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ORIGINAL ARTICLE

Simultaneous saccharification and bioethanol production from underutilized biomass, cowpea haulm using co-cultures of *Saccharomyces cerevisiae* (BY4743) and *Scheffersomyces stipitis* (PsY633)

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Abstract

Cowpea cultivation generates large quantities of biomass after pod harvest which are underutilized and could be exploited for energy generation. In this study, evaluation of sugar production by dilute sulfuric acid pretreatment of cowpea haulm for potential ethanolic fermentation was carried out. A central composite design (CCD) was used to investigate the effects of temperature (100–120 °C), time (30–90 min), and acid concentration (1.0–4.0%) on sugar yield and inhibitor formation. The model *F*-values of 25.42, 78.81, and 6.96 for xylose yield, glucose yield, and total inhibitor concentration, respectively, with low probability value ($p < 0.05$) suggest a high significance of the models. While the R^2-values of 0.9581, 0.9861, and 0.8424 indicated a satisfactory agreement of the quadratic models in predicting the responses. The quadratic model predicted optimum conditions for maximum xylose (76.5%) and glucose yield (28.6%), at minimum inhibitor concentration (2.34 g/L) and temperature (110 °C), with an acid concentration of 3.1% and reaction time of 55 min. These conditions were validated experimentally suggesting the effectiveness of experimental design towards process optimization. The resulting sugar-rich prehydrolysate was detoxified and fermented to ethanol using co-cultures of *Saccharomyces cerevisiae* BY4743 and *Scheffersomyces stipitis* (PsY633), while the recovered pretreated solid was subjected to simultaneous saccharification and fermentation with prehydrolysis (PSSF). A total ethanol titer of 15.67 g/L was obtained which corresponds to an overall conversion efficiency of 75%. This suggests that cowpea haulm could also be potentially exploited for bioethanol production either singly or in combination with other lignocellulosic biomass.

Keywords Bioethanol · Dilute acid pretreatment · Cowpea haulm · Response surface methodology · Simultaneous saccharification and fermentation

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1 Introduction

Bioethanol is a prominent biofuel and chemical feedstock that can be produced from lignocellulosic agricultural residues. Several lignocellulosic residues such as corncob, rice straw, soybean cake, sugarcane bagasse, and wheat bran have been studied for bioethanol production; however, many untapped potential sources are still not being explored or underutilized [1, 2]. Cowpea (*Vigna unguiculata* L.) is an important food legume grown in tropical and subtropical regions. Today, around 7.2 million tons of cowpea are produced worldwide, of which more than 95% is from Africa, and this average is maintained since 1994 to till date [3]. Cowpea haulm (CH) is the aboveground lignocellulosic biomass residue remaining after the cowpea pod harvest. It is an underutilized yet

promising candidate for bioethanol production due to its high sugar and low lignin content. Interestingly, another food legume which grows under similar conditions, viz., Bambara groundnut haulm, has recently shown tremendous potential for bioethanol production [4].

Typically, lignocellulosic biomass is a highly recalcitrant feedstock and therefore requires a pretreatment step prior to utilization for bioethanol production [4]. The pretreatment step helps to disrupt the recalcitrant matrix, solubilizing hemicellulose and lignin for increased enzyme access and digestibility of cellulose. Various pretreatments including steam, hydrothermal, extrusion, and ammonia-based methods have proven effective in increasing biomass digestibility [5]. Dilute acid pretreatment (DAP) is the most frequently studied chemical pretreatment method for agriculture biomass owing to its low cost and high sugar recovery (70–90%) [6]. DAP is influenced by several process parameters including temperature, pH, pretreatment time, solid loading, etc. [7]. The interaction between these factors affects the severity of the pretreatment. Under strong pretreatment conditions (high temperature or acid concentration), the hydrolyzed sugars are degraded into by-products that are inhibitory to the saccharification and fermentation process [8]. It is, therefore, necessary to optimally combine process parameters for maximum sugar recovery. Response surface methodology (RSM) is a multivariate statistical method based on the design of experiments that analyze the influence of the independent variables (factors) on the dependent variables (responses) and predict optimal process conditions by maximizing the dependent variables [9]. It has been used to optimize the effectiveness of pretreatment of lignocellulosic biomass [10, 11].

DAP usually results in a hydrolysate that contains a mix of pentose and hexose sugars which can be fermented sequentially using co-cultures of *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* [12]. In addition, the solid fraction recovered from DAP is usually hydrolyzed using enzymes and fermented into bioethanol. Separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) are the two commonly reported bioethanol fermentation strategies [13]. Additionally, reports on SSF processes have indicated that a prehydrolysis step could significantly enhance the fermentation process, ethanol titer, and conversion efficiency [14]. The aim of this work was to evaluate the potential of CH as feedstock for bioethanol production. To this end, response surface methodology was used to optimize dilute acid pretreatment for optimum sugar recovery, and the recovered solid fraction and liquid fractions were fermented to bioethanol by prehydrolysis with simultaneous saccharification and fermentation (SSF) and simultaneous hydrolysis and fermentation (SHF), respectively (Fig. 1).

2 Materials and methods

2.1 Feedstock

Cowpea (*Vigna unguiculata*) haulms were obtained from the Agricultural Research Council (ARC), VOPI, Pretoria, South Africa. The haulms were air-dried, milled using a hammer mill, followed by a laboratory blender (MRC SM–450L), and sieved to a particle size of ≤ 0.5 mm (Universal Test Sieve—117547, South Africa). Milled CH was stored at room temperature in airtight plastic containers.

2.2 Microorganisms and inoculum development

The pentose rich hydrolysate was fermented using *S. stipitis* wild type (PsY633) (CSIR, South Africa). *Scheffersomyces stipitis* culture was activated from frozen glycerol stock by two successive subculturing on malt extract agar and incubated at 30 °C. Malt extract agar contained (g/L) malt extract (20), dextrose (20), peptone (6), and agar (15) [15]. Glucose hydrolysate was fermented using *S. cerevisiae* BY4743 (Discipline of Microbiology, University of KwaZulu-Natal, South Africa) previously maintained on a yeast peptone dextrose (YPD) agar containing (g/L) yeast extract (10), bacteriological peptone (20), dextrose (20), and agar (15). *Scheffersomyces cerevisiae* BY4743 and *S. stipitis* wild type (PsY633) inoculum were grown on YPD and malt extract broth respectively at 30 °C for 18 h in an orbital shaker (120 rpm) to the early log phase (OD 0.8) prior to use. Cell growth was monitored regularly by measuring the optical density at 600 nm, using a spectrophotometer (Genesys 150 UV-Vis, Thermo Fischer Scientific, USA).

2.3 Experimental design and dilute acid pretreatment

A three-variable central composite design (CCD) was used to optimize the pretreatment conditions of CH with dilute H_2SO_4. The input variables were temperature (100–120 °C), acid concentration (1–4%), and pretreatment time (30–90 min), while the output variables were xylose yield (%), glucose yield (%), and total inhibitor concentration (g/L) in the prehydrolysate. The CCD was constructed using 8 axial points, 6-star points, and 6 replicates at the central point, giving a total of 20 experimental runs (Table 1). An optimization criterion that maximizes xylose and glucose yield in the prehydrolysate with minimal inhibitor production was set. Analysis of the experimental data was carried out using the statistical software package Design-Expert 11.1 (Stat Ease, Inc., Minneapolis, USA). CH (5 g) was mixed with H_2SO_4 (10% (w/v) substrate loading) in 250-ml Erlenmeyer flasks and pretreated in an autoclave according to the experimental design (Table 1). The pretreated slurry was cooled and filtered through a muslin cloth to separate the solid and liquid

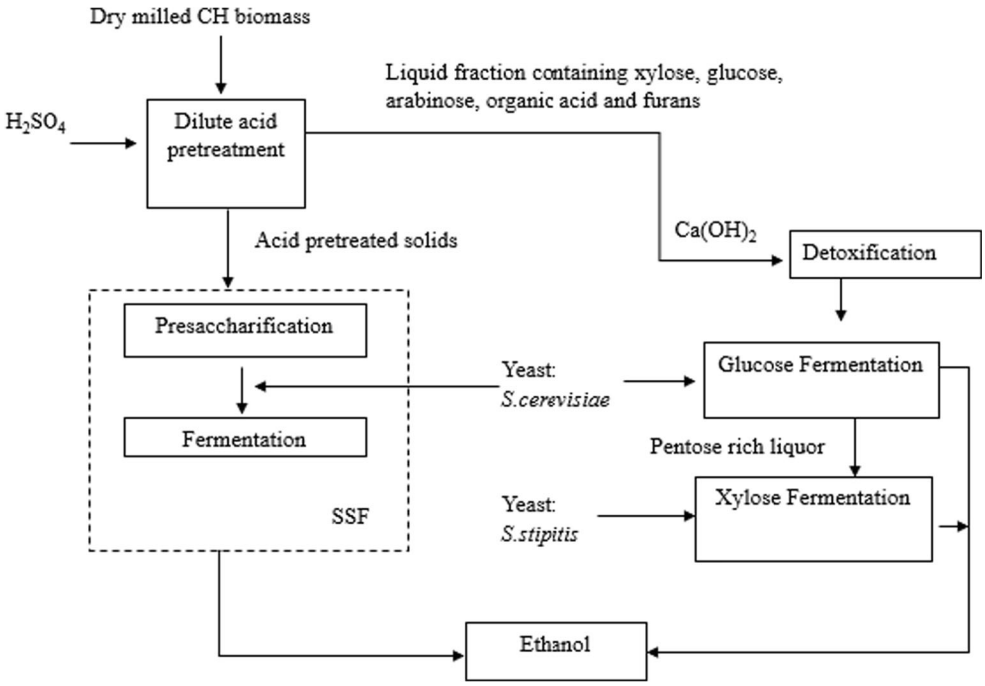

Fig. 1 Flow chart demonstrating the experimental process used for bioethanol production

fractions. The recovered solid residue was washed and dried at 30 °C for enzymatic hydrolysis and compositional analysis. The prehydrolysate and wash water were assayed for monomeric sugars and inhibitors.

Table 1 Central composite experimental design during the assessment of dilute acid pretreatment of cowpea haulm

Std	Run	A: temperature (°C)	B: pretreatment time (min)	C: acid concentration (%)	CSF
7	1	120	90	1	1.83
14	2	126.81	60	2.5	2.18
10	3	110	60	5.02	1.99
18	4	110	60	2.5	1.69
15	5	110	60	2.5	1.69
8	6	120	90	4	2.40
9	7	110	60	−0.02	-3.61
2	8	100	30	4	1.33
12	9	110	110.45	2.5	1.95
20	10	110	60	2.5	1.69
5	11	120	30	1	1.35
6	12	120	30	4	1.92
3	13	100	90	1	1.24
11	14	110	9.54	2.5	0.89
19	15	110	60	2.5	1.69
13	16	93.18	60	2.5	1.19
1	17	100	30	1	0.76
16	18	110	60	2.5	1.69
4	19	100	90	4	1.81
17	20	110	60	2.5	1.69

Acid concentration was kept at 0

The intensity of the pretreatment conditions was expressed in a combined severity factor (CSF). The CSF is generally defined as shown in Eq. 1

$$\log R_{\mathrm{o}}^{'} = \log\left(t \times e^{\left(\frac{T-100}{14.75}\right)} - \mathrm{pH}\right) \quad (1)$$

where log R'_O is the CSF, T is the hydrolysis temperature in °C, and t' is the reaction time in minutes.

2.4 Detoxification and fermentation of prehydrolysate

The prehydrolysate obtained under optimal DAP conditions was detoxified by treating with $Ca(OH)_2$ to reach a pH of 11 ± 0.1, kept at 30 °C for 1 h, and then adjusted to pH 6 ± 0.1 with 72% H_2SO_4, followed by centrifugation. The detoxified hydrolysate was supplemented with fermentation medium (g/L) composed of yeast extract (5), peptone (5), $Mg_2SO_4{\cdot}7H_2O$ (1), $(NH_4)_2SO_4$ (1), and KH_2PO_4 (2), at pH 6. After sterilization of the media, fermentation was initiated by inoculating first with *S. cerevisiae* BY4743, and *S. stipitis* was added after 24 h. Both the inocula were added in 1:1 ratio at 10% (v/v) level to the production medium (50 ml, pH 6) and incubated in an orbital shaker at 30 °C and 150 rpm. Samples were routinely taken for the analysis of glucose, xylose, and ethanol.

2.5 PSSF of dilute acid pretreated CH

Washed pretreated solids were subjected to prehydrolysis with simultaneous saccharification and fermentation (PSSF) in 250-ml Erlenmeyer flasks with 50 mL working volume. The prehydrolysis step was performed at 50 °C, 120 rpm for 24 h using the commercial enzyme complex Accellerase 1500 (20 mg/mL total protein content), and Accellerase XY (10 mg/mL total protein content), from Genencor (Netherlands). The hydrolysis solution was composed of the washed pretreated CH residue at 10% solid loading, sterile citrate buffer (pH 4.8, 0.05 M) supplemented with fermentation nutrients (g/L), yeast extract (5), peptone (5), $Mg_2SO_4{\cdot}7H_2O$ (1), $(NH_4)_2SO_4$ (1), and KH_2PO_4 (2) at pH 6, an enzyme load of 2 mg protein per gram of glucan and pentosan in the pretreated CH for Accellerase 1500 and Accellerase XC, respectively. The prehydrolyzed CH residue was subjected to simultaneous saccharification and fermentation process (SSF) for ethanol production in sterile 250-mL Erlenmeyer flasks. *Saccharomyces cerevisiae* BY4743 grown on YPD broth for 18 h was used as the inoculum. The flask was seeded aseptically with 10% (w/v) inoculum (OD 0.8) to make a final volume of 50 mL. The culture was incubated at 35 °C and 120 rpm for 48 h and routinely sampled for sugars and ethanol [16].

2.6 Analytical methods

Untreated and pretreated CH was characterized using compositional analysis, X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and scanning electron microscopy (SEM). The concentration of sugar and inhibitor in the hydrolysate was determined by high-performance liquid chromatography (HPLC). Ethanol concentration was determined using gas chromatography (GC); calculation of sugar and ethanol yield, fermentation efficiency, and component recovery were done according to equations S1–S8 and is provided along with a detailed description of the analytical methods employed in the supplementary material.

2.7 Statistical analysis

Results obtained were mean of three or more determinants. One-way ANOVA was done using Microsoft Office data analysis tool pack. A difference of $P \leq 0.05$ was considered significant.

3 Results and discussion

3.1 Feedstock composition

About 53.9% of the dry mass of CH was composed of carbohydrates, mainly glucans (43.9 ± 2.1%), xylans (7.2 ± 0.5%), and arabinans (3.1 ± 0.1%) while lignin content (4.5 ± 0.4%) was low. Hemicellulosic sugars represented 10.3% of the raw material with xylose as the main sugar (70%). The lignin content of CH used in this study was much lower than values reported for feedstock such as corn cob (14–15%), wheat straw (17–19%), sugar cane bagasse (20–42%), and rice husk (26–31%) [17]. Anele et al. [18] have reported 12.6–22.1% lignin in various varieties of cowpea haulm. However, low lignin values like those observed in this study have been reported for other lignocellulosic biomasses including water hyacinth (3.5%), orchard grass (4.7%), pineapple leaf fiber (1–5%), and bermudagrass (6.4%). The variation in lignin content could be attributed to the differences in variety, agronomic and physiological conditions of the plant, and the method of lignin measurement [19]. The particularly low lignin content (4.5%) and high sugar content (53.9%) observed for CH make it a promising feedstock for bioethanol. This is because cellulose and hemicellulose are the main sugar precursors for bioethanol production, while lignin provides a protective sheath over hemicellulose and cellulose by complex physical and chemical associations [20]. Therefore, low lignin content could ease the solubilization of polysaccharide fractions with environmentally friendly pretreatment conditions [21]. Moreover, the degradation of lignin during acid pretreatment leads to the formation of phenolic monomers which have

individual and synergistic inhibitory effects on bioethanol fermentation [22]. CH is also composed of a high mass fraction of extractives (33%). High extractive components have been reported for other agricultural residues including olive trees [23] and *Agave lechuguilla* [6]. Biomass extractives encompass a wide variety of high-value chemical compounds with reported therapeutic and industrial potentials that could be used to boost the economic viability of bioethanol production in a biorefinery process [24–26].

3.2 Yield and composition of solid and liquid fractions of pretreated CH

The yield and composition of the solid and liquid fractions are highly dependent on the pretreatment conditions. Generally, with increasing process severity (i.e., increase in acid concentration, temperature and pretreatment time), the hydrolysis of cellulose and hemicelluloses is accelerated, leading to a loss in solid and an increase in the amount of monomeric sugars that can be recovered in solution. The CSF for the dilute acid pretreatment in this study ranged from −3.62 to 2.40. Solid recovery exhibited a decline from 63 to 26% with increasing CSF corresponding to the highest (CSF = −3.62, run 7) and the lowest CSF (CSF = 2.4, run 6), respectively (Table 2). The experiments carried out in the center of the domain (runs 4, 5, 10, 15, 18, 20) with a CSF of 1.69 resulted in a solid recovery of around 36.38%. The solubilization of cellulose and hemicellulose was confirmed by examining the composition of the recovered solid fraction (Table 2). As expected, the hemicelluloses were most susceptible to dilute acid pretreatment. Under mild pretreatment conditions (CSF = −3.62), only 4.79% solubilization occurred, while in the most severe runs (CSF above 2), over 90% xylan solubilization was observed. The cellulose content in the pretreated solids ranged between 22 and 42%, amounting to a recovery of 52.14–96.46% of the initial cellulose content for the mildest and most severe runs, respectively.

Consequently, monomeric sugars in the hydrolysate increased up to a certain point and then began to decrease. Glucose was the most abundant sugar in the filtrates (0.2 to 15.6 g/L), followed by xylose (0.2 to 6.79 g/L), while arabinose was found in relatively minor concentrations (Table 3). The glucose obtained was attributed to the solubilization of amorphous portions of cellulose and even hemicellulose [7]. Some agricultural residues are known to be composed of glucuronoarabinoxylan as their hemicelluloses [27]. Xylose and arabinose could be released during hemicellulose hydrolysis. The decrease in the sugar yield at higher CSF is attributed to sugar degradation to secondary products. In addition to the sugars, furfural, 5-HMF, acetic, formic, and levulinic acids were detected in the prehydrolysate (Table 3).

Table 2 Yield and composition of solid fraction after dilute acid pretreatment

Composition of solids % (w/w)								
Run	CSF	Solid recovery	Glucan	Xylan	Arabian	Lignin	Cellulose recovery	Xylan solubilization
7	−3.62	62.8	42.34	7.8	2.254	4.28	96.46	4.79
17	0.77	50.5	32.25	6.05	1.333	4.55	73.47	26.15
14	0.89	41.4	32.6	3.15	1.02	3.64	74.27	61.51
16	1.2	42.4	30.81	2.42	1.082	3.95	70.19	70.49
13	1.24	43.6	33.99	4.09	1.15	4.49	77.44	50.09
8	1.34	42.3	33.94	2.00	1.65	3.4	77.32	75.53
11	1.36	40.5	31.59	3.57	1.054	2.96	71.96	56.42
15	1.69	38.6	34.63	1.75	0.88	3.01	78.89	78.57
4	1.69	37.6	33.52	3.18	0.874	3.13	76.37	61.13
10	1.69	37.2	33.28	1.75	0.962	4.24	75.81	78.58
5	1.69	36.3	32.65	2.25	0.941	2.42	74.39	72.52
18	1.69	34.6	33.71	1.68	0.875	3.48	76.78	79.52
20	1.69	34	32.96	1.6	0.827	3.58	75.08	80.49
19	1.81	37	33.01	2.17	0.913	3.12	75.21	73.55
1	1.83	35.1	31.97	2.48	0.934	2.73	72.82	69.72
12	1.93	33.3	32.52	1.51	0.759	4.04	74.08	81.55
9	1.96	33.3	33.11	1.12	0.818	2.53	75.42	86.31
3	1.99	35	34.83	1.1	0.882	2.5	79.34	86.61
2	2.19	31.1	33.22	0.77	0.738	2.45	75.67	90.63
6	2.4	26	22.89	0.65	0.62	2.91	52.14	92.06

Table 3 Sugar and inhibitor composition in the hydrolysate after pretreatment

Run	Acid conc (%)	Time (min)	Temp (°C)	pH	CSF	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Glucose yield (%)	Xylose yield (%)	Acetic (g/L)	Formic (g/L)	Levulinic (g/L)	Furfural (g/L)	5-HMF (g/L)	Total inhibitor (g/L)
7	−0.02	60	110	5.69	−3.62	1.28	0.85	0.10	2.63	10.38	ND	2.38	ND	ND	ND	2.38
17	1	30	100	0.71	0.77	0.23	0.21	0.18	0.48	2.531	ND	2.28	0.12	0.42	ND	2.82
14	2.5	9.55	110	0.38	0.89	5.89	4.22	1.41	12.08	51.51	0.03	1.09	0.23	0.63	ND	1.98
16	2.5	60	93.2	0.38	1.2	5.36	4.20	1.35	11.00	51.26	0.03	1.4	0.12	0.6	ND	2.17
13	1	90	100	0.71	1.24	3.54	2.50	1.27	7.26	30.51	0.02	1.02	0.11	0.52	ND	1.68
8	4	30	100	0.14	1.34	6.11	4.85	1.19	12.54	59.25	0.04	1.38	0.23	0.39	0.04	2.08
11	1	30	120	0.71	1.36	5.10	3.39	1.31	10.46	41.45	0.02	0.96	ND	0.63	ND	1.62
4	2.5	60	110	0.38	1.69	15.6	4.80	2.49	31.98	58.65	0.05	1.34	0.26	0.77	0.08	2.50
5	2.5	60	110	0.38	1.69	14.0	5.75	2.35	28.76	70.22	0.05	1.48	ND	0.73	0.06	2.33
10	2.5	60	110	0.38	1.69	13.3	6.17	2.27	27.34	75.38	0.07	1.73	0.47	0.85	0.11	3.23
15	2.5	60	110	0.38	1.69	13.8	6.11	2.18	28.39	74.63	0.03	0.84	0.24	0.81	0.14	2.07
18	2.5	60	110	0.38	1.69	13.8	6.26	2.09	28.39	76.4	0.04	1.05	0.14	0.57	0.19	2.00
20	2.5	60	110	0.38	1.69	13.6	6.26	2.16	27.79	76.5	0.03	0.79	0.24	0.71	ND	1.91
19	4	90	100	0.14	1.81	7.21	5.99	1.25	14.78	73.12	0.04	1.08	0.24	0.75	0.18	2.29
1	1	90	120	0.89	1.83	6.74	5.28	1.52	13.92	64.46	0.05	1.18	0.4	0.91	0.18	2.71
12	4	30	120	0.14	1.93	7.52	6.61	1.2	15.41	80.74	0.05	0.62	0.24	0.79	0.41	2.11
9	2.5	110	110	0.38	1.96	7.61	6.79	1.31	15.61	82.96	0.04	1.47	0.31	0.7	0.39	3.04
3	5.02	60	110	0.08	1.99	7.08	6.01	1.11	14.52	73.41	0.07	1.09	0.45	0.54	0.49	2.64
2	2.5	60	127	0.38	2.19	7.42	5.98	1.08	15.22	73.10	0.08	1.60	0.65	0.63	0.73	3.70
6	4	90	120	0.14	2.4	7.75	5.96	1.27	15.89	72.79	0.1	2.39	1.20	0.45	0.79	4.92

3.3 Modeling and optimization of dilute acid pretreatment conditions

From the experimental results (Tables 2 and 3), second-order polynomial regression equations were obtained (Eqs. 2–4) for each dependent variable.

$$\begin{aligned}\text{Xylose yield} = {} & 72.0636 + 18.5229 \times A + 8.04102 \times B \\ & + 9.57308 \times C + -5.63569 \times AB \\ & + \quad -6.46357 \times AC + -3.34878 \times BC \\ & + -11.2849 \times A^2 + -2.32457 \times B^2 \\ & + -4.11168 \times C^2 \end{aligned} \quad (2)$$

$$\begin{aligned}\text{Glucose yield} = {} & 28.7573 + 3.40559 \times A + 1.38401 \\ & \times B + 2.03142 \times C + -0.939293 \\ & \times AB + -1.58358 \times AC + -0.635929 \\ & \times BC + -7.03096 \times A^2 + -5.16809 \\ & \times B^2 + -5.42675 \times C^2 \end{aligned} \quad (3)$$

$$\begin{aligned}\text{Total inhibitor concentration} = {} & 2.34244 + 0.2193 \times A + 0.347797 \times B \\ & + 0.370734 \times C + 0.385377 \times AB + 0.353631 \\ & \times AC + 0.602266 \times BC + 0.0312669 \times A^2 \\ & + 0.0306822 \times B^2 + 0.180194 \times C^2 \end{aligned} \quad (4)$$

where A is the acid concentration, B is pretreatment time, and C is temperature.

The model adequacy and the significance of each coefficient were checked by the analysis of variance (ANOVA) (Tables S1–S3, supplementary material). The significance of the models and each coefficient was determined by F- and P-values. The model F-values of 25.42, 78.81, and 6.96 with low probability value ($P < 0.0001$), ($P < 0.0001$), ($P = 0.0028$) for xylose yield, glucose yield, and total inhibitor concentration, respectively, imply a high significance of the model. Furthermore, the lack of fit was also insignificant for all the models. The high R^2-values of 0.9581, 0.9861, and 0.8424 indicated a satisfactory agreement of the quadratic model to the experimental data.

Acid concentration, temperature, and time exerted significant positive individual effects on xylose and glucose yield, whereas the interactive and quadratic terms all had a negative

effect (Eqs. 4 and 5). On the other hand, both linear and interaction terms caused an increase in the total inhibitor concentration (Eq. 6). A similar observation was made by Gonzales et al. [28] during the dilute acid hydrolysis of oil palm empty fruit bunch.

The quadratic model predicted that the optimum conditions for maximum xylose (76.5%) and glucose yield (28.6%) at minimum inhibitor concentration (2.34 g/L) were temperature (110 °C), acid concentration (3.1%), and time of reaction (55 min). To validate the predicted conditions and check the adequacy of the model equation, pretreatment experiments were conducted in triplicate under these conditions, and the mean values were presented. The experimental values of 79%, 27.2%, and 2.53 g/L for xylose yield, glucose yield, and minimum inhibitor concentration, respectively, matched well with the predicted values demonstrating the accuracy of the model. In a previous study, Botella et al. [29] also reported xylose and glucose yield in the range of 35–79% and 21–24%, respectively, from α-hydroxyethane sulfonic acid (HESA) pretreatment of ensiled sweet sorghum. While in another experiment, 68.35 and 8.31% of xylose and glucose were obtained from cotton stalk treated with sulfuric acid under the optimized conditions comprising of temperature (157 °C), acid concentration (1.07%, w/w), and time (20 min) [30]. In contrast, a higher yield of xylose (90.95%) was obtained from pinewood sawdust under the following optimized conditions, viz., a reaction temperature of 106.7 °C, a reaction time of 4.57 h and phosphoric-acid concentration 4.49 wt% [31]. Considering the yield and process parameters involved, optimized process conditions in this study were comparatively eco-friendlier and more cost-effective than the previous reports.

3.4 Interactive effect of process variables on responses

The response models were mapped against two experimental factors while the other factor was maintained constant at its central value. In view of the parameters investigated in this study, the maximum xylose and glucose yield in the prehydrolysate was attained.

The maximum xylose yield (76%) could be obtained when the acid concentration ranged from 2.67 to 3.9% and the pretreatment time ranged from 63 to 110 min. On the other hand, the maximum glucose yield (28.6%) could be obtained when the acid concentration ranged from 1.8 to 3.9% and the pretreatment time ranged from 40 to 80 min. It was observed that higher xylose and glucose yields could be obtained at either short pretreatment time and high acid concentration or long pretreatment time and low acid concentration (Figs. 2a and 3a). Too low or too high values of both factors resulted in low sugar yield. The observation is attributed to the fact that, in an aqueous environment, dilute acids dissociate into protons (H^+) which diffuse through the lignocellulosic matrix, cleaving the ether bonds between hemicellulose and lignin and the glycosidic linkage between sugar monomers. This results in the solubilization of hemicelluloses, amorphous cellulose into glucose, and acid-soluble lignin [7]. Under low acid conditions, fewer protons (H^+) are available to catalyze the reaction. Hence, longer contact time with the lignocellulosic material is required to instigate bond cleavage and improve sugar yield. At a higher acid concentration, more protons (H^+) are available to cleave the ether and glycosidic bonds, reducing the required contact time. Therefore, long pretreatment time with high acid concentration enables further catalytic reaction of the solubilized sugars by the free protons (H^+) to by-products, leading to lower xylose and glucose yield [32]. The positive effect of acid concentration and time was also reported during dilute acid pretreatment of dried distillers grains with soluble DDGS [33].

Also, for this study, a similar trend was observed in the interaction between acid concentration and temperature. Under low-temperature conditions, the increase in acid concentration enhanced xylose and glucose yield, while at high temperatures, high sugar yield was obtained at low acid concentration (Figs. 2b and 3b). Contour lines indicate that the maximum xylose yield was obtained when the acid concentration and the temperature were in the range of 2.9–3.99% and 110–122 °C, respectively. At less than 2.9% acid concentration, there was a steady increase in xylose yield with temperatures up to 122 °C. A similar trend was observed for glucose yield. The observed trend could be because temperature influences the rate of reaction during DAP. Hence, increasing the temperature facilitates faster diffusion of protons and hydrolysis of bonds leading to an increase in sugar yield even at low acid concentration. However, as the acid concentration increases with temperature, the solubilized sugars are speedily converted to inhibitors leading to a decrease in the sugar yield [7]. This trend was also observed by Cao et al. [31], who reported enhanced xylose yields when the phosphoric-acid concentration was high and the temperature was low, or the phosphoric-acid concentration was low and the temperature was high.

The interaction between temperature and time in this study was insignificant for sugar yield. The contour plot (Fig. 3c) indicates that, under similar temperatures, increasing the time above 1 h did not lead to any significant increase in sugar yield. This may be attributed to the fact that the temperature range within the scope of this study was not sufficiently high to instigate hemicellulose solubilization without the effect of the acid catalyst (≥2.9%). Hemicellulose and lignin decomposition had been shown to occur above 120 °C [34]. Hence, several dilute acid pretreatment studies were carried out at a temperature greater than 120 °C [35]. Therefore, heating alone irrespective of the duration of pretreatment did not lead to significant sugar yield.

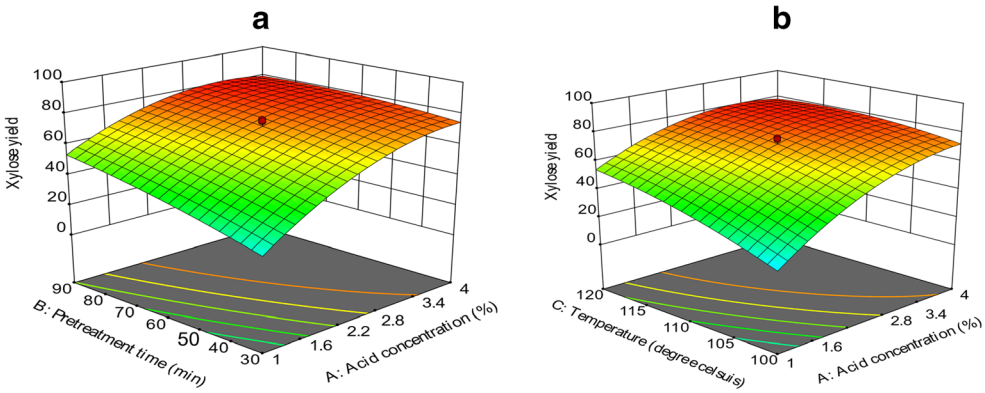

Fig. 2 Response surface graph showing the interactive effect of **a** acid concentration and time; **b** acid concentration and temperature on xylose yield

The concentration of inhibitors increased steadily when any two interacting factors increased from the lowest to the highest values. Maximum inhibitor concentrations were formed when any two factors were at the maximum (Fig. 4a–c). This trend is related to the severity of the pretreatment which is influenced by acid concentration, temperature, and time [35]. When any two factors were at a maximum, the severity of the pretreatment was high, and under severe conditions, the conversion of solubilized sugars into inhibitors (formic acid, acetic acid, furans) was accelerated [22].

3.5 Characteristics of solid residue pretreated at optimal conditions

The solids recovered after dilute acid pretreatment of CH at optimal conditions contained 31% cellulose, 2.5% hemicellulose, and 4.1% lignin. This is equivalent to about 72% of the initial cellulose remaining in the pretreated solids which could be saccharified enzymatically and fermented to bioethanol.

3.6 X-ray diffraction

X-ray diffraction was done to determine crystallinity differences in untreated and dilute acid pretreated CH samples. Some studies indicate that crystallinity imparts recalcitrance to biomass hindering digestibility [36]. Compared with the untreated CH (NCH), diffractogram results show that the crystalline peak present at an angle 2 = 22.5 ° (crystalline cellulose) becomes extended and sharper, in addition to multiple new crystalline peaks in the pretreated sample (PCH) (Fig. 5). The crystallinity index increased from 55 to 65% in untreated and pretreated CH, respectively. This increasing trend in the crystallinity index is consistent with previous studies and has been attributed to the removal of amorphous portions in the biomass leading to an improvement in digestibility. The crystallinity index of dilute phosphoric acid pretreated (4% v/v) cauliflower wastes was slightly increased from 41.31 to 45.28% and 30.95 to 32.57% for cauliflower stalk and leaf, respectively [37]. Also, the crystallinity index and crystallite size of dilute acid pretreated rice straw increased from 40.84 to 51.49% and 2.60 to 3.08 nm compared with native samples [38].

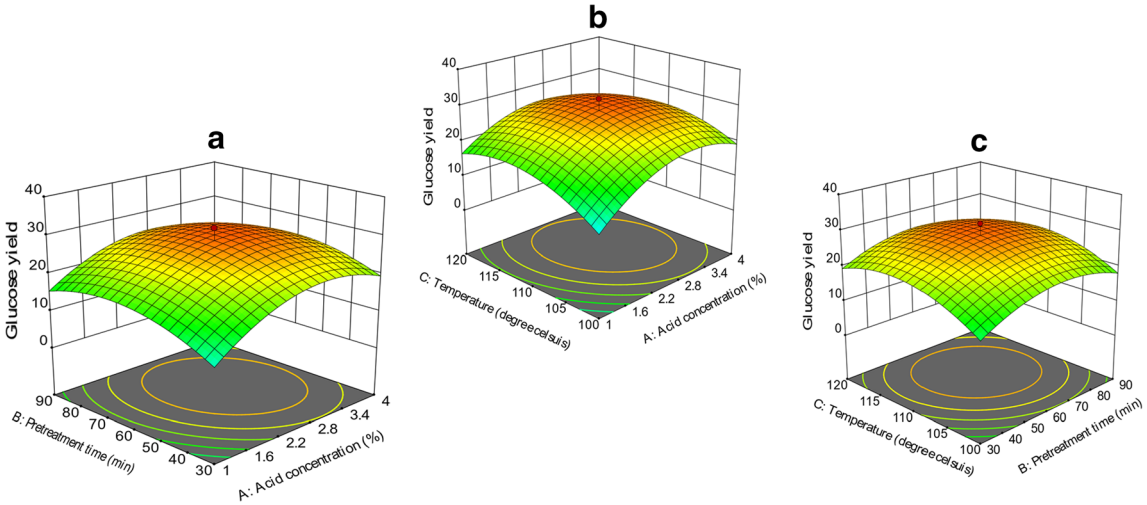

Fig. 3 Response surface graph showing the interactive effect of **a** acid concentration and time; **b** acid concentration and temperature; **c** temperature and time on glucose yield

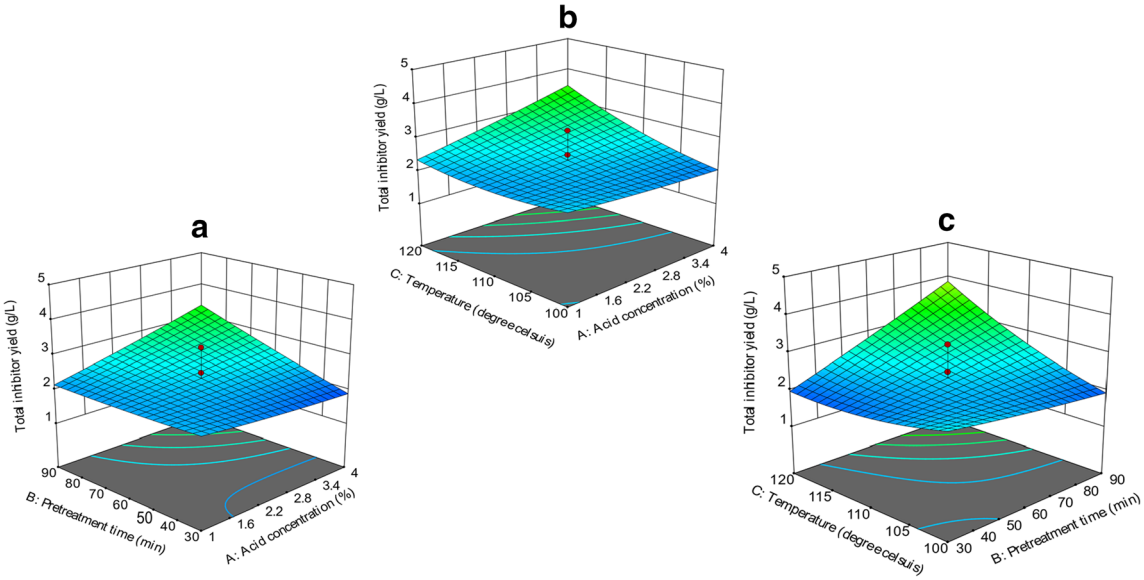

Fig. 4 Response surface graph showing the interactive effect of **a** acid concentration and time; **b** acid concentration and temperature; **c** temperature and time on inhibitor concentration

3.7 FTIR analysis

Chemical changes in the native and pretreated samples were observed using FTIR (Fig. 6). The peak at 1507 cm^{-1}, attributed to the C–C widening in the aromatic ring of lignin, occurred only in the pretreated sample. Other characteristic lignin bands at 1600 cm^{-1} (C=C stretching vibration in lignin), 1372 cm^{-1} (phenolic hydroxyl group), 1320 cm^{-1} (C–O stretching of the syringyl ring) as well as ether (ar–C–O–C–al) and ester linkage between hemicellulose and lignin assigned to peaks 1234 cm^{-1} and 1727 cm^{-1} respectively exhibited weaker bonds in the pretreated sample compared with native samples, indicating cleavage of the linkages between lignin and carbohydrates and a disruption in the lignin structure due to dilute acid treatment [39]. Absorption peaks at 897 cm^{-1} (β-1,4-glycosidic linkages associated with crystalline cellulose) and 1153 cm^{-1} (asymmetric elongation of C–O–C within cellulose) occurring only in the pretreated sample indicate damage to β-1,4-glycosidic linkages within the cellulose structure [40]. Other peaks associated with cellulose and hemicellulose observed around 1027 cm^{-1} (C–O, C=O stretching of hemicellulose or cellulose), 1364 cm^{-1} (C–H bending vibration in cellulose and hemicellulose), 1422 cm^{-1} (CH_2 scissoring motion in cellulose), 2900 cm^{-1} (C – H and CH_2 stretching), and 3200–3300 cm^{-1} (OH stretching) also exhibited weaker bonds in the pretreated sample compared with the native sample.

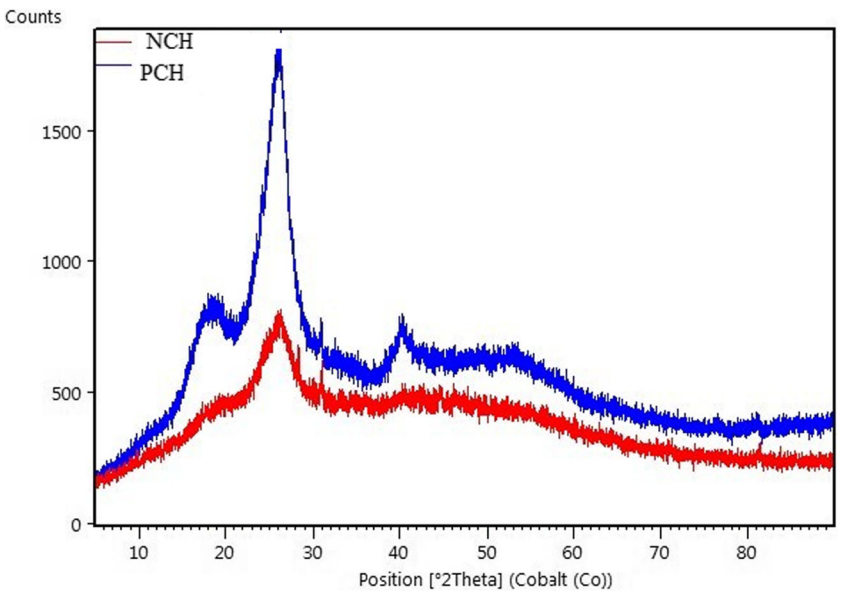

Fig. 5 X-ray diffraction peaks for native CH (NCH) and pretreated CH (PCH)

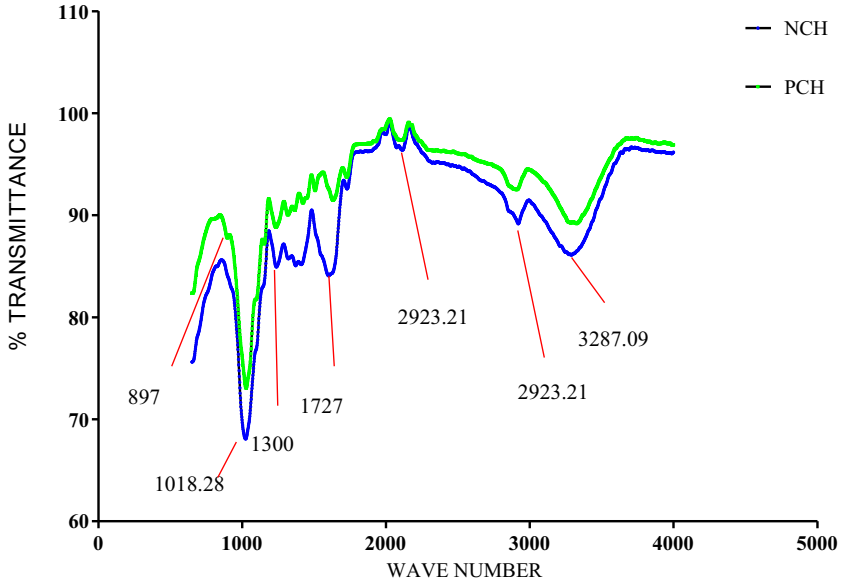

Fig. 6 Fourier transform infrared spectroscopy of native and pretreated CH

3.8 Scanning electron microscopy

SEM images revealed a complete and compact lignocellulosic structure in the untreated cowpea haulm (Fig. 7a). Multiple cracks and fiber porosity of cowpea haulm was observed in the SEM image after DAP pretreatment under the optimized conditions (Fig. 7b). The abrasion and fiber disruption in dilute acid pretreated biomasses may be attributed to the solubilization of the amorphous portion correlated with the enhanced acid effects on the biomasses with the help of high temperature and pressure from the pretreatment [41]. Similarly, dilute sulfuric acid pretreated cassava residues exhibited fragmented surfaces compared with the untreated residues which were compact and intact [42].

3.9 Detoxification and fermentation of prehydrolysate

The prehydrolysate obtained under optimal conditions contained glucose 13 g/L, xylose 6.5 g/L, and arabinose 2.03 g/L. The inhibitors such as furfural 0.8 g/L, 5-HMF 0.2 g/L, formic acid 1.44 g/L, and acetic acid 0.05 g/L were also detected. The concentration of inhibitors obtained in this study was lower than inhibitory concentrations for ethanologens. Acetic acid is inhibitory to *S. cerevisiae* when the concentration exceeds 1.5 g/L while concentrations of furfural and HMF > 1 g/L would inhibit the bioethanol production process [43]. However, inhibitors exert varying synergistic effects on ethanologens depending on the inhibitor composition [44, 45]. Moreover, the highly varied composition of inhibitory com-

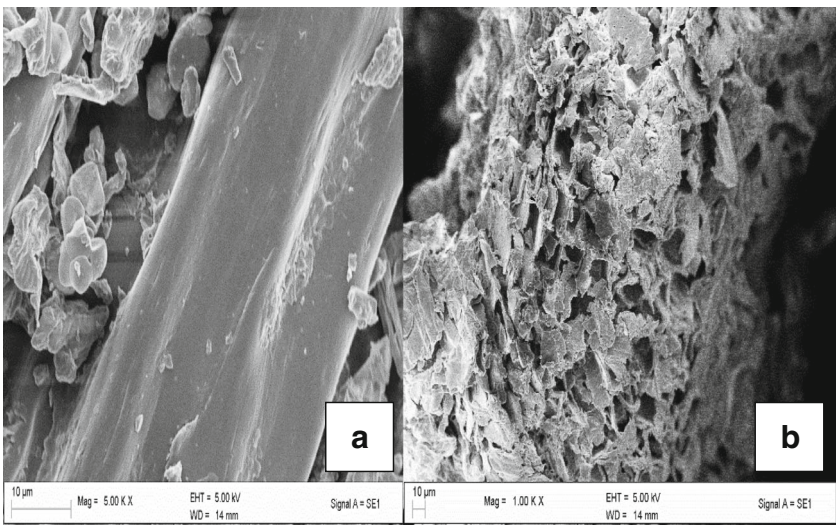

Fig. 7 Scanning electron micrograph of (**a**) native and (**b**) pretreated cowpea haulm

pounds that could be produced during pretreatment hinders an accurate quantification of all inhibitors [46]. Hence, detoxification is an essential and commonly applied step after DAP to remove these inhibitory substances. Overliming is a standard procedure to reduce the toxicity of the prehydrolysate by the addition of alkali ($Ca(OH)_2$ or NaOH) to increase the pH up to 12, followed by the adjustment of pH to slightly acidic conditions resulting in the precipitation of inhibitors which can be removed by centrifugation [47–49]. The alkaline conditions facilitate aldol reactions between ketones and aldehydes, and the oxidation of carbonyl compounds mitigates their toxicity [46]. The detoxified hydrolysate in this study contained glucose 10.8 g/L, xylose 4.6 g/L, arabinose 1.03 g/L, furfural 0.1 g/L, and formic acid 0.29 g/L. There was complete removal of acetic acid, 5-HMF, and 80% removal of furfural and formic acid after the detoxification step. Deshavath et al. [50] reported a 23.7%, 20%, 11.9%, and 12.9% reduction in furfural, 5-HMF, formic acid, and acetic acid, respectively, after overliming of dilute acid pretreated sorghum stalks. However, there was approximately 23% and 29% loss of glucose and xylose. In the studies of Zhang et al. [46], 7.2% sugar loss was reported following overliming of poplar prehydrolysate. Mohagheghi et al. [51] also observed that xylose loss in corn stover hydrolysate rose with increasing overliming pH from 7% at pH 9 to 34% at pH 11.

Fermentation of detoxified acid hydrolysate using a co-culture of *S. cerevisiae* BY4743 and *S. stipitis* wild type (PsY633) produced a maximum ethanol titer of 6.22 ± 0.17 g/L (Fig. 8), after 36 h incubation, which corresponds to an ethanol yield of 0.38 g/g sugar consumed, with 0.17 g/L/h productivity and 74% fermentation efficiency. About 88% of the initial glucose was consumed within 24 h and 3.7 ± 0.3 g/L ethanol was produced, which represents 59% of the total ethanol produced. Xylose utilization started after glucose was exhausted. The results obtained in this study are comparable with previous literature. In a study conducted on sugarcane straw, about 12.67 g ethanol/L was produced from 33.45 g/L glucose, corresponding to 62.74% fermentation efficiency after dilute acid pretreatment and enzymatic hydrolysis [52]. Keshav et al. [53] reported a maximum ethanol yield of 11.64 ± 0.48 g/L from the fermentation of detoxified acid pretreated cotton stalk hydrolysate using a co-culture of *S. cerevisiae* VS3 and *P. stipitis* NCIM3498. In this study, the maximum ethanol yield of 0.47 g/g with a productivity of 0.24 g/L/h was attained at 48 h incubation. Similar studies by Láinez et al. [54] reported maximum ethanol concentrations of 22.79 g/ L after 24 h fermentation by *S. cerevisiae* from 50.88 ± 0.54 g /L glucose, corresponding to 87.63% fermentation efficiency.

3.10 Simultaneous saccharification and fermentation with prehydrolysis

Saccharomyces cerevisiae BY4743 was introduced following a 24 h presaccharification that resulted in an initial sugar concentration of 14.8 g/L glucose (53% of the theoretical yield) and 2.01 g/L xylose (90% of the theoretical yield) accumulated in the fermentation broth. The maximum ethanol titer of 9.45 g/L obtained in this study within 18 h of fermentation corresponded to an ethanol yield of 0.42 g/g glucose and productivity of 0.53 g/L/h (Fig. 9).

The results obtained in this study agree with earlier reports which have indicated that the application of a prehydrolysis step prior to SSF facilitated an ethanol yield ≥ 50% of the theoretical yield [55, 56]. Following a 24-h prehydrolysis and SSF of sugar straw hydrolysate, Mesa et al. [52] observed an ethanol concentration of 14.47 g/L corresponding to 72.37% glucose utilization. Fernandes et al. [57] observed an ethanol concentration of 5.7 g/L corresponding to 58% of

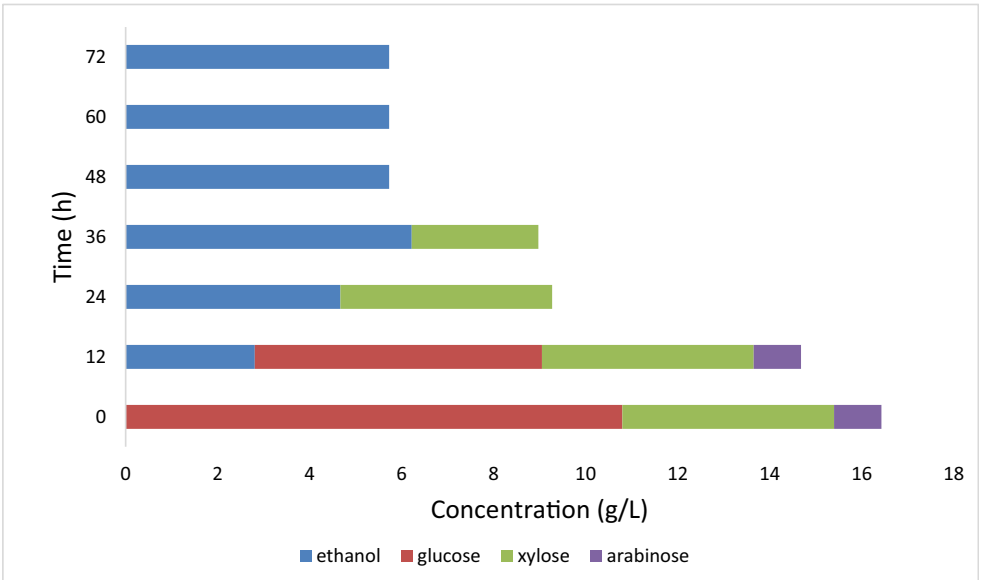

Fig. 8 Time course for sugar consumption and ethanol production in the SHF process

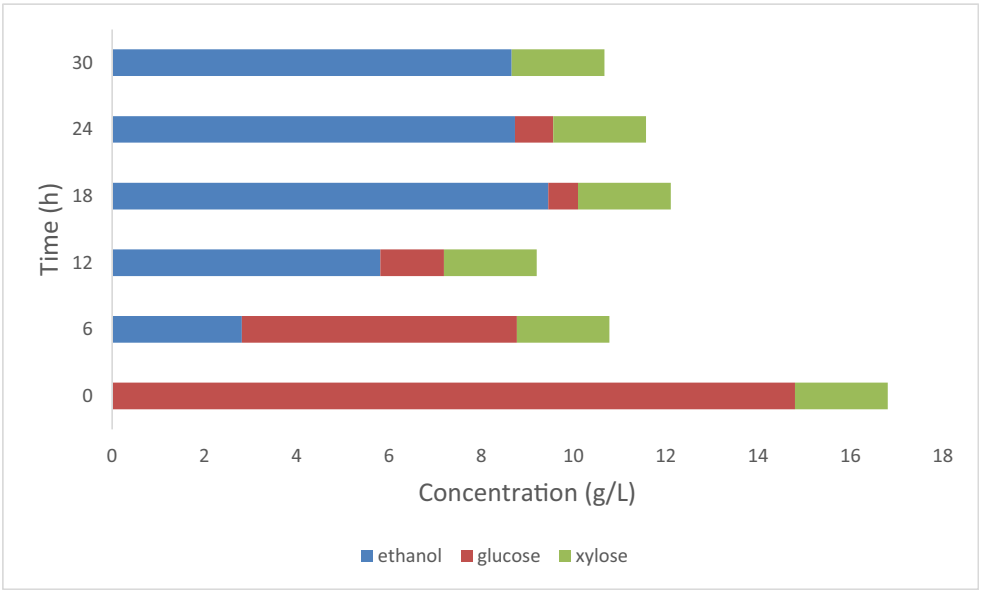

Fig. 9 Time course for sugar consumption and ethanol production in the SSF process

ethanol yield from PSSF of dilute acid pretreated olive pomace. Bioethanol concentrations and bioethanol conversions for the PSSF of sequential alkalic salt and dilute acid pretreated corncob were 36.92 g/L and 62.36 %, respectively [14]. The variation in the ethanol concentration in various studies is due to the differences in the amount of fermentable sugar subjected to the fermentation process [58].

Considering the pretreatment and enzymatic hydrolysis step in this study, a total sugar concentration of 44.9 g/L corresponding to 93% of the theoretical sugar in CH was hydrolyzed, indicating the effectiveness of DAP. However, only 84.5% of the hydrolyzed sugar was recovered for fermentation, due to sugar loss in the pretreatment and detoxification stage. High sugar yields above 90% have been reported for DAP of biomasses such as cotton, agave, etc. [6, 30]. The fermentation of both the hydrolysates from DAP and the enzymatic stage in this study gave a total ethanol titer of 15.67 g/L corresponding to 75% conversion efficiency. This was similar to the ethanol yield of 73.3% observed from the fermentation of mixed sugars obtained from dilute acid hydrolysis and enzymatic hydrolysis of *Agave lechuguilla* using *Escherichia coli* MM160 [6]. The conversion of both pentose and hexose sugars has been shown to improve ethanol titers [59, 60].

4 Conclusion

The physicochemical composition, pretreatment, and fermentation of CH were investigated in this study. Our results suggest that CH is a promising feedstock for bioethanol due to its high polysaccharide and low lignin composition. The pretreatment conditions for CH was optimized by response surface methodological approach. The quadratic model was validated and resulted in maximum xylose and glucose yield with minimum inhibitor concentration at optimum conditions. In addition, 85% of the theoretical sugar yield in CH could be recovered with dilute acid hydrolysis indicating the effectiveness of this optimized pretreatment strategy for biosugar production from CH. Furthermore, the fermentation of sugar hydrolysates obtained from both the pretreatment and enzymatic stage was useful to improve bioethanol yield from CH. However, subsequent studies should focus on the optimization of the enzymatic saccharification and fermentation conditions, which could further enhance the bioethanol yield.

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Author contribution Somiame Itseme Okuofu: methodology, formal analysis, investigation, writing—original draft; Prashant Bhagwat: writing—review and editing; Abe Shegro Gerrano: resources, writing—review and editing; Suren Singh: writing—review and editing, co-supervision; Santhosh Pillai: conceptualization of idea, project leader, funding acquisition, supervision, writing—review and editing.

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Availability of data and material The data that support the findings of this study are available from the corresponding author upon reasonable request.

Code availability Not applicable

Declarations

Ethics approval Not applicable

Consent to participate Not applicable

Consent for publication Not applicable

Conflict of interest The authors declare that they have no conflict of interest.

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