	Solid: Liquid (g: mL) (X_2)	UAE Time (min) (X_3)	Predicted	Experimental
1	0.5	40	10	9.81	10.21
2	0	10	25	0.11	0.56
3	1	40	25	11.70	11.25
4	0.5	10	10	7.99	7.71
5	0.5	25	25	10.02	10.19
6	1	25	10	10.10	10.15
7	0	25	40	0.78	0.73
8	1	10	25	8.35	8.57
9	0.5	10	40	7.86	7.46
10	0	25	10	0.97	0.80
11	1	25	40	10.61	10.78
12	0.5	25	25	10.02	10.20
13	0	40	25	1.00	0.77
14	0.5	40	40	10.27	10.56
15	0.5	25	25	10.02	9.67

576

577 **Table 2.** Comparison of xylose consumption, xylitol yield and xylan recovery by different
578 starter cultures at 72 h of fermentation

Starter Culture	Xylose consumption (%)	Xylitol Concentration (g/L)	Xylitol yield, Yp/s (g/g)	Xylitol recovery of total xylan (%)
C. guilliermondii	67.71 ± 2.52[c]	2.39 ±0.13[c]	0.43 ± 0.02[c]	8.97 ± 0.48[c]
Corynebacterium glutamicum	25.76 ± 0.95[a]	0.26 ±0.03[a]	0.13 ± 0.02[a]	0.99 ± 0.10[a]
C. guilliermondii & *Corynebacterium glutamicum*	48.70 ± 3.79[b]	1.53±0.09[b]	0.38 ± 0.02[b]	5.73 ± 0.34[b]

579 Different superscript letters (a-c) within a column indicate significant differences ($p < 0.05$)
580 between mean observations.

581

Figures

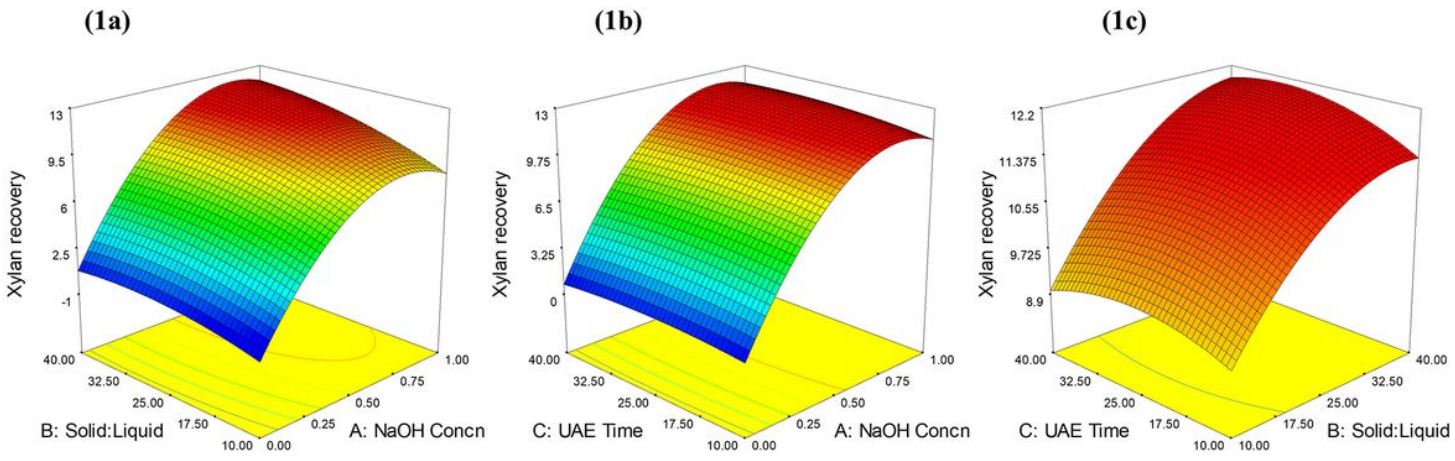

Figure 1

Response Surface 3D graph (1a, 1b and 1c) showing interactive effects of NaOH concentration, solid to liquid ratio and ultrasonication time on xylan recovery %

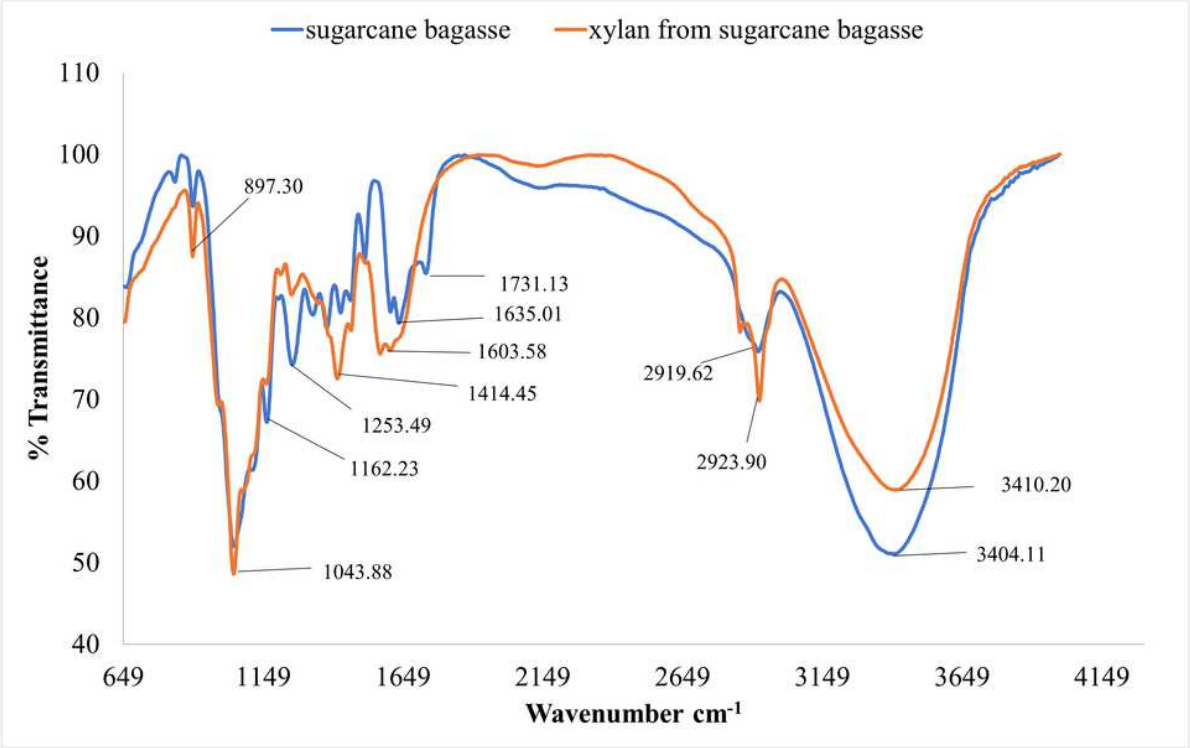

Figure 2

Comparison of FTIR spectra of xylan and sugarcane bagasse powder

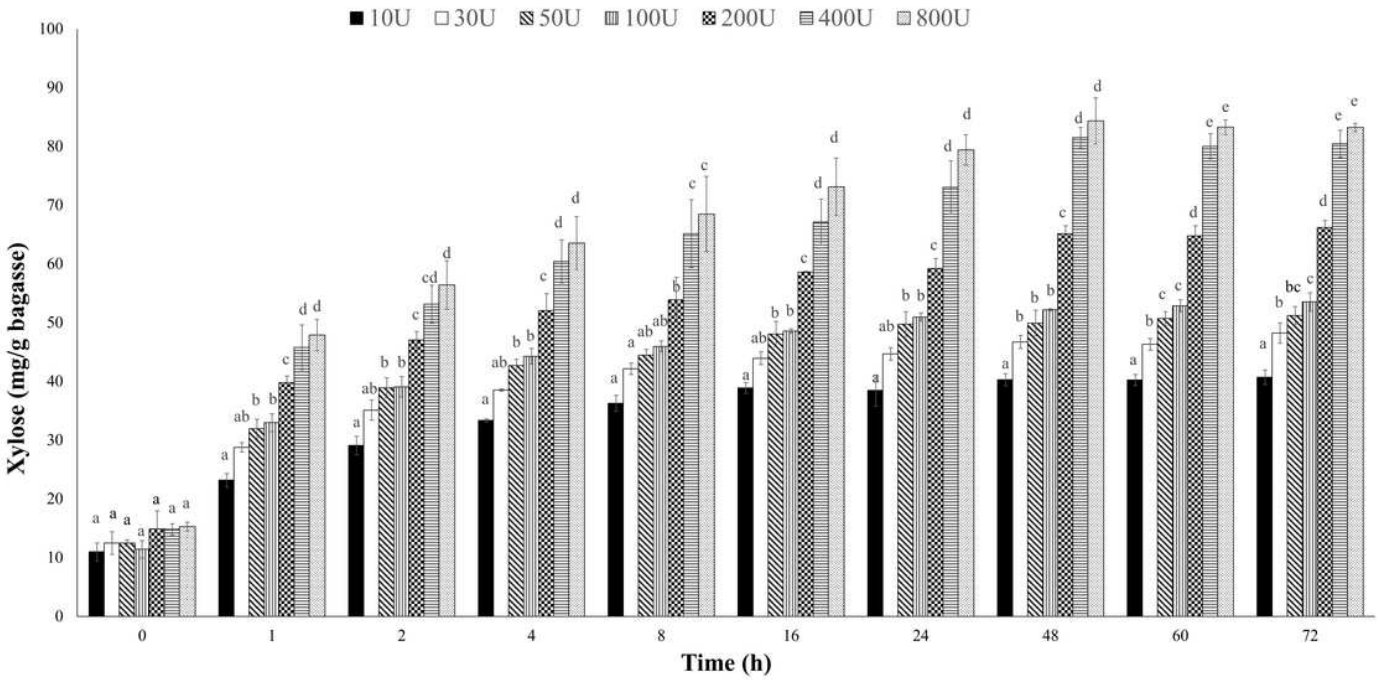

Figure 3

Effect of different enzyme concentrations on xylose recovery at different hydrolysis period. Different superscript letters (a-e) indicate significant difference ($p < 0.05$) between different enzyme dosage rate (10-800 U/g) at a particular time.

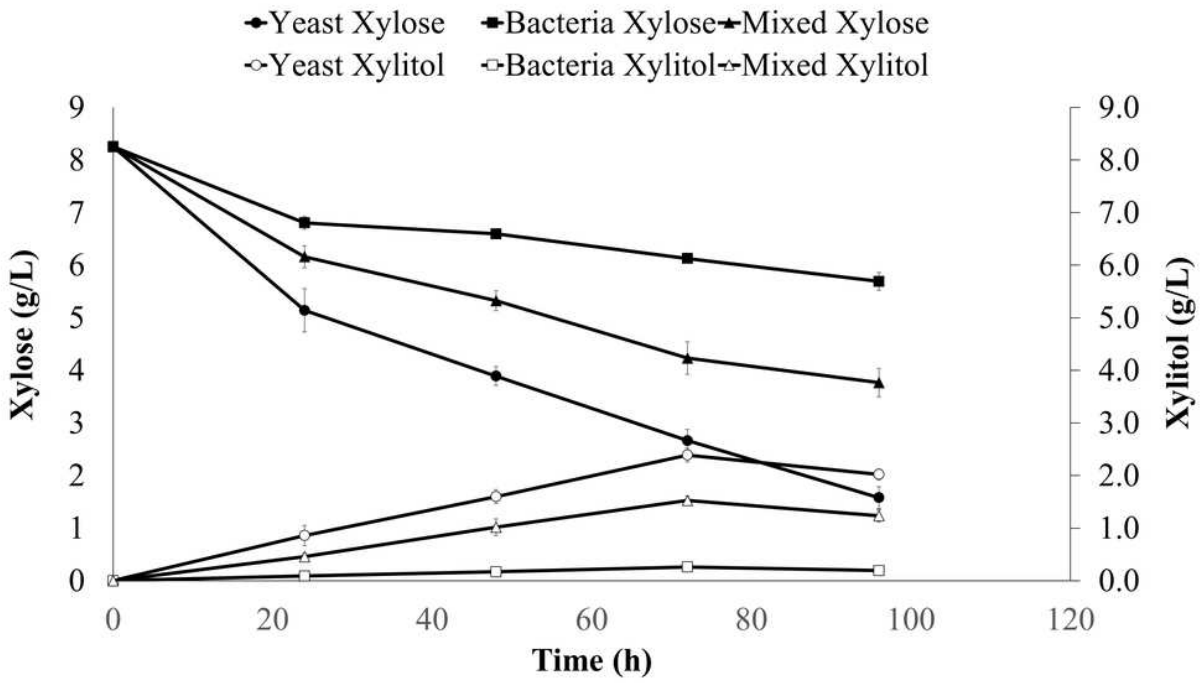

Figure 4

Xylitol formation and xylose consumption (black symbol) at different time periods during fermentation of sugarcane bagasse hydrolysates by Candida guilliermondii Corynebacterium glutamicum and their mixed culture.

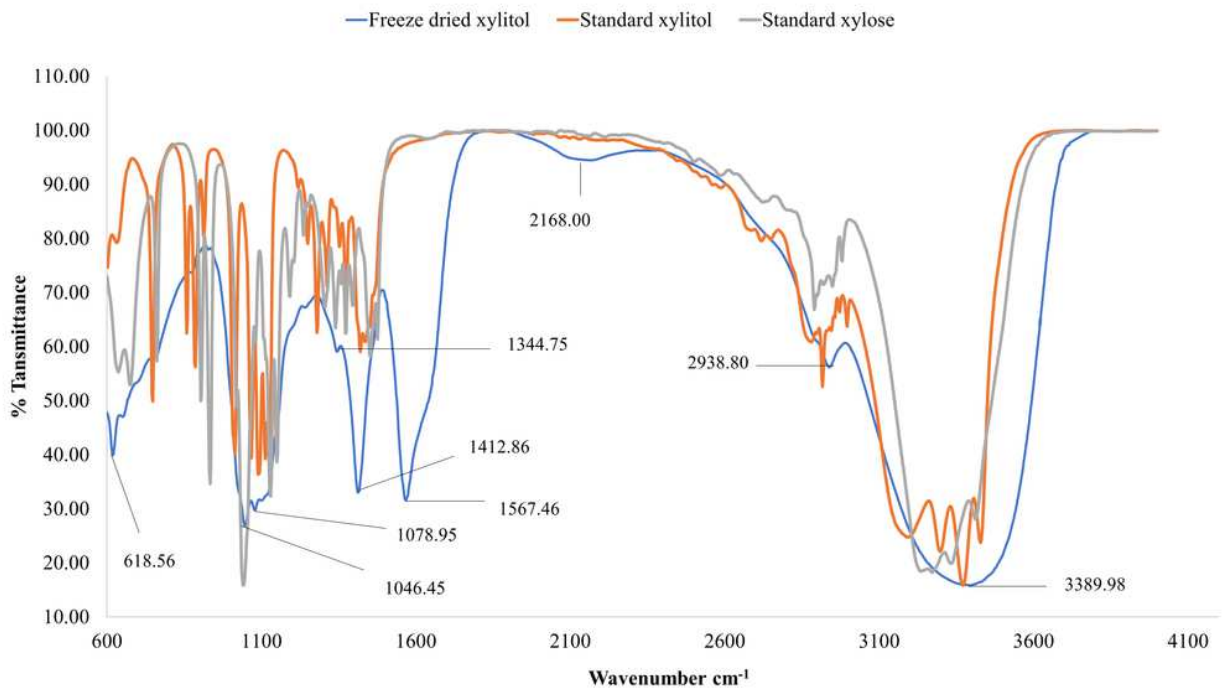

Figure 5

FTIR spectra of partially purified freeze-dried xylitol, pure xylitol and xylose standard.

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