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SCIENTIFIC PAPER

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IMPROVING ENZYMATIC SACCHARIFICATION OF CASSAVA STEM USING PEROXIDE AND MICROWAVE ASSISTED PRE-TREATMENT TECHNIQUES

Article Highlights

- Impact of peroxide and microwave pre-treatment on enzymatic saccharification of cassava stem was evaluated
- Microwave assisted pre-treatment was found to be effective in enhancing enzymatic saccharification of cassava stem
- Optimal factors were 1.5% NaOH, 31 min and 132 W microwave power
- Spectral and SEM studies give the information of physicochemical structural changes in the stem after pre-treatment

Abstract

The effectiveness of microwave assisted alkali (MAA) and alkaline hydrogen peroxide (AHP) pre-treatment methods in improving the enzymatic saccharification of cassava stem was investigated. Ground cassava stems were by MAA method by varying microwave power, NaOH concentration and pre-treatment time. AHP method was performed at various H_2O_2 concentrations, pre-treatment temperatures and times. The results showed that reducing sugar yield was higher from MAA pretreated stem when compared with AHP pre-treatment, which demonstrated that MAA pre-treatment was effective in releasing sugars. SEM studies on the pre-treated samples revealed extensive distortion of fibres in MAA pre-treated than AHP pre-treated samples, which showed pores and cracks in the fibrous structure. Spectral studies showed the change in the chemical structure of pre-treated samples. The work revealed that the studied pre-treatment methods were effective in improving the enzymatic saccharification of cassava stem.

Keywords: cassava stem, pre-treatment, microwave, hydrogen peroxide.

There is an increasing worldwide interest in using fuels from renewable resources, for instance ethanol, due to a rising alarm about the depletion of our world's fossil fuel reserves and its greenhouse effect [1]. The production of value added fuels and chemicals from biomass through bioconversion offers potential, economic, environmental and planned advantages over products from fossils [2]. Lignocellulosic biomass, such as agro-industrial residues, which are cheap and abundant in nature, seems to be potential feedstock for the production of second generation biofuels [3]. Conversion of lignocellulosic biomass to fermentable sugars can be accomplished by dilute acid or cellulase [4]. As enzymatic hydrolysis is milder and more specific and does not produce byproducts [5], it seems to be the most promising approach for ethanol production. However, the hydrolytic process is affected by two categories of factors such as structural substrate factors (degree of crystallinity, degree of polymerization, structural composition and available surface area) and mechanical factors (thermal inactivation, cellulase adsorption, and synergism) [6,7]. Consequently, prior to enzymatic hydrolysis, lignocel-

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lulosic biomass require pre-treatment to increase their digestibility and accessibility by cellulolytic enzymes to cellulosic fibres [8].

Cassava (*Manihot esculenta* Crantz.) crop is cultivated in an area of 20 million ha, with a total production of 256.53 million t in approximately 102 countries of the world and it meets approximately 6% of the world's dietary energy. In India, with a total production of 9.62 million t, it is cultivated in 0.28 million ha. The mature cassava plant contains 50% roots, 44% stems and 6% leaves. The primary agricultural residues from cassava include leaves and stems and the secondary processing waste comprises the cassava peels. The annual yield of dry primary and secondary processing waste in India come to 2.24 and 0.48 million t, respectively [9]. Cassava stem, after harvesting of the starchy roots, is mostly abandoned or burned in the wild. Only 10-20% of the stems are used as mushroom substrates and for propagation, or recycled to maintain soil fertility. As cassava stem is lignocellulosic in nature and to fully utilize it as a potential feedstock for bioethanol production, pre-treatment is required in order to render the cellulosic fibers more accessible to enzymatic hydrolysis. The studies on pre-treatment of cassava stem by hydrothermal treatments [9], dilute acid hydrolysis at high temperatures [10] and wet oxidation [11] makes the process expensive because of the use of steam and there arises a need for the use of corrosion resistant reactors [12].

Microwave irradiation has been widely used for process operations because of the shorter processing time and higher heating efficiency [13]. Microwave (MW) pre-treatment of rice straw and bagasse slurry has been found to improve enzymatic hydrolysis [14]. It has been reported that microwave irradiation could modify the ultra-structure of cellulose, degrade lignin and hemicellulose and thereby increase its enzymatic degradability [15,16]. Alkaline pre-treatments at low temperatures have been reported to be effective although most of the pre-treatment studies have focused on acidic conditions and temperatures greater than 150 °C. AHP pre-treatment have been applied to a number of lignocellulosic biomass and conditions. Following SO_2 steam pre-treatment of softwood or dilute acid pre-treatment of corn stover, AHP has also been studied as a post-treatment for delignification [17]. Hydrogen peroxide has the advantages of not leaving residues in the biomass, as it degrades into oxygen and water and practically there is no formation of secondary products [8].

In this work, the efficacy of microwave assisted alkali pre-treatment (MAA) and alkaline hydrogen peroxide (AHP) pre-treatment technologies were studied in improving the release of reducing sugars from cassava stem after enzymatic saccharification. These two processes were selected because both are alkaline processes, and are predicted to cause less sugar loss than acid processes [18]. The ultra-structural changes occurring during pre-treatment are also studied.

MATERIALS AND METHODS

Substrate

Cassava stems were collected from a farm in Chennimalai, Tamilnadu, India, and washed manually using tap water to remove dirt, dried at 50 °C in a hot air oven for 5 days, milled, screened to select the fraction of particles with a size of 800 μm, homogenized in a single lot and stored at 4 °C until needed.

Pre-treatment

The pre-treatment methods evaluated were AHP and MAA. The effect of hydrogen peroxide concentration (0.9-6%), pre-treatment time (9.54-110.4 min) and temperature (23.1-56.8 °C) were evaluated during AHP method. The pre-treatment solution of alkaline peroxide was prepared by dissolving H_2O_2 in distilled water and adjusting the pH to 11 with sodium hydroxide. Cassava stem (5 g) was treated with 100 ml of the pre-treatment solution in 250 ml flasks in an orbital shaker (REMI CIS-24BL) agitated at 100 rpm. In MAA pre-treatment method, the effect of sodium hydroxide concentration (0.6-2.3%), microwave power (106-173 W) and pre-treatment time (13-46 min) were evaluated under atmospheric pressure. Cassava stem (5 g) was treated with 100 ml sodium hydroxide solution with solid liquid ratio of 1:5 (g/ml) and placed in microwave (ENERZI Microwave Systems Pvt. Ltd, 2.45 GHz, max. output 800 W). After pre-treatment, the contents were filtered through Whatman No. 1 filter paper and the supernatant was subjected to sugar, furfural and acetic acid analyses. The alkali in the solid residue was removed by washing with distilled water repeatedly. The residues were dried at 100 °C until constant weight was observed and used to calculate the mass loss during pre-treatment. The wet residue was further subjected to enzymatic hydrolysis. The effect of variables on reducing sugar yield was evaluated using central composite design (CCD) using 20 experimental runs.

Enzymatic hydrolysis

The pre-treated cassava stem was suspended in 100 ml of 50 mM sodium citrate buffer (pH 4.8) containing amylase, amyloglucosidase, cellulase and β-glucosidase supplemented with 0.02% sodium

azide under aseptic conditions. As cassava stem contains considerable amount of starch, it was treated with amylase (Himedia® Laboratories, 200U/g biomass) and amyloglucosidase from *Aspergillus niger* (70 U/mg, Sigma-Aldrich Co, St. Louis, MO). Cellulases (SRL, India) loading was 20 FPU/g biomass. *β*-Glucosidase (SRL, India) was added to completely convert cellobiose to glucose, loading 1.00 IU/g biomass. The reaction mixture was incubated in a shaking incubator at 50 °C and 120 rpm [19]. Aliquots were taken regularly at intervals and analyzed for reducing sugars after deactivating the enzymes by boiling. The value of reducing sugar yields used for the statistical analysis was picked at the reaction time after which no significant changes in the variable was noticed. Experiments were conducted in triplicate and the mean values were used in the analyses.

Analytical methods

The biomass under study was analysed for the biochemical composition. The total starch content in cassava stem was determined by using the hydrolytic enzymes such as amylase and amyloglucosidase. Cassava stem slurry (5%) was treated with amylase (50.0 mg) at 90 °C for 45 min at pH 5.5. After 45 min, the slurry was brought to room temperature (30±1 °C) and its pH was lowered to 4.6. Amyloglucosidase (70 U) was then added to it and kept for incubation at room temperature for 24 h and the glucose released was assayed by anthrone method of Sadasivam and Manickam [20]. Since glucose was originally present in the samples, enzyme blank was also kept to nullify the values. Starch content was calculated from the glucose value using the Morris factor, 0.9. The crude protein content of the cassava stem was determined by Kjeldahl method using automatic Nitrogen digestion and distillation system (Biokjel, TechnoReach). The percentage of protein was calculated by multiplying nitrogen content with 6.25.

Ash content was determined by igniting the sample in muffle furnace at 550 °C for 7 h. The ash percentage was calculated from the weight of ash. Cellulose was estimated by the method of Sadhasivam and Manickam [20] using acetic/nitric reagent. Cassava stem (0.5 g) was boiled with 3 ml acetic/nitric reagent for 20 min and cooled. The suspension was centrifuged at 4000 rpm for 20 min and the supernatant was discarded. The residue was washed with distilled water and suspended in 10 ml of 67% H_2SO_4 for 1 h during which hydrolysis takes place. The hydrolysate was appropriately diluted and was added to the diluted aliquot 10 ml anthrone reagent. The colour developed was read spectrophotometrically at 630 nm. The cellulose content was calculated from the optical density of pure cellulose.

Hemicellulose content was determined by detergent extraction method of Goering and Van Soest [21] by taking the difference of neutral detergent fibre (NDF) and acid detergent fibre (ADF). NDF was determined by boiling 1 g of cassava stem with 10 ml of cold neutral detergent solution, 2 ml decahydronaphthalene and 0.5 g sodium sulphite for 1 h. After 1 h, the mixture was filtered, washed with acetone and dried at 100 °C for 8 h. The weight of the dry residue is noted as NDF. ADF was determined by heating 1 g cassava stem with 100 ml acid detergent solution for 1 h. After 1 h, the mixture was filtered, washed and dried at 100 °C for 8 h. The weight of the dry residue was noted as ADF. Lignin content was determined by subtracting cellulose and ash content (%) from *ADF* (%).

The reducing sugar concentration was determined using 3,5-dinitrosalicyclic acid (DNS) method [22]. Glucose, xylose, arabinose and acetic acid were determined using high performance liquid chromatography (HPLC) coupled with UV and refractive index detector (Shimadzu, Japan) and C18 column (Enable C18G, 5 µm, 250 mm×4.6 mm) using acetonitrile and water as mobile phase at a flow rate of 0.6ml/min. Furfural was detected at 280 nm [23]. The morphological differences of untreated and pre-treated cassava stem were examined using scanning electron microscopy (JEOL Ltd., Tokyo, Japan). The changes in the functional groups of cassava stem before and after pre-treatment were analyzed by Fourier-transform infrared spectroscopy (FTIR) (Bruker ALPHA, Germany). The spectra were recorded between 4000 and 600 cm^{-1}.

Statistical analysis

ANOVA and regression analyses were executed to study the direct and interactive effects of variables. Coefficients of the model developed were analyzed for their significance and the insignificant ones were eliminated from the model. The optimal levels of the variables and predicted response were obtained by solving second-order polynomial model for maximization of response using Design Expert software (version 8.0.7.1, Stat-Ease, Inc., USA).

RESULTS AND DISCUSSION

Pre-treatment of lignocellulosic resources are regularly studied to enhance the hydrolysis of cellulose by reducing the crystallinity of cellulose fibre and, lignin and hemicellulose contents, and increasing the porosity of cellulose [24]. The type of pre--treatment method adopted influences the structural

modifications, based on which the extent of enzymatic hydrolysis of the biomass depends [25]. CCD with five replicates in the central point was performed for each pre-treatment considered to study the influence of process parameters.

Composition of cassava stem

Characterization of the lignocellulosic biomass is the key step in designing appropriate pre-treatment techniques and the production of bioethanol depends on biomass quality. Raw cassava stem contained 18.4% starch, 23.3% cellulose, 27.6% hemicelluloses, 21.8% lignin, 3.8% protein and 3.5% ash (% dry weight). The results indicated that cassava stem could be a potential feedstock as the major constituents are carbohydrates.

Effect of pre-treatment on composition of liquor

It is necessary to analyze the sugars and the degradation products in order to identify the mechanism by which the pre-treatment methods influence the process. It has been established that furfural is the direct degradation product of xylose. Moreover, acetic acid is produced from the decomposition of O-acetyl groups in the branched chain of hemicellulose [26]. The more the acetic acid recovered, the more the hemicellulose decomposed. Xylose was the major product in all experiments (Table 1). Furfural concentrations ranged from 0.26 to 0.63 g/L in MAA method and it was negligible in AHP method. Acetic acid release was below the inhibitory concentration (6 g/L) which ranges from 1.10 to 5.56 g/L in AHP method and from 1.42 to 6.43 g/L in MAA method, above this level may affect the activity of cellulase and production of ethanol [27].

Effect of pre-treatment on mass loss

Table 2 shows the solid loss during AHP and MAA pre-treatment of cassava stem. In AHP method, H_2O_2 concentration and pre-treatment time had a significant effect on mass loss. The mass loss ranged from 14.37 mass% at low concentration of H_2O_2 to 41.87% at high concentration of H_2O_2. Although both H_2O_2 concentration and pre-treatment time contributed to the mass loss, H_2O_2 concentration was found to have the greatest impact on mass loss. Banerjee [17] observed a 35% decrease in total insoluble biomass after AHP pre-treatment of corn stover. In MAA method, NaOH concentration was found to have significant effect on mass loss, which ranged from 21.47 to 45.33 mass%. The mass loss increased as the concentration of NaOH was increased. McIntosh and Vancov [28] have found loss of hemicelluloses following exposure to alkaline substances during the pre-treatment process. Alkali is effective at solubilizing grass xylans although much of the solubilised

Table 1. Composition (g/L) of liquor obtained after the pre-treatment of cassava stem

Run	AHP				MAA				
	Glucose	Arabinose	Xylose	Acetic acid	Glucose	Arabinose	Xylose	Acetic acid	Furfural
1	0.24	0.82	4.15	3.75	0.32	0.98	4.56	5.42	0.54
2	0.31	0.92	4.24	4.45	0.41	1.26	6.54	5.42	0.62
3	0.26	1.23	6.45	4.21	0.24	1.34	6.89	5.32	0.42
4	0.26	0.87	4.24	3.54	0.42	0.96	4.36	4.34	0.34
5	0.28	0.94	5.67	3.23	0.45	1.06	6.01	4.32	0.58
6	0.25	1.02	6.34	3.56	0.38	1.24	6.52	5.67	0.32
7	0.32	0.84	4.27	1.27	0.41	1.56	6.32	5.83	0.46
8	0.26	0.78	3.45	1.43	0.37	0.86	3.89	4.45	0.42
9	0.24	1.12	6.24	2.67	0.34	0.98	5.28	4.76	0.54
10	0.38	1.34	5.45	3.74	0.4	0.89	4.63	6.43	0.48
11	0.35	0.87	3.43	2.45	0.29	1.24	5.41	5.42	0.36
12	0.32	0.96	5.67	2.32	0.39	0.92	5.26	6.41	0.41
13	0.31	1.32	7.24	5.56	0.38	1.22	4.92	5.62	0.5
14	0.24	0.86	4.01	1.1	0.32	0.74	3.21	6.43	0.63
15	0.21	0.94	4.21	3.54	0.45	0.82	5.01	5.42	0.52
16	0.25	0.96	5.32	1.62	0.35	0.78	3.46	3.21	0.26
17	0.27	0.92	5.32	2.48	0.25	0.98	5.43	6.34	0.32
18	0.24	1.02	6.83	4.65	0.21	0.92	6.28	2.85	0.54
19	0.31	1.23	6.1	3.21	0.46	1.13	5.89	5.43	0.64
20	0.26	0.92	5.31	2.83	0.18	0.86	3.23	1.42	0.21

Table 2. Design matrix presenting reducing sugar yield after hydrolysis and percentage of mass loss for the pre-treated cassava stem with alkaline hydrogen peroxide (AHP) and microwave assisted alkali (MAA)

Run	AHP Experimental factors			MAA Experimental factors			Reducing sugar yield, mg/g pulp		Mass loss, %	
	H_2O_2 conc., %	Temperature, °C	Pre-treatment time, min	NaOH conc., %	Microwave power, W	Pre-treatment time, min	AHP	MAA	AHP	MAA
1	3.5	40	60	2.3	140	30	409.0	397.7	38.54	38.23
2	3.5	40	60	1	160	40	412.7	321.7	34.63	25.36
3	2	50	30	1.5	140	30	286.3	456.0	25.32	44.24
4	6.0	40	60	1.5	140	30	442.7	451.7	41.87	43.97
5	2	50	90	1.5	140	30	297.3	457.3	21.54	45.14
6	2	30	90	2	120	20	286.0	431.0	20.32	35.36
7	0.9	40	60	1.5	140	30	243.7	457.3	14.37	36.22
8	3.5	40	60	2	120	40	421.7	431.3	36.49	38.28
9	3.5	40	60	2	160	20	417.7	289.0	37.25	32.34
10	5	30	90	1	160	20	447.7	263.7	39.43	31.23
11	3.5	40	60	1.5	140	30	415.7	459.0	36.14	44.55
12	2	30	30	1.5	140	46.8	274.3	416.7	24.59	44.24
13	5	50	90	1.5	140	13.1	416.0	331.3	36.22	31.22
14	3.5	40	9.54	1.5	173.6	30	254.7	318.0	18.31	41.37
15	5	30	30	1.5	140	30	367.0	453.0	30.95	45.33
16	3.5	23.1	60	1	120	40	367.3	354.0	30.23	35.39
17	3.5	40	60	2	160	40	396.3	378.0	36.88	38.42
18	3.5	56.8	60	1.5	106.3	30	413.7	420.7	35.77	33.24
19	5	50	30	1	120	20	387.3	361.3	31.24	35.32
20	3.5	40	110.4	0.6	140	30	347.3	274.7	35.39	21.47

xylan may reprecipitate when pH is decreased for hydrolysis [17].

Effect of alkaline hydrogen peroxide pre-treatment

Alkali pre-treatment incorporates the use of alkaline solutions to remove various uronic acid substitutions on hemicellulose and lignin that reduces the enzyme accessibility to cellulose and hemicellulose. Generally, alkaline pre-treatment is found to be more effective on herbaceous crops and agricultural residues than on wood materials. Peroxide pre-treatment reduces cellulose crystallinity by oxidative delignification and enhances enzymatic conversion. Increased cellulose availability and lignin solubilisation were observed during the peroxide pre-treatment of wheat straw, Douglas fir and oak [29].

Table 2 shows the design matrix, reducing sugar yield after hydrolysis and percentage of mass loss for the pre-treated cassava stem with AHP. An analysis of variance was performed and the results are shown in Table 3. The results specified that the fitting model ($p < 0.0001$) was highly significant. The coefficient of determination (R^2) was 0.959, which indicated that the model could explain 95.9% variability of the response variable. The adjusted value of coefficient of determination ($R^2 = 0.944$) was also satisfactory, indicating the significance of the model.

Analysis of the response trend showed that the model could explain the effect of hydrogen peroxide concentration, temperature and pre-treatment time on reducing sugar yield satisfactorily. The coefficients calculated for each variable by regression analysis are presented in Table 3. The regression coefficients of linear and squared terms for the variables such as, H_2O_2 concentration and pre-treatment time were highly significant ($p < 0.05$). The interactive effect of H_2O_2 concentration and pre-treatment time was significant for reducing sugar yield at the 90% confidence level ($p < 0.1$). The temperature had no significant effect on reducing sugar yield. The positive coefficients of H_2O_2 concentration and pre-treatment time suggest that these factors had a positive influence on reducing sugar yield. Alkaline peroxide pre-treatment of wheat straw has been studied and found to remove 65.97% lignin and 20.10% hemicelluloses of raw materials. AHP pre-treated wheat straw (1.5% NaOH, 50 °C, 6h and 0.3% H_2O_2, 6 h) showed 97.01% enzymatic hydrolysis rate [4]. Rabelo *et al.* [8] have obtained 494.7 mg/g reducing sugar on enzymatic hydrolysis from sugarcane bagasse pre-treated at 20

Table 3. ANOVA for quadratic model[a] and regression coefficients of the model for reducing sugar yield after hydrolysis of AHP pre-treated cassava stem; coefficient of determination, R^2 = 0.959; adjusted R^2 = 0.944; coefficient of variation (CV): 4.28%; SS, sum of squares; DF, degree of freedom; MS, mean square; A: H_2O_2 concentration (%), B: temperature (°C), C: pre-treatment time (min); p value less than 0.05 indicates model terms are significant

Source	*SS*	*DF*	*MS*	Coefficient estimate	Standard error	*F* value	probability (*P*) > *F*
Intercept	-	-	-	406.5295	5.422901	66.11762	< 0.0001
A	-	-	-	59.22131	4.238588	195.2151	< 0.0001
C	-	-	-	21.07616	4.238588	24.72523	0.0002
AC	-	-	-	10.8375	5.537983	3.829614	0.0706
A^2	-	-	-	-22.7882	4.10576	30.80575	< 0.0001
C^2	-	-	-	-37.7081	4.10576	84.34954	< 0.0001
Model	81111.12	5	16222.22	-	-	66.11762	< 0.0001
Residual (error)	3434.956	14	245.354	-	-	-	-
Lack of fit	3038.908	9	337.6564	-	-	4.262818	0.0624
Pure error	396.0483	5	79.20967	-	-	-	-
Total	84546.07	19	-	-	-	-	-

°C with 5% H_2O_2 for 24 h. Cotton stalk pre-treated with 2% H_2O_2 for 60 min at 121 °C and 15 psi showed 49.8% of cellulose conversion [29].

The optimal pre-treatment conditions for maximum reducing sugar yield considering efficiency were H_2O_2 concentration of 4.91%, temperature of 40.3 °C and pre-treatment time of 76.7 min, which would result in a predicted reducing sugar yield of 447.71 mg/g cassava stem. To confirm the result of the predicted value, experiments were performed at the optimal condition which was suggested as 5% H_2O_2/77 min at 40 °C. Another trial was conducted at room temperature, since the temperature was found to be an insignificant factor. The reducing sugar yield from the experiment conducted at 40 °C and room temperature were found to be 445.6±2.5 and 441.3±1.5 mg/g cassava stem respectively, which does not have a significant difference. The optimized condition was found to be 5% H_2O_2 and 77 min.

Effect of microwave assisted alkali pre-treatment

Some studies have reported that microwave irradiation could degenerate lignin and hemicelluloses in lignocellulosic biomass and change the ultra-structure of cellulose, thereby augmenting the enzymatic susceptibility of lignocellulosic biomass [13]. MAA pre-treatment of wheat straw was found to lower sugar losses and enhance hydrolysis rates than conventional alkali pre-treatment methods [30]. A significant increase in the conversion of starch materials to glucose, on pre-treating with microwave irradiation has been reported [15,30]. Combination of microwave treatment with either acid or alkali or combined acid/alkali, an option for pre-treatment of lignocellulosic biomass, has been recently explored [12,30,31].

Table 2 shows the design matrix, reducing sugar yield after hydrolysis and percentage of mass loss for the pre-treated cassava stem with MAA method. An analysis of variance was performed and the results are shown in Table 4. The results specified that the fitting model ($p < 0.0001$) was highly significant. The coefficient of determination (R^2) was 0.987, which indicated that the model could explain 98.7% variability of the response variable. The model was considered as significant, as the adjusted value of coefficient of determination (R^2 = 0.978) was satisfactory. Analysis of the response trend was rational and the model explained the effect of NaOH concentration, microwave power and pre-treatment time, on reducing sugar yield satisfactorily. Model coefficients were determined by regression analysis and their significance was tested. The values for linear, square and interaction effects are presented in Table 4. The regression coefficients of linear and squared terms were highly significant ($p < 0.0001$) and the interactive effects of NaOH concentration and microwave power, and microwave power and pre-treatment time were significant ($p < 0.05$). Positive coefficients indicate a linear increase in reducing sugar yield while negative coefficients indicate a linear decrease in reducing sugar yield. Among the various factors, microwave power had a negative effect on reducing sugar yield during MAA pre-treatment. It supports previous studies that hemicellulose removal and cellulose digestibility has been enhanced by increasing severity (pre-treatment time and temperature) of pre-treatment process [32]. An extended pre-treatment time with a higher microwave power could also lead to a decrease in reducing sugar yield, because increase in pre-treatment time and microwave power increases

Table 4. ANOVA for quadratic model and regression coefficients of the model for reducing sugar yield after hydrolysis of MAA pre-treated cassava stem; coefficient of determination (R^2) = 0.987; adjusted R^2 = 0.978; coefficient of variation (CV): 2.5%; SS, sum of squares; DF, degree of freedom; MS, mean square; A: NaOH concentration (%), B: microwave power (W), C: pre-treatment time (min); p value less than 0.05 indicates model terms are significant

Source	*SS*	*DF*	*MS*	Coefficient estimate	Standard error	*F* value	probability (*P*) > *F*
Intercept				455.7259	3.987539	110.7765	< 0.0001
A				31.88586	2.645641	145.2561	< 0.0001
B				-36.4594	2.645641	189.9136	< 0.0001
C				20.76797	2.645641	61.62062	< 0.0001
AB				-8.175	3.456697	5.593104	0.0375
BC				19.25	3.456697	31.01265	0.0002
A^2				-42.3156	2.57546	269.955	< 0.0001
B^2				-30.5953	2.57546	141.1237	< 0.0001
C^2				-28.9513	2.57546	126.3648	< 0.0001
Model	84899.26	9	9433.251			109.024	< 0.0001
Residual (error)	865.2453	10	86.52453				
Lack of fit	825.857	5	165.1714			20.96705	0.0023
Pure error	39.38833	5	7.877667				
Total	85764.5	19					

the pre-treatment temperature, which could activate degradation of sugars in the pre-treatment process [32,33]. Alkali cause less degradation of sugars as it do not favour dehydration [34]; however, alkali release acetyl and other acidic groups on hemicelluloses and produce organic acids that lower the pH [35]. For example, at high temperature, furfural, an undesirable end-product of xylose decomposition is formed, resulting in a much lower total xylose yield [36].

The optimal pre-treatment conditions for highest reducing sugar yield considering efficiency were NaOH concentration of 1.48%, microwave power of 131.37 W and pre-treatment time of 30.96 min, which would result in a predicted reducing sugar yield of 464.9 mg/g cassava stem. To confirm the result of the predicted value, experiments were conducted at the optimal conditions (1.5% NaOH, 132 W and 31 min), displaying the reducing sugar yield of 463.3±1.5 mg/g cassava stem.

Spectral characterization

The FTIR spectra of native and treated cassava stem at optimized conditions of AHP method and MAA method are shown in Figure 1. The most rep-

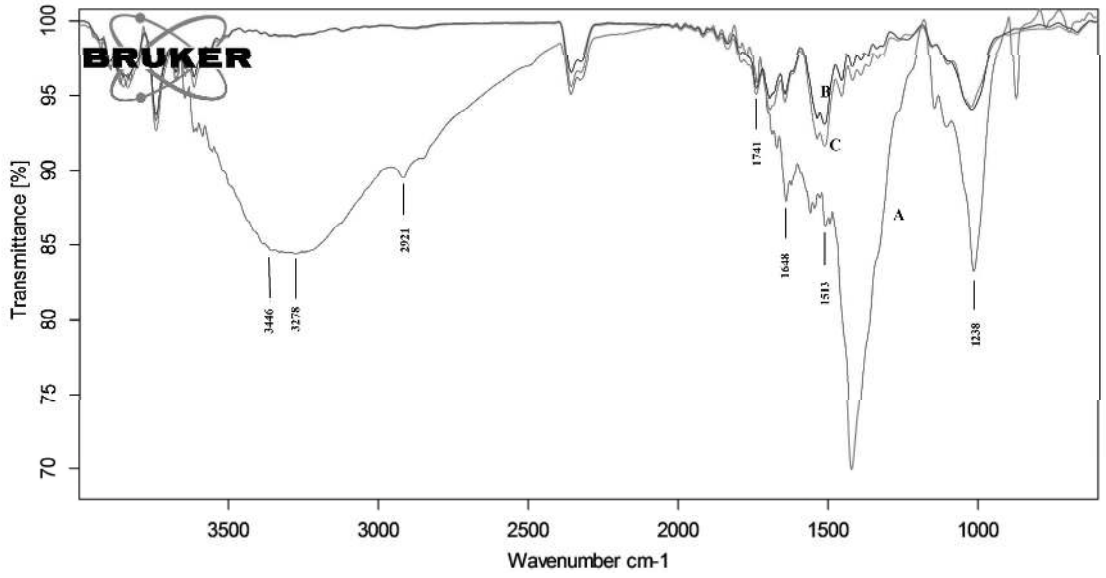

Figure 1. FTIR spectra of native cassava stem (A), MAA pre-treated (B), 1.5% NaOH, 132 W, 31 min, and AHP pre-treated cassava stem (C), 5% H_2O_2, 40 °C, 77 min.

resentative bands can be briefed as follows. The broad absorption at 3278-3446 cm^{-1} related to the stretching of H-bonded OH groups, and one at 2921 cm^{-1} to the C-H stretching [37,38]. The absorption peak at 1741 cm^{-1} could be attributed to the acetyl groups of hemicelluloses or to the ester linkage of carboxylic stretching group of ferulic acid [39]. The occurrence of peak at 1513 cm^{-1} was attributed to the aromatic skeletal vibrations of the associated lignin in the hemicelluloses [40]. The band region between 1650 and 1410 cm^{-1} indicates the stretching of the aromatic phenyl ring of lignin. Absorption at 1000--1200 cm^{-1} was related to the structural features of cellulose and hemicelluloses. The peak between 1019 and 1028 cm^{-1} interprets the C-O-(H) stretching, which was found with cellulose containing higher C=O compounds. The peak at 1238 cm^{-1} indicates C-O-C stretching at β-(1-4) glycosidic linkages of cellulose [23]. The profile of FTIR spectra of native AHP and MAA pre-treated cassava stem were different. This illustrated that there were structural changes in the cassava stem and partial removal of lignin and hemicellulose. The low signal intensity at 1744 cm^{-1} in AHP and MAA pre-treated cassava stem imply that alkali have cleaved the ester bond of hemicelluloses. The absorption peak at 1513 cm^{-1} was found to be smaller in MAA pre-treatment than in AHP pre-treatment. This implies that MAA pre-treatment was effective in solubilizing lignin. The MAA pre-treatment gave maximum reducing sugar yield on enzymatic hydrolysis compared to AHP pre-treatment. The pore size of the substrate compared to the size of enzyme is one of the major factors restricting the enzymatic hydrolysis of lignocelluloses. There are reports that removal of hemicelluloses could increase the pore size thereby, permitting accessibility for the degrading enzymes to reach cellulose [9]. The broadness of the peak at 3280 cm^{-1} was reduced in pre-treated cassava stem compared to native cassava stem. It might be due to microwave irradiation and alkali which enhances the saponification of intermolecular ester bonds cross-linking xylan hemicelluloses and lignin or other hemicelluloses, and hence O-H absorption band intensity lowers due to its involvement in this reaction [13].

Scanning electron microscopy analysis

Scanning electron microscopy (SEM) analysis was performed to establish the structural changes and surface characteristics of cassava stem after pre-treatment. The cassava stem pre-treated at optimized conditions of AHP method and MAA method were imaged by SEM. As shown in Figure 2, the pre-treatment resulted in significant physical changes. The SEM images of native cassava stem showed the presence of starch granules along with fibrous materials. The native stem appears to have an even and smooth flat surface, revealing a rigid surface structure (Figure 2a), while the pre-treated samples had a rugged, rough, and broken surface (Figure 2b and c). The images clearly demonstrate that the pre-treatment could change the lignocellulosic structure and distorted the fibers of cassava stem. These alterations of

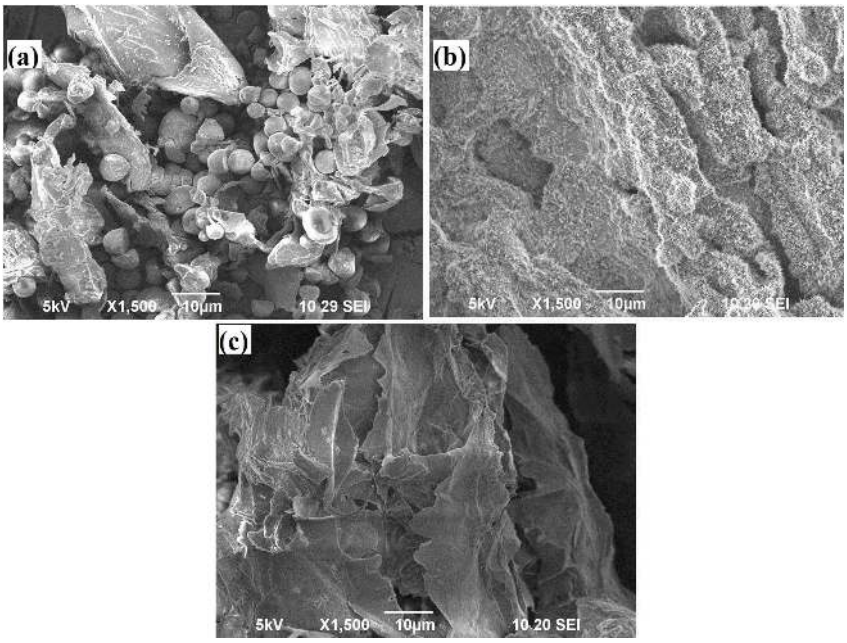

Figure 2. Scanning electron micrographs. a) native cassava stem, b) MAA pre-treated cassava stem (1.5% NaOH, 132 W, 31 min) and c) AHP pre-treated cassava stem (5% H_2O_2, 40 °C, 77 min).

structure and reduction in crystallinity could be expected to increase the enzyme accessibility to cellulosic fibres by increasing surface area. Cassava stem pre-treated by MAA method was found to show extensive distortion of the structure and increase in surface area when compared with AHP pre-treated biomass. The pre-treatment of cassava stem by microwave irradiation together with alkali seems to be disintegrating the fibers completely.

Comparative analysis of pre-treatment effect on reducing sugar yield

Untreated cassava stem yielded 123 mg/g of reducing sugar after enzymatic saccharification whereas after AHP and MAA pre-treatment the yield was 445.6 and 463.3 mg/g, respectively. The results indicated that pre-treatment methods have improved the hydrolysis yield fourfold when compared to native cassava stem. MAA pre-treatment method has enhanced the performance of saccharification considerably than AHP pre-treatment of cassava stem. The reducing sugar yield after AHP pre-treatment was found to be slightly lower than reducing sugar yield after MAA pre-treatment. SEM analysis has indicated extensive disintegration of fibres in MAA pre-treated stem than AHP pre-treated stem. A degradation byproduct (acetic acid) was formed in MAA pre-treatment but its concentration was less than the inhibitory limit (6 g/L) for cellulase activity and fermentation. The profile of FTIR spectra of native, AHP and MAA pre-treated cassava stem were different. This indicates that there were structural changes of cellulose and partial removal of lignin and hemicelluloses. The spectra of MAA pre-treated cassava pulp have shown low intensity of absorption peaks at 1516 indicating the removal of lignin effectively compared to AHP method. There is a statistically significant difference in the reducing sugar yield from cassava stem pre-treated by MAA and AHP methods. Hence, pre-treatment with microwave assisted alkali can be preferred as it takes less time and is efficient in enhancing the reducing sugar yield.

CONCLUSION

The effectiveness of AHP and MAA pre-treatment in improving cassava stem amenability to enzymatic hydrolysis was evaluated. The processing conditions for the pre-treatment methods were optimized and their influence on the reducing sugar yield was studied using CCD. Good correlations between the experimental and predicted reducing sugar yields were found. Pre-treatment of cassava stem resulted in an improved reducing sugar yield (four fold) on enzymatic hydrolysis when compared to native cassava stem. The changes in chemical structure as well as physical characteristics made the pre-treated cassava stem more susceptible to enzymatic saccharification. Both AHP and MAA pre-treatment methods were found to be effective in enhancing saccharification of cassava stem, however, the latter was significantly superior in improving the reducing sugar yield than the former.

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NAUČNI RAD

POBOLJŠANJE ENZIMSKE SAHARIZACIJE STABLJIKI KASAVE PRETHODNOM OBRADOM PEROKISODOM I MIKROTALASIMA

Ispitivana je efikasnost prethodne obrade mikrotalasima (MAA) i vodonik-peroksidom (AHP) u poboljšanju alkalne enzimske saharizacije stabiljki kasave. Kod MAA metode, nadzemni delovi kasave su tretirani različitim intenzitetima energije mikrotalasnog zračenja, koncentracijama NaOH i vremenima trajanja predtretmana. AHP metoda je izvedena pri različitim koncentracijama H_2O_2, temperaturama i vremenima trajanja predtretmana. Rezultati su pokazali da je smanjenje prinosa šećera bilo veće kod stabiljki tretiranih MAA nego AHP metodom, što je pokazalo da je MAA metodom bio efikasan u oslobađanju šećera. SEM fotografije prethodno tretiranih uzoraka otkrile su veće izobličenje vlakana u uzorcima prethodno tretiranih MAA metodom, dok uzorci tretirani AHP metodom imaju pore i pukotine u fibroznoj strukturi. Spektroskopska proučavanja pokazale su promenu hemijske strukture prethodno tretiranog uzorka. Rad je otkrio da su metode predhodnog tretmana bile efikasne u poboljšanju enzimske saharizacije stabiljki kasave.

Ključne reči: stabiljke kasave, prethodni tretman, mikrotalasi, vodonik-peroksid.