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Full Length Research Paper

Effect of chemical pretreatment of some lignocellulosic wastes on the recovery of cellulase from *Aspergillus niger* AH3 mutant

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Lignocellulosic biomass holds remarkable potential for conversion into commodity products presenting dual advantage of sustainable resource supply and environmental quality. Though their utilization does not compete with food and feed demand, its bioconversion and utilizability is facilitated by pretreatment. The effect of the substrate pre-treatment using acid and alkali at two different concentrations (0.5 and 2 M) for two different residence timings (1 and 3 h) on cellulase production from corncob, corn straw and bagasse was studied using *Aspergillus niger* AH3. The strain was inoculated into 10 g/L of the processed pre-treated lignocellulosic substrates previously added to batches of the Mandels basal medium. The pH of the medium was adjusted to optimum (4.8) and the flasks with the contents autoclaved, thereafter fermentation begun. Samples of each flask were taken aseptically at regular interval (24 h) throughout the growth phase until the enzyme activity peaked off (between 110 and 170 h), centrifuged and the clear supernatant was used for the enzyme assay. Enzyme expression in all the pretreated biomass increased steadily from day one and peaked off at day four or five for the alkali pretreated residues whereas it was at day six for acid pretreated residues. Generally for the alkali treated residues irrespective of residence time, maximum cellulase yield was at day 5 while for the acid treated residues, maximum cellulase yield was at day 6. Enzyme yield from residues treated for longer period (3 h) using alkali when compared to those using acid under the same condition of fermentation was highly significant. The alkali treated residues showed higher cellulase yield than the acid treated residues. Highest cellulase activity (0.06777 IU/ml/min) was displayed by the organism grown on bagasse substrate pretreated with 2M NaOH for one hour. The proximate analysis of the cellulosic residues differed from one substrate to another, with the bagasse being the best. Pulverized substrates syndicated with alkali pretreatment using 2 M NaOH for one hour was optimal for cellulase production from the cellulosic residues.

Key words: Lignocellulosic biomass, utilization, pretreatment, cellulase, substrates, *Aspergillus niger* AH3, fermentation, supernatant, substrate

INTRODUCTION

Nigeria, like many other developing countries in Sub Sahara Africa, is an agricultural country and, as such, produces several hundred million tons of agricultural products, annually. The crop production is associated with waste generation and the exploitation of these waste materials as a renewable resource for bio-product development could be a major challenge for biotechnology (Bahrim, 2004). Large quantities of such wastes accumulate every year which results not only in the deterioration of the environment, but also in the loss of potentially valuable materials which can be processed to yield a number of value added products such as enzyme, food, fuel, feed and a variety of chemicals (Someet et al*.,* 2001). The agricultural wastes are composed essentially of cellulosic or lignocellulosic matter which are available on a renewable or recurring basis and can be grown, collected and replenished fairly quickly without perma-

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nently depleting the natural resources (Geoffroy et al., 2006). Lignocellulosic material is a class of biomass that is rich in lignin, hemicellulose and cellulose, including wood, agricultural residues and paper wastes (Chang, 2007).

Advances in microbial, biotechnology, and genetic engineering are leading to a new manufacturing concept for converting renewable biomass to valuable products (Ragauskas et al., 2006), and the integration of these wastes into the manufacturing technologies will provide the possibility for the development of sustainable model for the production of commodity products. The challenge of overcoming the recalcitrance of cellulosic biomass is an impediment to converting them into reactive intermediates and useful products (Chandra et al., 2007). Therefore, the utilization of cellulosic biomass will include developing microorganisms and pretreatment steps likely to accelerate its bioconversion. In view of this, the study was aimed to investigate the ability of Aspergillus *niger* AH3 strain to produce cellulase from different agricultural residue hydrolysates obtained via acid and alkali pretreatment.

MATERIALS and METHODS

Source of *Aspergillus niger*

Aspergillus niger AH3 was obtained from the Department of Microbiology, Ahmadu Bello University, Zaria. The microorganism was a mutant obtained via hydroxylamine constitutive for cellulase production. The cultures were maintained on potato dextrose agar (PDA) slants and sub-cultured fortnightly at 28 - 30^{o}C.

Substrate

The lignocellulosic biomasses used for the study were corn cob (CC), corn straw (CS) and sugarcane bagasse (SB). The corn cob and corn straw were obtained from harvest waste dump site of IAR, A.B.U. research farm Shika, Nigeria. The bagasse was obtained from Savannah sugar company, Bauchi, Nigeria.

Preparation of substrate

The substrates were sun-dried for a period of three weeks and subsequently oven-dried slowly at 50^{o}C for two days. The dried substrates were chopped into bits, pulverized into coarse particle sizes and then washed in several changes of hot water in order to remove the residual sugars as described by Rezende et al. (2002). One kilogram (1 Kg) each of the dried substrates was ball milled for ten minutes using Christy Laboratory Mill machine (Christy and Norris, Ltd England). The ball milled substrates were individually screen analyzed to pass through 0.8 mm (850 µ) of Endecotts test sieve (Griffin and George, Edinburgh).

Proximate analyses of the substrate samples

Each of the pulverized samples that had been screened with sieve to 850 µ particle size was analyzed proximately using the AOAC (1990) method, for dry matter, crude protein, crude fiber, crude fat, ash content and nitrogen free extracts.

Chemical pretreatments

In order to prepare hydrolysates for fermentation, 500 g of each dry milled lignocellulosic material was mixed with either 0.2 or 5 M of sulphuric acid solution or sodium hydroxide solution as the case may be and left in the digester for varying time of one or three hours for both concentrations. After the lapse of the required residency, sufficient water was added to each Erlenmeyer flask to dilute the acid or the alkali and the digested samples recovered by filtration. The particles were returned into the flasks and repeatedly washed with sterile distilled water until the pH of the wash water was neutral, then dried overnight at 60^{o}C using the method described by Fan et al. (1982) and Rajoka and Malik (1997). The dried hydrolysates were packaged in sterile polypropylene bags until use.

Cultivation medium and enzyme production

Submerged culture fermentation based on the method of Liming and Xueliang (2004) was adopted. The fungus was cultivated at ambient temperature of 28°C in Mandel's medium (Mandels et al., 1981) consisting of (gL^{-1}): yeast extract, 0.2; peptone, 1; $(NH_4)_2SO_4$, 4; KH_2PO_4, 2; urea, 0.3; $MgSO_4$. $7H_2O$, 0.3, $CaCl_2$, 0.3; $FeSO_4$. $7H_2O$, 0.5; Mn SO_4. $4H_2O$, 0.16; $ZnSO_4$, 0.14; $CoCl_2$, 2; Tween-80, 0.1%. 10 g of the appropriate lignocellulosic hydrolysates (serving as carbon source) was added and the pH adjusted to 6 using 0.05 M sodium hydroxide solution. The medium was then autoclaved for 15 min at 121^{o}C, allowed to cool and spore suspension diluted to concentration of 1.0 x 10^6 using the method of Adhikari and Shrestha (1989) was seeded aseptically into the flask. The cultivation was carried out for 168 h in 250 ml capacity Erlenmeyer flasks in a rotary shaker (Bellco Glass Inc. USA) at ambient temperature of 28^{o}C and 350 rpm at 60 rpm. The experiments were conducted in duplicate.

Enzyme assays

During the growth process, samples were withdrawn for analysis of cellulase at every 24 h using the supernatant of centrifugates until enzyme activity peaks off. The pH was also recorded at each time.

Cellulase activity, termed saccharifying cellulase, was determined for all the substrates using filter paper as the substrate as proposed by Ghose (1987). It was assayed by incubating 0.5 ml of each culture supernatant with a rolled '1 by 6 cm' filter paper strip (Whatman No. 1) (Whatman, UK) in one millilitre (1 ml) of 0.05 M citrate buffer (pH 4.8) contained in test tube at 50^{o}C for 60 min. Cellulase activity was calculated and expressed in International Units (IU) as described by Janas et al. (2002). One unit of cellulase corresponded to the amount necessary to form 1 milligram (1 mg) of glucose per minute at 50^{o}C.

RESULTS

Proximate analysis of substrate samples

The result of the proximate analysis of the cellulosic residues is presented in Table 1. The crude protein content in the different residues varied from one substrate to another and was generally low. Corn cob had the highest (6.69%), followed by bagasse (5.38%) then corn straw (4.13%). High crude protein content in the corn cob in a medium (Mandels) containing inorganic (ammonium sulphate) and organic (yeast extract) sources can account for increased protein content which

Table 1. Proximate composition of the cellulose residues.

Residue	%DM	%CP	%CF	% Oil	% Ash	%NFE
Corn straw	86.56	4.13	23.14	15.15	7.02	37.40
Corn cob	95.68	6.69	22.27	7.90	2.75	60.31
Bagasse	96.19	5.38	36.30	8.93	2.35	60.18

DM = Dry matter, CP = crude protein, CF = crude fiber, and NFE = nitrogen free extract.

served as nitrogen source required for growth and efficient enzyme expression by the organism. Relatively high crude fiber content in bagasse correlated positively with increase in xylose content (common sugar in hemicelluloses) unlike the straw with waxy cuticle and high silica content. Cell wall of corncob was characterized by a low content of cellulose.

There was a negative correlation between the crude fiber content and crude protein content in all the residues, which suggests that an increase in oil content in associated with a reduction in crude protein content. The ash content varied enormously from 2.35% for bagasse to 7.02% for corn straw. The negative impact of high inorganic constituents (ash) in the biomass may be aggravated in biochemical conversion systems where the use of inorganic chemicals during pretreatment adds to the total amount of non-convertible inorganic residues. High value in both corn cob (60.31%) and bagasse (60.18%) is an indication of high content of fermentable portion of carbohydrate in the biomass. Bagasse contained substantial quantity of dry matter (96.19%) with good content of nitrogen free extract (60.18%) which are contained in the biomass needed by the organism for fermentation.

Effects of chemical pretreatment on cellulase yield from *A. niger* AH3

The effect of alkali and acid pre-treatment on cellulase yield from the biomass residues fermented with the organism is presented in Figures 1 to 4. Enzyme expression in biomass pretreated with 0.5 M NaOH for one hour increased steadily from day one and peaked off at day four (Figure 1) with high cellulase activity of 0.05041 IU/ml/min obtained from the bagasse. Next in sequence was corn cob (0.04922 IU/ml/min) and then corn straw (0.04692 IU/ml/min). The cellulase yield from the biomass pretreated with 0.5 M H_2SO_4 for one hour is also illustrated in Figure 1. There was a gradual increase in enzyme expression from day one and peaked off at day six. The highest cellulase activity (0.05024 IU/ml/min) for this treatment (0.5 M H_2SO_4) was expressed in the bagasse.

In comparison with the biomass pretreated with 0.5 M NaOH for three hours, enzyme expression rose steadily from day one and peaked off at day five (Figure 2) with bagasse exhibiting highest cellulase activity of 0.05985 IU/ml/min. Biomass pretreated using 0.5 M H_2SO_4 for residence time of three hours on cellulase yield is presented in Figure 3. Sharp increase in enzyme expression from day one that peaked off at day six was observed with highest enzyme activity, 0.04724 IU/ml/min

Figure 3 depicts the effect of biomass pretreatment using 2 M NaOH of the alkali for one hour on the yield of cellulase from the organism. Very high cellulase expression of 0.06777 IU/ml/min was exhibited by the mutant fermented on bagasse. Activity of the enzyme peaked off at day five. Biomass digested in 2M H_2SO_4 for a residence time of one hour is shown in Figure 3. There was a gradual increase in enzyme expression from day one which peaked off at day six, though the strain fermented in the straw showed a decline in activity between days two and three and thereafter began to show increase in enzyme activity which peaked off at day six. Highest cellulose activity (0.04676 IU/ml/min) was displayed in the bagasse.

The cellulase yield from from the biomass pretreated with 2 M NaOH for three hours is illustrated in Figure 4. The cellulase expression by the mutants in the pretreated biomass are in the same range (0.04922 to 0.04973 IU/ml/min), the difference between them accounting for only one percent. Enzyme activity peaked off on the fifth day for the strain. Similarly, enzyme expression by strain grown on the biomass pretreated with 2 M H_2SO_4 solution in the digester for three hours is illustrated in Figure 4. Enzyme activity increased from day one and peaked off at day six. Highest enzyme expression, 0.04953 IU/ml/min, was recorded however in the straw rather than bagasse as observed in other treatments.

Generally for the alkali treated residues irrespective of residence time, maximum cellulase yield was at day 5 while for the acid treated residues, maximum cellulase yield was at day 6. Enzyme yield from residues treated for longer period (3 h) using alkali when compared to those using acid under the same condition of fermentation was highly significant (0.001). The alkali treated residues showed higher cellulase yield than the acid treated residues. Enzyme expression in biomass irrespective of the chemical pretreatment applied increased steadily from day one and peaked off at day five. Highest cellulase activity (0.06777 IU/ml/min) was displayed by the organism grown on bagasse substrate pretreated with 2 M NaOH for one hour.

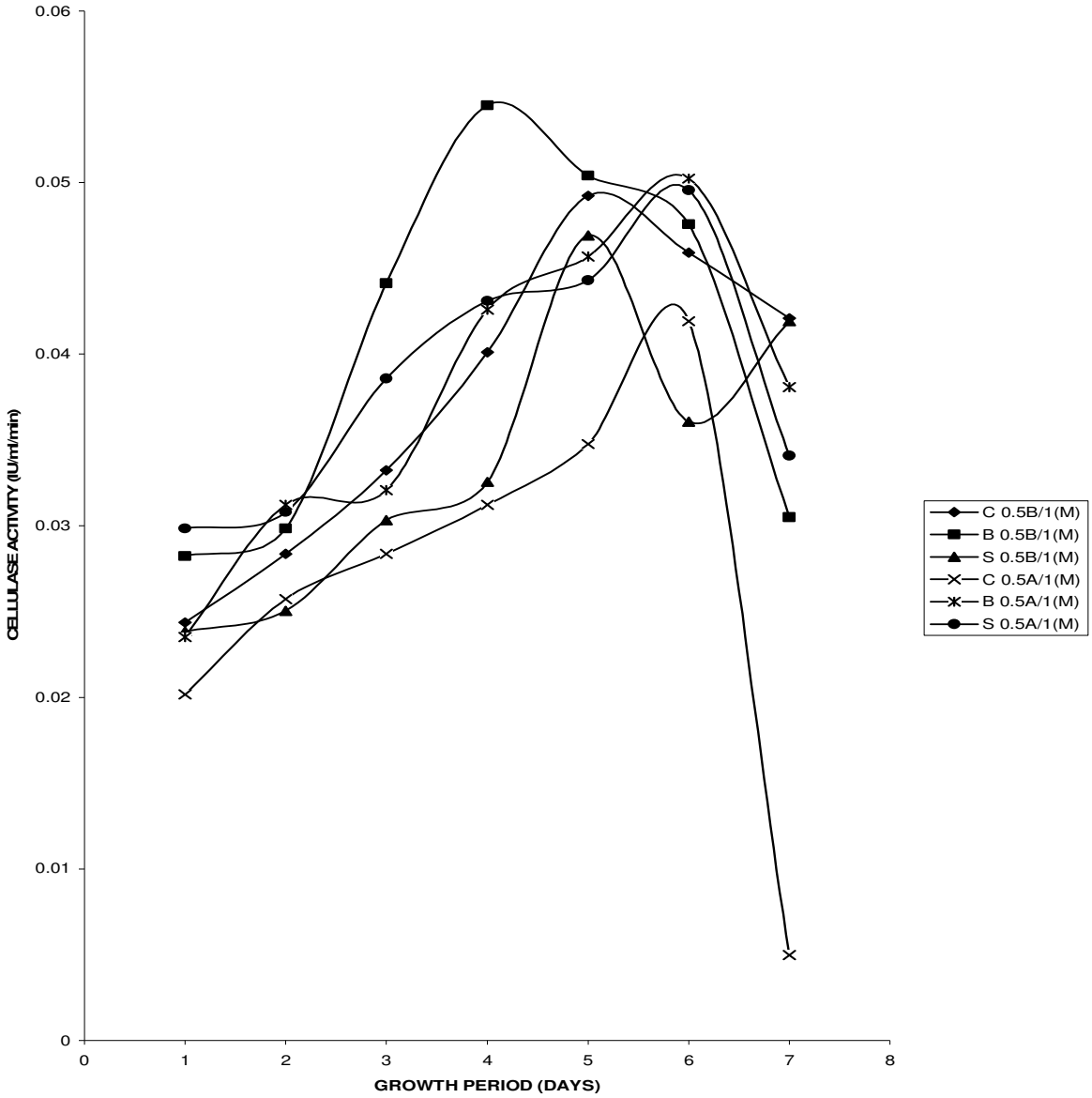

Figure 1. Effect of biomass pretreatment using 0.5 M NaOH for 1 h (0.5 B/1) and 0.5 M H_2SO_4 for 1 h (0.5 A/1) on cellulase yield. C = Corn cob, B = bagasse, and S = corn straw.

DISCUSSION

Analysis of variance on the effect of chemical pretreatment on cellulase yield showed that there is a significant difference ($P<0.05$) in the enzyme yield by the strain between the chemicals used and in residence time. The chemical pretreatment of lignocelluloses causes swelling leading to an increase in internal surface area, decrease in the degree of polymerization, decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, thus increasing the cellulose hydrolysis (Kleinert, 1966). Alkali pretreatment appears to be a more subtle choice for the pretreatment purposes. For the three residues studied, paired samples statistics show that there is no significant difference in enzyme yield between all substrates treated with 0.5 or 2 M sulphuric acid irrespective of the residence time. Generally, the alkali treated residues showed higher cellulase yield than the acid treated residues. Pretreating the substrates with sodium hydroxide may have resulted in the swelling of the particles causing easy removal of the lignin and cellulose depolymerization occasioned by the separation of hydrogen bonds of the cellulose. The swelling of the particles have been interpreted to be due to the signification of inter medullar ester bonds (Fan et al., 1982).

According to Bharathi and Ravindra (2006), delignification and depolymerization of rice bran was needed in order to utilize it as a potential substrate in solid state fermentation (SSF) for the production of cellulase. Milling of the substrate followed by alkali treatment and subse-

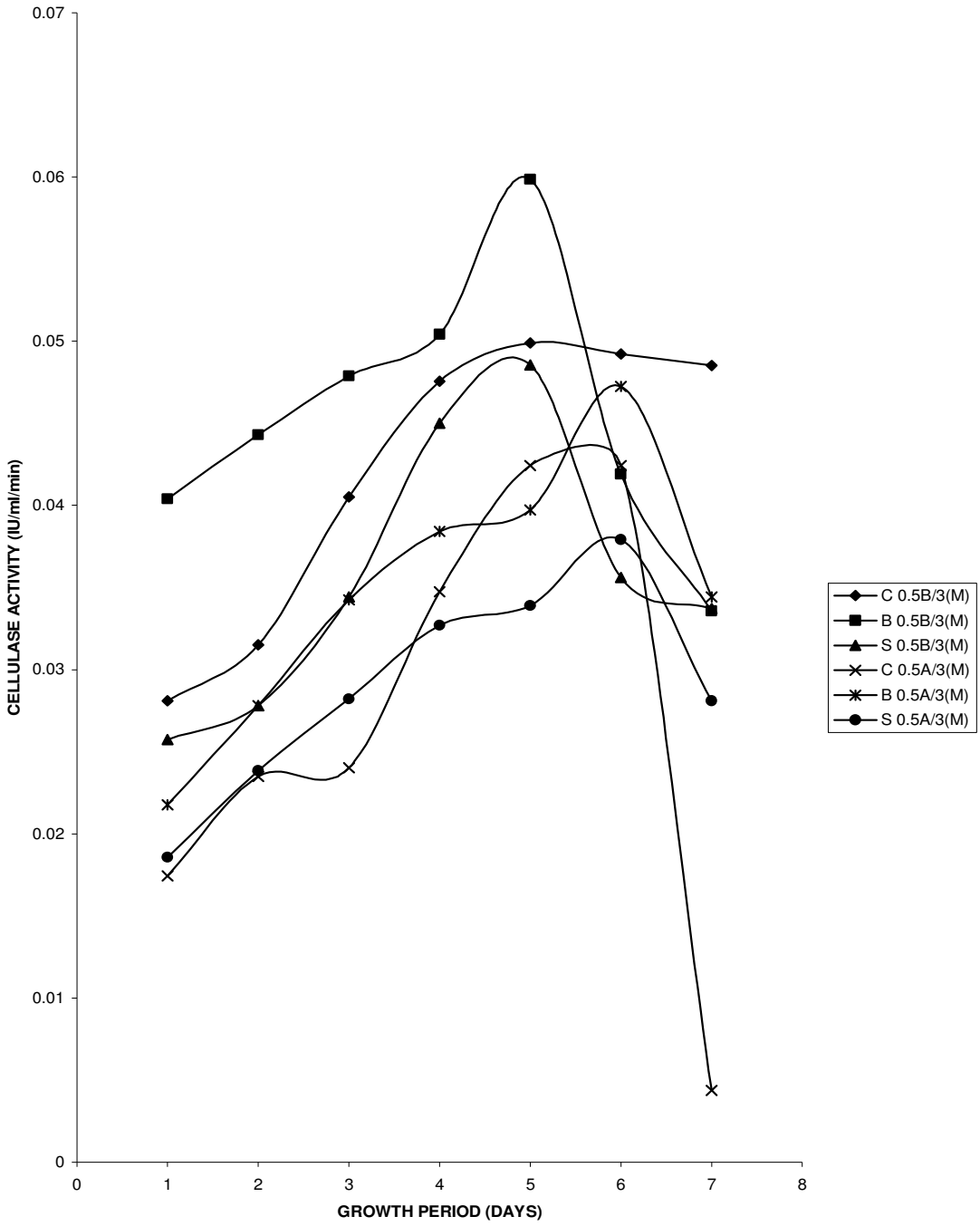

Figure 2. Effect of biomass pretreatment using 0.5 M NaOH for 3 h (0.5 B/3) and 0.5 M H_2SO_4 for 3 h (0.5 A/3) on cellulase yield. C = Corn cob, B = bagasse, and S = corn straw.

quent steam explosion for a residence time of 10 min at 121ºC and 15 psi were found to be the optimized conditions for the productions of cellulose by from rice bran by *Scopulariopsis* sp. in an SSF process. Acid pre-treatment of the residues yielded lower cellulase activity because it does not remove lignin from the substrate but only modifies the lignin carbohydrate linkage according to the findings of Ghosh and Singh (1984). Pretreating with higher concentration (2 M) of acid even for prolonged period as in Figure 4 may have resulted in the loss of polysaccharides and formation of secondary reaction. This may have led to the accumulation of degradation products of pentoses and hexoses i.e. furfural and other complexes which act as inhibitions that hinder the micro-

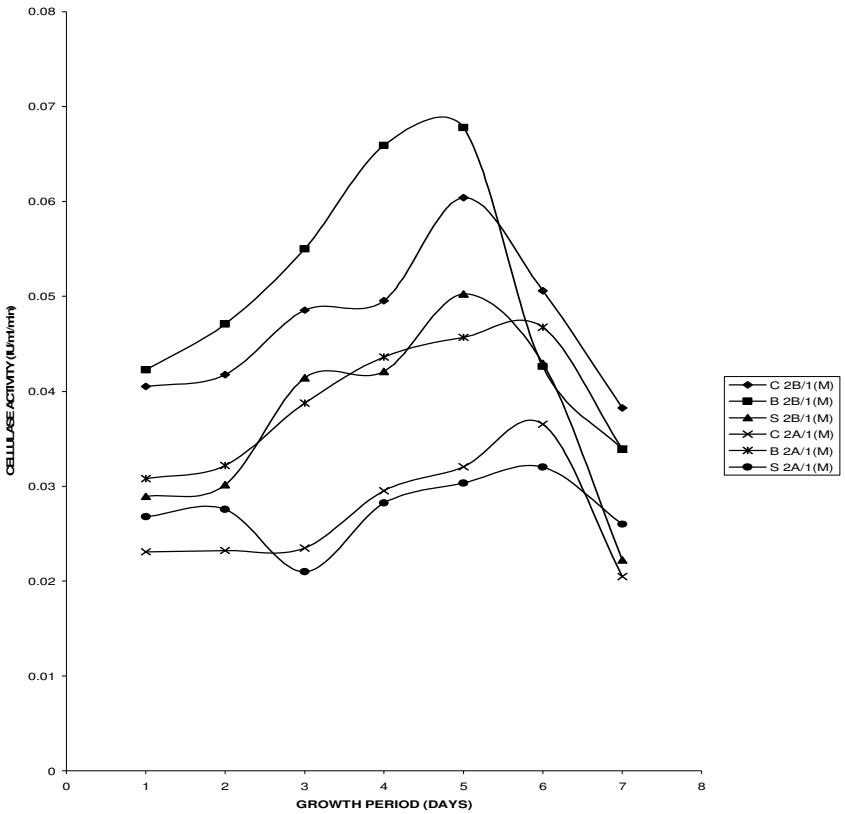

Figure 3. Effect of biomass pretreatment using 2 M NaOH for 1 h (2 B/1) and 2 M H_2SO_4 for 1 h (2 A/3) on cellulase yield. C = Corn cob, B = bagasse, and S = corn straw.

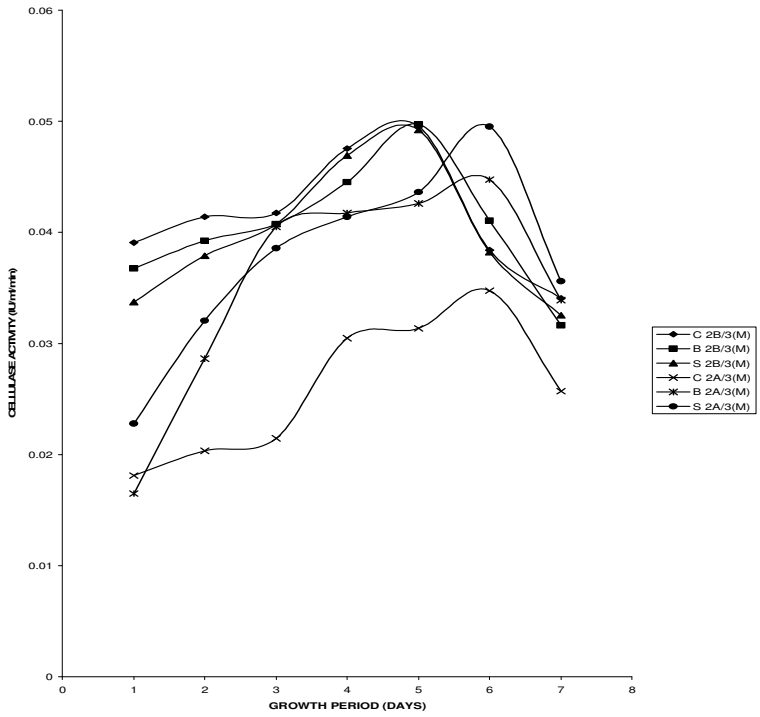

Figure 4. Effect of biomass pretreatment using 2 M NaOH for 3 h (2 B/1) and 2 M H_2SO_4 for 3 h (2 A/3) on cellulaose yield. C = Corn cob, B = bagasse, and S = corn straw.

bial fermentation process in the subsequent steps (Bharathi and Ravindra, 2006). Acid chemical pretreatment may permit cellulose to reanneal leading to horrification of cellulose in micro fibrils (Houghton et al, 2006).

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