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Cellulase Producing Potential of *Aspergillus terreus* Uv2 on Cellulosic Wastes Pretreated with Acid and Alkali

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Authors' contributions

This work was carried out in collaboration between all authors. Author DD designed the study, protocol and draft of the manuscript. Author FAK sourced for the cellulosic wastes and the pretreatment processes. Author OPA managed the statistical analysis. Author JDB isolated and characterized the Aspergillus terreus and maintained the stock culture. Author NEE managed the literature searches and the mutation experiment. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Cellulases offer very wide applications in biotechnology and enzymes from microbial origins present inexpensive source. Production of value added chemicals from wastes will be an exciting translation from waste to wealth and an eco-friendly initiative instead of the incineration option often given to cellulosic wastes.
Study Design: Sulphuric acid and Sodium hydroxide solutions were prepared at 0.5 M and 2 M concentrations to pretreat three cellulosic wastes that had been made neutral prior to fermentation with a known cellulase producing mold
Place and Duration of Study: All experiments were conducted in the laboratory of the Department of Microbiology, Federal University of Technology, Minna, Nigeria for a period of six weeks.
Methodology: Hypercellulase producing *Aspergillus terreus UV2* strain was used to ferment pretreated cellulosic wastes: Corn cob, corn straw and bagasse, using submerged fermentation in

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Mandel basal medium. The crystalline lignocelluloses were milled and fractionated into 850 µ particle size and pretreated in two concentrations (0.5 M and 2 M) of both acid (sulphuric acid) and alkali (sodium hydroxide) independently and were left for varying residence time of one hour or three hours in the digester at ambient temperature, Optimum spore concentration of 1.0 x 10^6 spores/ml and pH of 4.8. Supernatants of crude enzyme were taken and assayed at 24 hours interval.

Results: Cellulase activity peaked at 96 hours. Enzyme secretion in the cellulosic wastes was highest in sugarcane bagasse, followed by the corn cob and then the corn straw corresponding to 51%, 40% and 16% respectively. Alkali pretreated cellulosics gave higher yield of cellulase than its counterpart acid. Non-pretreated residues gave only low enzyme titers. Bagasse produced optimum cellulase yield of 0.068 IU/ml/min within 120 hours when subjected to 2 M NaOH digestion for one hour before fermentation. This translated to 39% increase in enzyme expression when compared with non-treated bagasse of 0.049 IU/ml/min.

Conclusion: Sugarcane bagasse therefore when digested with mild alkali (2 M NaOH) for a pretreatment period of one hour holds a great possibility for cellulase production using a mutant mold, *Aspergillus terreus UV2*. Production of value added chemicals from cellulosic wastes will be an exciting translation from waste to wealth.

Keywords: Cellulase; enzyme; cellulose; wastes; Aspergillus terreus; bagasse, pretreat.

1. INTRODUCTION

Aspergillus species produces a spectrum of extracellular enzymes but selection of a particular strain, however, remains a tedious task, especially when commercially competent enzyme yields are to be achieved. *Aspergillus* genera produce myriads of enzymes [1] including cellulases. Thus, the selection of a suitable strain for the required purpose depends upon a number of factors, in particular upon the nature of the substrate and environmental conditions. Bioconversion of lignocellulosic wastes therefore could make a significant contribution to the production of biochemical that may be highly treasured in biotechnology [2]. Lignocellulosic materials represent an underutilized renewable resource available in huge amounts, approximately 3,720 million tonnes per annum [3] and are continually being replenished by the carbon cycle [4]. Lignocellulosic wastes though are potential substrates for cellulose production must be pretreated for effective conversion by the organism. Many physical, chemical and enzymic pretreatment methods for enhancing bioconversion of cellulosic materials have been reported [5-7]. Among all the methods that have been adopted for treatment of cellulosic wastes, enhanced biodegradation by genetically engineered microorganisms have been acknowledged as the most appealing one [8]. Although the type of enzymes expressed by these microorganisms is very similar, the actual mechanism of degradation of the crystalline cellulose may be different for the different enzyme systems. Currently industrial demand for cellulases is being met by production methods using submerged fermentation processes, employing generally, genetically modified species of *Trichoderma.* There are several reports describing co-culturing of two cultures for enhanced enzyme production. Gupta and Madamwar [9], cultivated two species of *Aspergillus: Aspergillus ellipticus* and *Aspergillus fumigatus* and reported improved hydrolytic and β-glucosidase activities compared to when they were used separately using SSF system. The present study was therefore aimed at comparing the performance of a mutant mold on the expression of cellulase in lignocellulosic wastes that had been pretreated with acid and alkali separately at two different residence times. Results may give credence as to the possibility of converting waste to useful biochemical and forestalling the incineration of this wastes whose gasses are environmentally unfriendly.

2. MATERIALS AND METHODS

2.1 Microorganism

Aspergillus terreus UV2 employed for this work was a mutant with high capacity to produce cellulase obtained from UV irradiation. It was obtained following the previous work reported by Damisa et al. [10].

2.2 Lignocellulosic Materials

The lignocellulosics used for the experiment were corn cob, corn straw and sugarcane bagasse. The sun-dried materials were ground into bits, and further pulverized into smaller particle sizes of 850 µ. The pulverized substrates were washed with hot water several times to remove all residual sugars using the method described by Rezende et al. [11].

2.3 Chemical Pretreatment of Substrates

The balled milled, 850 µ sieved, samples were used as the standard to assess the effect of both acid and alkali pretreatments on the cellulase yield. The effect of acid pretreatment of the lignocellulosics on the enzyme yield was tested by soaking the pulverized samples in 0.5 M and 2 M sulphuric acid solution for varying residence time (one hour or three hours) in conical flasks which served as the digester. The procedure was repeated for alkali pretreatment using sodium hydroxide solution at 0.5 M and 2 M concentrations. Four types of treatment were applied for each substrate as summarized in Table 1.

After the soaking period had lapsed, copious amount of water was added to each flask content to dilute the alkali or the acid and all digested samples were recovered through filtration. The digested particles were washed repeatedly washed with water until neutral pH was recorded for the wash water and thereafter oven-dried at 60ºC overnight as described by Rajoka and Malik [12]. All dried samples were packaged in polythene bags and labeled.

2.4 Cellulose Fermentation

The production of extracellular enzymes in all treatments using the crystalline cellulose (corn cob, corn straw and bagasse) for all fermentation runs were carried out in submerged cultures using the *A. terreus* UV2 in batch units using the method described by [13]. The substrates were fermented in Mandel's medium as proposed by Mandels et al. [14] with the addition of ten gram per litre of the appropriate carbon source (corn cob, corn straw or baggase as the case may be). The medium consisted of (gL^{-1}): Tween-80, 0.1%; peptone, 1; yeast extract, 0.2; Urea, 0.3; $(NH_4)_2$ SO_4, 4; $MgSO_4$. $7H_2O$, KH_2PO_4, 2; 0.3, $CaCl_2$, $0.3ZnSO_4$, 0.14; $FeSO_4$. $7H_2O$, 0.5; Mn SO_4. $4H_2O$, 0.16; $CoCl_2$, 2; pH 6.

The medium was divided into batches of 100ml into 250 ml Erlenmeyer flasks and cotton plugged, sterilized at 121ºC for 15 minutes and cooled to room temperature before 10% of the inoculum spore suspension that had been diluted to 1.0×10^6 cells per ml was seeded aseptically into the contents of the flask. All fermentations were accomplished in a rotary shaker (Bellco Glass Inc. USA) at room temperature and 350 pm. In the course of the fermentation, samples were taken for analysis of cellulase at 24 hours interval throughout the period using the crude enzyme supernatant until the activity peaks off. The pH of the medium was also recorded at per time.

2.5 Saccharifying Cellulase Activity Determination

The saccharifying cellulase activity (total cellulase), was determined for all the substrates using the method described by Ghose [15].

Table 1. Summary of the pretreatments using sulphuric acid and sodium hydroxide

Sl. no.	Type of agricultural wastes	Physical state and quantity	Concentrations of NaOH or H_2SO_4 used (100 ml)	Treatment time (hours)
1	CC	P(850 µ), 20 g	0.5 M	1
2	CC	P(850 µ), 20 g	0.5 M	3
3	CC	P(850 µ), 20 g	2 M	1
4	CC	P(850 µ), 20 g	2 M	3
5	CS	P(850 µ), 20 g	0.5 M	1
6	CS	P(850 µ), 20 g	0.5 M	3
7	CS	P(850 µ), 20 g	2 M	1
8	CS	P(850 µ), 20 g	2 M	3
9	SB	P(850 µ), 20 g	0.5 M	1
10	SB	P(850 µ), 20 g	0.5 M	3
11	SB	P(850 µ), 20 g	2 M	1
12	SB	P(850 µ), 20 g	2 M	3

CC= corn cob; CS= corn straw; SB= sugarcane bagasse; P(850 µ)= pulverized and screened to 850 µ particle size

Aliquots of 0.5 ml of each culture supernatant was incubated with a '1 cm by 6 cm' rolled filter (Whatman No. 1) paper strip (Whatman, UK) in 1ml of 0.05 M citrate buffer (pH 4.8) in a test tube immersed in a waterbath at 50ºC for 60 minutes. Each tube was removed from the water bath and the reaction ended by adding 3.0 ml Dinitrosalicylic acid (DNS) reagent. The tubes were boiled vigorously for five minutes in a boiling water bath containing sufficient water to cover the portions of tubes occupied by the reaction mixture plus the reagent. The tubes were transferred to cold water bath and diluted with 2.5 millilitre of distilled water. The absorbance was determined using U.V spectrophotometer (Cecil 1000 series, Cambridge England) against a blank at 540nm and the quantity of reducing sugar read from a sugar curve. Saccharifying cellulase activity was estimated and expressed in International Units (IU) according to the method of Janas et al. [16]. One unit of cellulase is the amount necessary to form 1 mg of glucose per minute at 50ºC.

2.6 Statistical Analysis

Pearson correlation, Paired Sample T Test, Duncan Multiple Range Test and least significant difference test were performed using SPSS 22.0 statistical software to determine the effect of chemical pretreatment and residence time on enzyme yield from the various lignocellulosic substrates.

3. RESULTS AND DISCUSSION

3.1 Effects of Acid and Alkali Pretreatment of the Cellulosic Wastes on Cellulase Recovery from *Aspergillus terreus* UV2

The effect of acid and alkali pre-treatment of the cellulosic Wastes on cellulase recovery fermented with the mutant mold, *Aspergillus terreus UV2*, is shown in Figs. 1 to 4. All alkali pretreated residues regardless of the residency time, maximum yield of cellulase was observed at day 120 hours while the acid pretreated residues displayed maximum yield of cellulase at 150 hours. All residues pretreated for 3 hours using alkali when compared with those of acid under similar fermentation conditions were highly significant (*P*=0.001). Generally, the residues pretreated with alkali showed higher cellulase activity than the residues pretreated with acid and is highly significant when compared using Pearson correlation at P=0.05. Enzyme recovery in residues pretreated with 0.5M NaOH for 1 hour increased from 24 hours and peaked at 120 hours apart from the bagasse whose activity peaked off at 96 hours. Maximum enzyme activity of 0.05041 IU/ml/min was showed by bagasse, then corn cob (0.04922 IU/ml/min) and finally by corn straw (0.04692 IU/ml/min).

The effect of acid pre-treatment (2 Molar)(2 M) and residency time on Cellulase yield from the cellulosic sources is presented in Fig. 1. Straw treated for a longer residence time (3 hours) showed highest cellulase activity of 0.04953 IU/ml/min. The peak of expression for all substrates was at 144 hours (day six).

Similarly, in the residues pretreated with 0.5 M NaOH for 3 hours, enzyme yield rose from day one and peaked off at 120 hours (fifth day) (Fig. 2) save the bagasse. Highest enzyme expression of 0.05985 IU/ml/min was recorded for bagasse pretreated with 0.5 M NaOH for 3 hours' time of residency.

Fig. 3 depicts the effect of substrate pretreatment (2M NaOH) for either 1hour or 3hours residency time on the cellulase expression by the mutant mold. High enzyme yield of 0.06777 IU/ml/min was recorded from bagasse fermented by the *Aspergillus terreus UV2* that had been pretreated for 1 hour residency time. Pearson correlation statistics at P=0.05 revealed that there is a strong correlation between concentration of the alkali used in the pretreatment and enzyme yield.

The enzyme expression from the organism used to ferment the cellulosic substrates pretreated with 0.5 M H_2SO_4 for either 1 or 3 hours is presented in Fig. 4.

The effect of cellulose pretreatment with 0.5 M H_2SO_4 for 3 hours residency time on cellulase activity from the mold is presented in Fig. 4. Sharp increase in enzyme yield was recorded from day one and peaked off at 144 hours regardless of the substrate type. High enzyme yield of 0.05024 IU/ml/min, was observed in the bagasse pretreated at residency time of 1 hour. Long residency time with acid pretreatment appears to subvert the cellulase yield and decreased it as compared to long residency time in alkali that appears to have potentiated the production of the cellulase by the mold in the substrate.

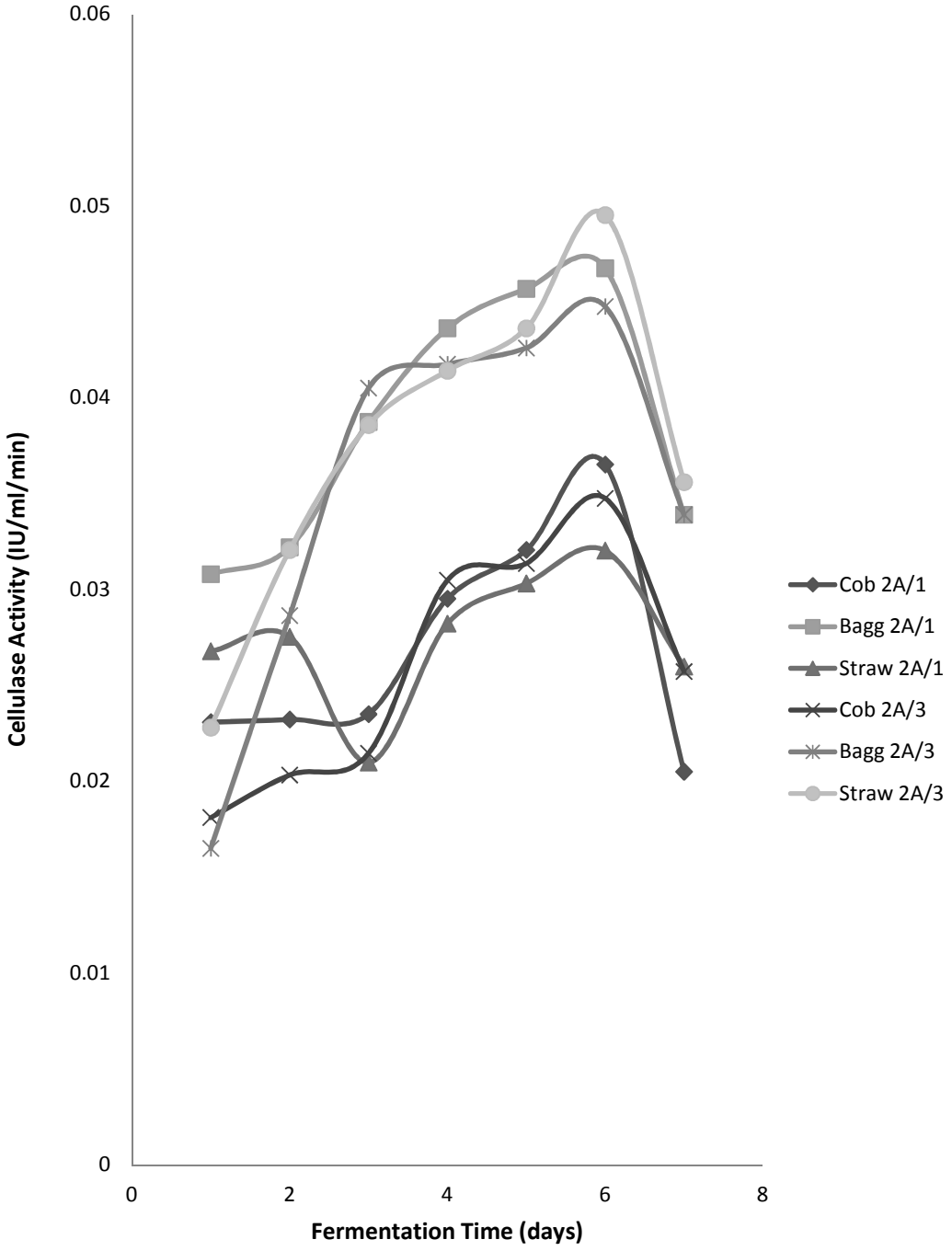

Fig. 1. Effect of acid pre-treatment (2 M) and residence time on cellulase yield from the cellulosic wastes

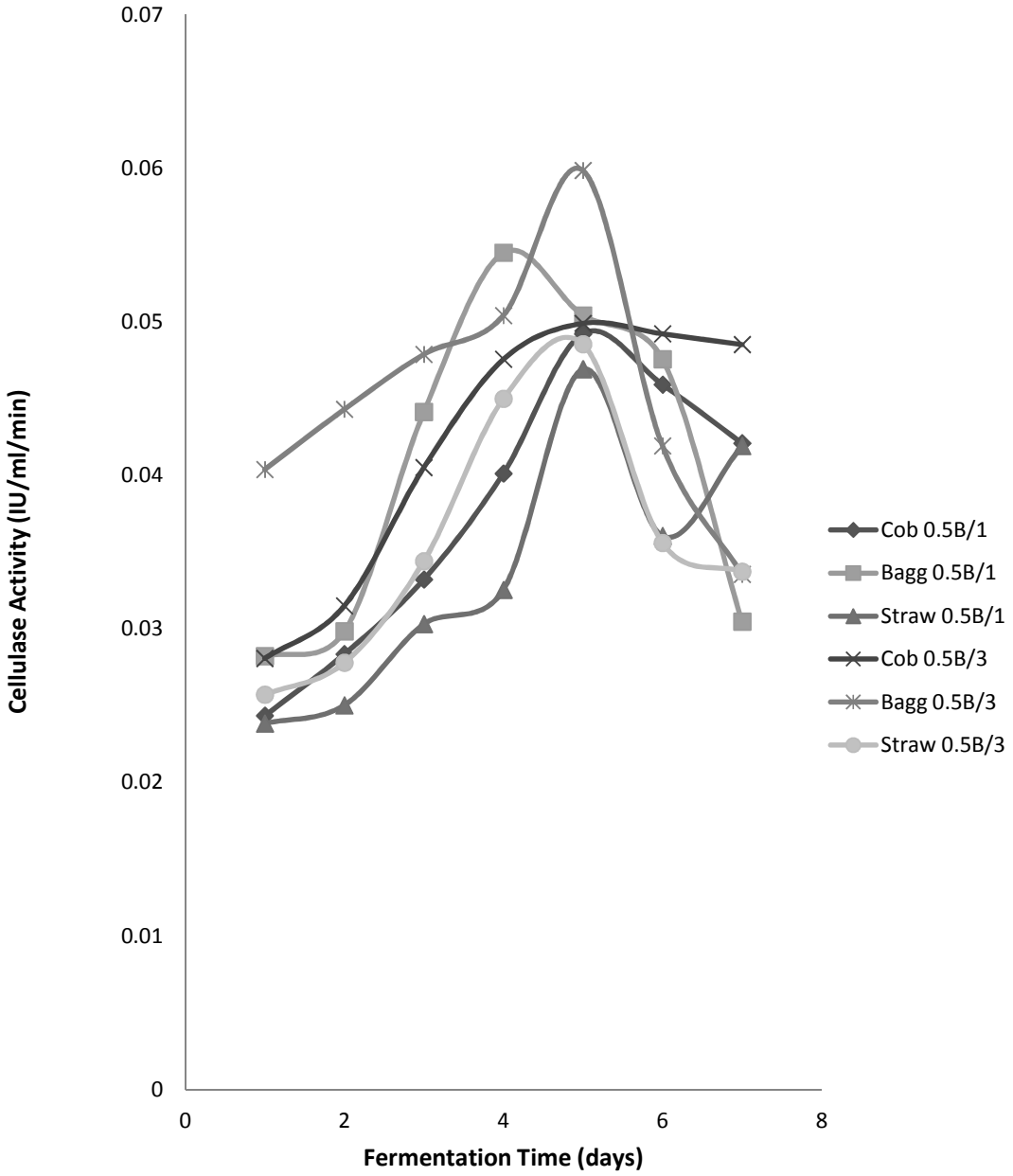

Fig. 2. Effect of alkali pre-treatment (0.5 m) and residence time on cellulase yield from the cellulosic wastes

Though concentrated acids are powerful agents for cellulose hydrolysis, they are toxic and harder to ferment [17] and must be recovered after hydrolysis to make process feasible [18]. H_2SO_4 when mixed with biomass to hydrolyze hemicellulose to xylose and other sugars continue to break xylose down to form furfural [19]. Furfural inhibits microbial growth and

fermentation. This explains the low output of cellulase fermentation in acid treated cellulosic wastes. Hemicellulose is removed when H_2SO_4 is added and this enhances digestibility of cellulose in the residual solids. Similar observation was recorded by [19]. Use of acid may increase crystallinity index of the substrates and may be responsible for the low cellulase yield observed. Dilute acid pretreatment has been observed to have a negative influence on the fermentation processes by microorganisms. According to Selig et al. [20], such pretreatment causes formation of spherical droplets made up of lignin and carbohydrate complexes that enhances nonproductive binding of enzymes to lignin.

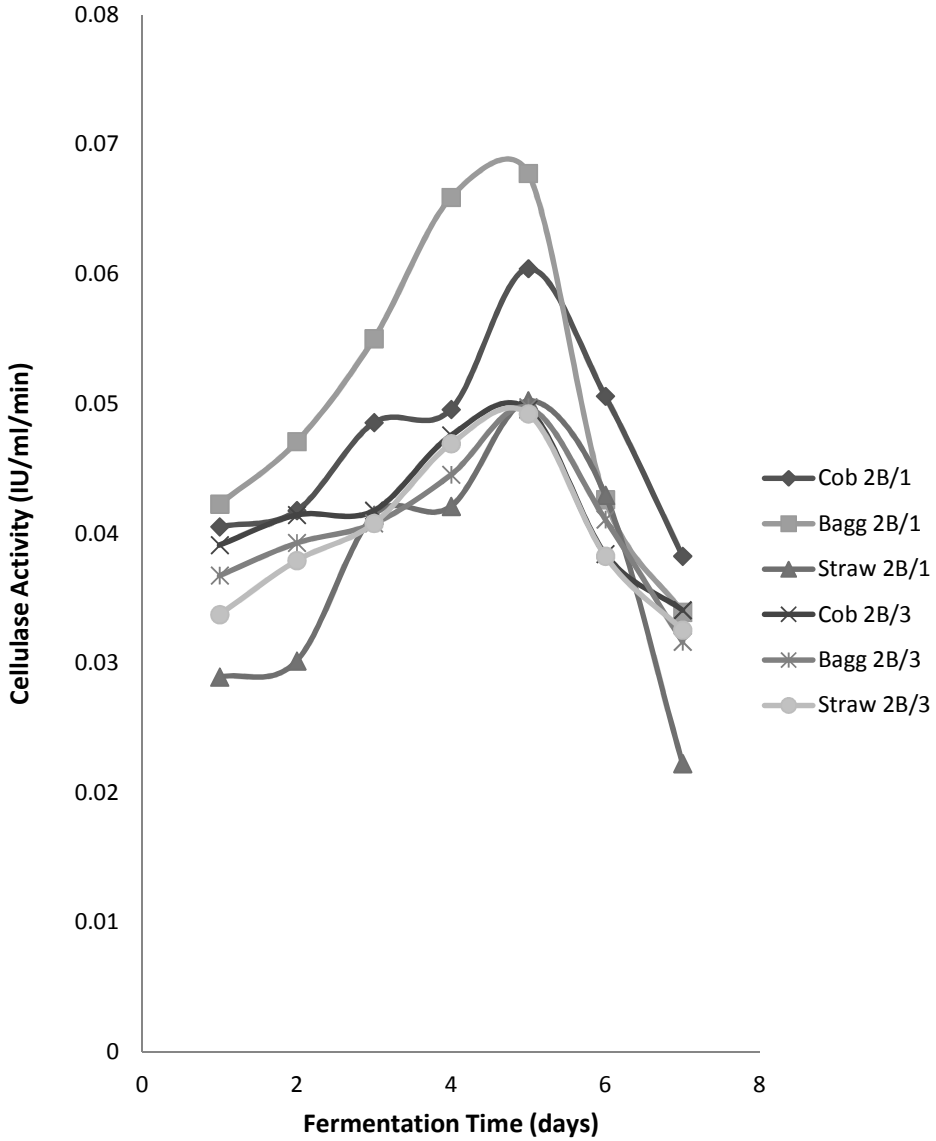

Fig. 3. Effect of alkali pre-treatment (2 m) and residence time on cellulase yield from the cellulosic wastes

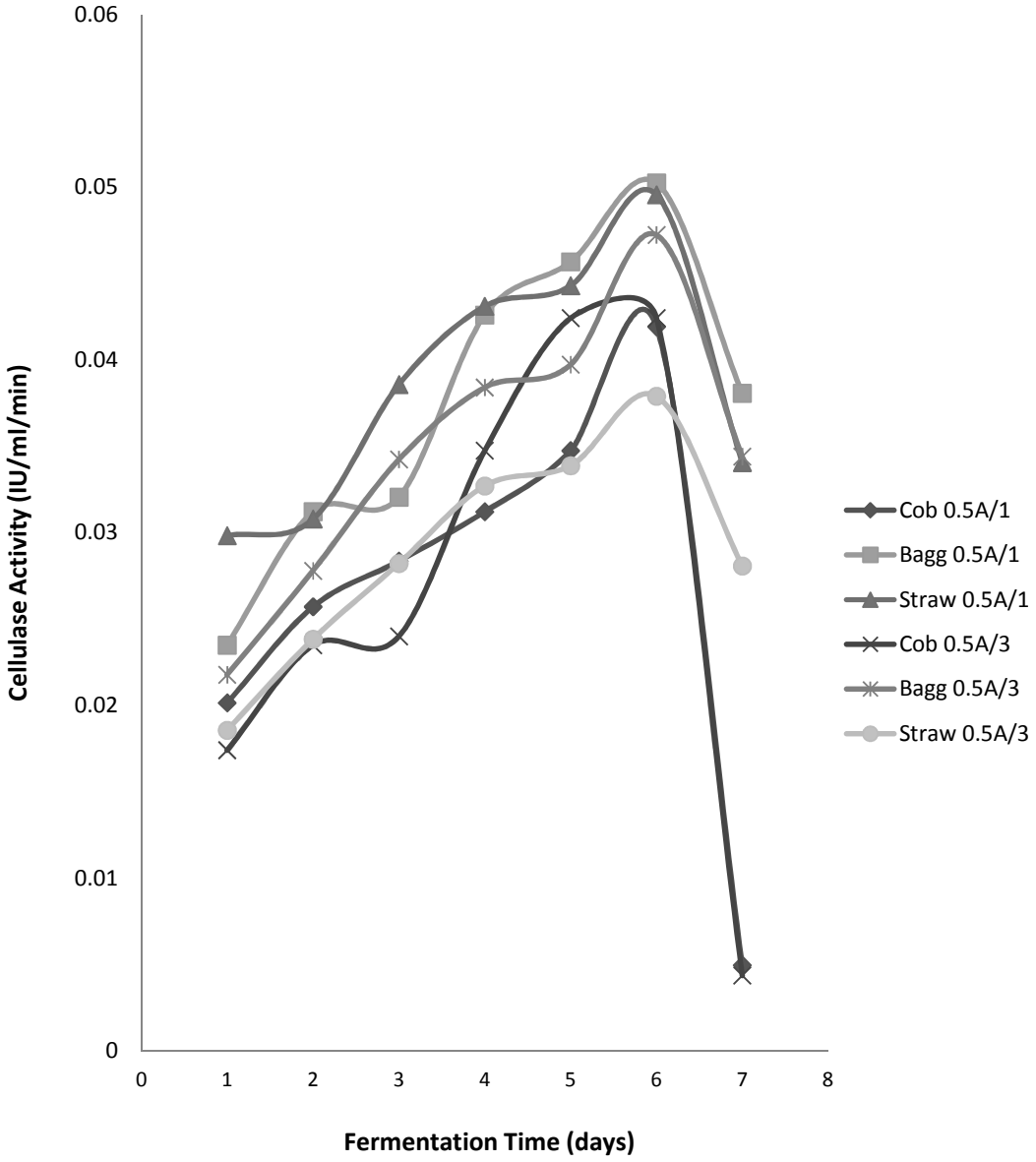

Fig. 4. Effect of acid pre-treatment (0.5 m) and residence time on cellulase yield from the cellulosic wastes

On the other hand, the effect of alkaline pretreatment on the cellulosic materials depend on the lignin content of the material. Alkaline pretreatment in this case may have exposed the cellulose to enzymatic hydrolysis. The removal of lignin enhanced the effectiveness of the enzyme by eliminating non production adsorption sites and increasing access to cellulose and hemicellulose complex. According to Kong et al. [21], alkali removes acetyl groups from hemicellulose and reduces the commonly observed stearic hindrances of hydrolytic enzymes and greatly enhancing carbohydrate digestibility.

4. CONCLUSION

Statistical analysis using ANOVA procedure on the effect of acid and alkali pretreatment on the yield of cellulase showed that there is a significant difference (P=.001) in the enzyme yield between the chemicals and between the cellulosic residues. There was a strong correlation between the enzyme yield and pretreatment type. In the three substrates considered for this work, paired sample T Test statistics at 95% confidence interval demonstrated that there was no significant difference in enzyme activity between the substrates pretreated with either concentration of the acid regardless of the residency time. Cellulase yield of 0.068 IU/ml/min was obtained when bagasse digested with 2 M NaOH for one hour was fermented. Pre-treating the cellulosic materials with sodium hydroxide may have allowed the particles to swell and caused the removal of the lignin so that the cellulose fibers were depolymerized as a result of the hydrogen bond separation. Acid pre-treatment of the cellulosic wastes gave significantly lower expression of cellulase activity than the alkali pre-treated counterparts when compared using Duncan Multiple Range Test at P=0.05. Acid pretreatment has no effect on the lignin found in the substrate but only alters the lignin-carbohydrate bonding. Pretreating the substrates with high concentrations of acid (2 M) even for extended residency time only resulted in loss of the polysaccharides and the formation of secondary reaction product. Acid pretreatment may allow cellulose to re-anneal causing hornification of the cellulose in micro fibrils.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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