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Mutagenic treatment of aspergillus niger with hydroxylamine for improved cellulase synthesis from cellulosic wastes

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Abstract

Cellulases present exciting possibilities for use in lignocellulosic bioconversion. Since microbial sources of cellulases appear to be the cheapest option, the search for strains of microorganisms with robust characteristics is open ended. Conidia of parental strains of Aspergillus niger isolated from soil were mutagenised with hydroxylamine. The selected mutant coded Aspergillus niger AH3, obtained after treatment with hydroxylamine was tested for the production of cellulase on carboxymethylcellulose, bagasse, corn cob and corn straw in shake flask incubated at 320C after ball milling the crystalline lignocelluloses to 250µ particle and the pH maintained at 4.8. Cellulase were evaluated and compared with Trichoderma reesei cellulase preparation (ECONASE CEP) as control. Maximum enzyme activity was at 96 and 120 hours for both mutant and wild strains fermenting amorphous and chrystalline cellulose respectively. Generally, enzyme expression in the lignocellulosics was in the order: bagasse > corn cob > corn straw. Maximal cellulase yield, 0.04888IU/ml/min., was obtained from bagasse fermented with the mutant than with wild, 0.04158, on same substrate thus translating to 18% increase in activity. The candidate mutant and sugarcane bagasse therefore holds great potential as chemical factory and raw material for commercial production of cellulases.

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Introduction

Cellulases are a group of hydrolytic enzymes capable of hydrolyzing natural as well as modified celluloses to smaller sugar components like glucose units. True cellulase activity is defined as the ability of the cellulase preparation to degrade crystalline cellulose extensively (Johnson et al., 1992). Cellulolytic enzymes play an important role in nature's biodegradation processes where plant lignocellulosic materials are efficiently degraded by cellulolytic fungi and bacteria. Cellulase production has attracted a worldwide attention due to the possibility of using this enzyme complex for conversion of abundantly available renewable lignocellulosic biomass for the production of carbohydrates for numerous industrial applications (Hayward et al., 2000). Among microorganisms, fungi are of great interest for enzyme production because they excrete their enzymes extracellularly (Bollok and Reczey 2000). In the fungal family, Trichoderma reesei is regarded as the most efficient producer of cellulase (Miettinen and Suominen 2000). However, the fungus does not excrete sufficient amount of β-glucosidase for efficient enzymatic hydrolysis (Grohmann 1993), for which Aspergillus strains are known to be good producers (Juhasz et al., 2003). Aspergillus niger is a member of the genus Aspergillus, which includes a set of fungi that are generally considered asexual, although perfect forms (reproduce sexually) have been found. Aspergillus is filamentous, cosmopolitan and ubiquitous fungus found in nature. They are geographically widely distributed and have been observed in a broad range of habitats because they can colonize a wide variety of substrates. Aspergillus niger produces a spectrum of extracellular enzymes. Selection of a particular strain, however, remains a tedious task, especially when commercially competent enzyme yields are to be achieved. 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. Industrial bioprocesses with filamentous fungi embrace the production of a majority of commercially important products of biotechnology, in the sense of quality as well as the diversity of metabolites (Znidarsic and Pavko 2001). These are mainly the submerged culture processes, where a dynamic relationship exists between the environmental conditions and the growth pattern of these microorganisms (Znidarsic and Pavko 2001). Hypercellulolytic mutant strains secrete large amounts of cellulases, the largest published amounts being 40g/litre (Durand et al., 1988). Isolation and screening of a hyper producer strain therefore plays a key role in the production of the cellulase enzyme. The vast reservoir of genetic resources of microorganisms is increasingly being tapped and harnessed for greater productivity. Strain improvement is a vital part of process development in most fermentation industries. In strain development, classical mutagenesis is one of the most powerful techniques used to increase enzyme yield from microorganisms (Okonko et al., 2006). A variety of chemicals are known to be mutagenic and these may be classified into three groups according to their modes of action.

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Materials and Methods

The Test Organism

Strain of Aspergillus niger employed for this work was isolated from the various soil environments collected from the six experimental locations. The strains were obtained using the dilution plate count technique described by Dhingra and Sinclair (1995).

Preparation of 0.003 M Hydroxylamine Solutions

The method of Freese et al. (1961) was adopted: Hydroxylammonium chloride solution of 1M was obtained by dissolving 69.49g of the salt in one litre of distilled water. Therefore, to obtain 0.003M solution, 0.2085g of the hydroxylammonium chloride salt was dissolved in a buffer containing: 17.7g of Na2HP04.12H2O ; 3g of KH2PO4 ; 0.001M of MgSO4.7H2O (0.2463g salt per litre), and one litre of distilled water. The pH was adjusted to 7.5 with concentrated (1M) sodium hydroxide solution using Hanna digital pH meter as the guide. The preparation was labeled and stored at refrigeration temperature of 40C.

Qualitative Determination of Cellulolytic Activity

From the various isolates obtained, screening for cellulolytic A. niger strains was made in a synthetic modified Mandels mineral agar medium (Mandels et al., 1974) which served as the selective and expression medium. The screening medium consisted of (gL-1) yeast extract 0.2, carboxymethyl cellulose 10, peptone 1, (NH4)2SO4 4, KH2PO4 2, Urea 0.3, MgSO4.7H2O 0.3, CaCl2 0.3, FeSO4. 7H2O 0.5, MnSO4.4HzO 0.16, ZnSO4 0.14, CoCl2 2, L-Sorbose 6, Congo red 0.025, Tween -80 0.1%, Triton – X100 0.1% and Agar 17.5. The pH was adjusted to 6 using 0.05M sodium hydroxide, and then sterilized at 1210C for 15 minutes. The medium was dispensed into petri plates, allowed to gel and then seeded with spores from the isolates. The cultures were incubated at ambient temperature of 320C for 90 hours; thereafter they were transferred to an incubator set at 500C for 18 hours. The cultures were flooded with 1M NaCl solution. Cellulolytic species had a clear zone around the colony on a reddish pink agar background in form of a halo. Further confirmation of the cellulolytic species was done using the Congo red assay technique as described by Teather and Wood (1982). The medium comprised (w/v): ball-milled filter paper 0.5%, Congo red powder 0.1%, Agar 2%, pH adjusted to 6. The cellulolytic species showing zone of clearing around the colony on red background were selected and stored on slopes of PDA at 40C.

Induction of Mutants

Conidia of cellulolytic Aspergillus niger strains were grown for one week on PDA plates and used for the mutation studies.

Chemical Mutagenesis Using Hydroxylamine

Hydroxylamine does come in the form of hydroxylammonium chloride. Therefore to obtain hydroxylamine from hydroxylammonium chloride, the salt was reacted with 1M NaOH solution using the method of Freese et al., (1961). The hydroxylamine mutagen solution was prepared to give a concentration of 0.003M. Five milliliters of 0.1% Tween 80 in sterile distilled water was added onto the surface of the wild cellulolytic cultures on PDA plates with sterile inoculating loop was used to loosen the spores to form a suspension which was collected in a sterile test tube and concentrated using digital centrifuge at 8000rpm for five minutes. The concentrated spores were re-suspended in 5ml of the 0.003M mutagen solution and then transferred aseptically into sterile bijou bottles. The mixtures were incubated at 370C for 18 hours and then subsequently cloned on plate cloning medium described by Morikawa et al., (1985). The inoculated plates were incubated at room temperature of 320C for four days. Conidia from the mutant clones were further subjected to three cycles of mutation using conidia from previous hydroxylamine treated cells. To determine the success or otherwise of the mutations using hydroxylamine, cloned conidia were replicated on modified plate screening medium described by Mandels et al.(1974) and the plates incubated at ambient temperature (320C) for three days, followed by 18 hours at 500C. The cultures were removed from the incubator and flooded with 1M NaCl. Hypercellulolytic mutants were selected based on the diameter of the zone of hydrolysis (in mm) surrounding the colonies in comparison with the control (wild). Controls were not introduced into the mutagen solution. Mutants were stored on slopes of PDA and kept at 40C until required for analysis.

Inoculum Preparation

Pure cultures of Aspergillus niger strains of both wild and the best mutant from slopes were grown on PDA plates at ambient temperature for 5 days and 100ml of 0.1% Tween 80 was used to wash and resuspend the spores in sterile 100ml capacity Erlenmeyer flasks. The spore suspension was diluted to concentrations of 1.0 x 106 cells per ml by counting in Neubauer chamber using the method of Adhikari and Shrestha (1989). The inoculum ratio was 10% v/v of the entire volume. Inoculations into the fermentation flasks containing the mash were achieved using sterile pipettes.

Preparation of Substrate for Fermentation

The production of extracellular enzymes in all treatments using the carboxymethyl cellulose (control) and crystalline cellulose (corn cob, corn straw and bagasse that had been ball milled to particle size of 250μ) for all fermentation runs were carried out in submerged cultures using the wild type isolate and the selected mutant strains in batch units based on the method of Liming and Xueliang (2004). The substrates were

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fermented in Mandel's medium as proposed by Mandels et al., (1981) with the addition of ten gram per litre of the appropriate carbon source. The medium consisted of (gL-1): yeast extract, 0.2; peptone, 1; (NH4)2 SO4, 4; KH2PO4, 2; Urea, 0.3; MgSO4. 7H2O, 0.3, CaCl2, 0.3; FeSO4. 7H2O, 0.5; Mn SO4. 4H2O, 0.16; ZnSO4, 0.14; CoCl2, 2; Tween-80, 0.1% and pH adjusted to 6 using 0.05M sodium hydroxide solution. The medium was divided into batches of 100ml into 250ml Erlenmeyer flasks and cotton plugged which served as the fermentors. The media were sterilized at 1210C for 15 minutes, allowed to cool and inoculum ratio as obtained bove seeded aseptically into the flask. Fermentations were performed in a rotary shaker (Bellco Glass Inc. USA) at ambient temperature of 320C and 350rpm. During the growth process, samples were withdrawn for analysis of cellulase every 24 hours throughout the growth phase using the supernatant of centrifugates until enzyme activity peaked off. The pH was also recorded at each time.

Cellulase Production on Amorphous and Crystalline Cellulose Using Wild and Mutant Strains

Growth studies were carried out using carboxymethyl cellulose and the crystalline cellulosic wastes as sole carbon source for both the wild strain and the best mutant selected using ANOVA statistical analysis. The initial pH value of the medium was adjusted to the optimum, the medium sterilized and the inocula seeded into the flasks and growth begun at ambient temperature of 320C. Samples were withdrawn for analysis of cellulase at 24 hour interval.

Enzyme Assay (Saccharifying Cellulase Activity Assay)

The total cellulase activity, termed saccharifying cellulase, was determined for all the substrates used using filter paper as the substrate as proposed by Ghose (1987). This method estimates overall cellulolytic activity. It was assayed by incubating 0.5ml of each culture supernatant with a rolled '1cm by 6cm' filter paper strip (Whatman No. 1) (Whatman, UK) in one millilitre (1ml) of 0.05M citrate buffer (pH 4.8) contained in test tube at 500C for 60 minutes. Whatman No.1 filter paper was used as a substrate in this regard because it is widely available and very similar to 'real' process substrate – not too resistant, and not too susceptible (Mandels et al., 1976). Each assay tube was removed from the 500C water bath (Grant J.B. Series, U.K) and the enzyme reaction stopped by addition of 3.0ml 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. Thereafter, the tubes were transferred to cold water bath and each diluted subsequently with 2.5millilitre of sterile distilled water. The absorbance was determined using U.V spectrophotometer (Cecil 1000 series, Cambridge England) against the reagent blank at 540nm and the quantity of reducing sugar read from a glucose standard .Cellulase activity was calculated and expressed in International Units (IU) as described by Janas et al.(2002); and Ul-Haq et al., (2002) using Whatman No 1 filter paper.

Results and Discussion

Cellulolytic Activity Determination (Qualitative) of the Wild Isolates

The result of the cellulolytic determination of the A. niger isolates showed that the isolate from rice growing field exhibited the highest cellulolytic ability and coded as 'A'.

Table 1: Zone of Clearing on Mandel's Medium Exhibited by the Wild and Mutant Strains of A. niger on the Screening Medium (in mm)

	Wild	Mutant	
Isolate	Untreated Strain	Hydroxyamine (0.003M)	% Modification
F (Garden soil)	05	06	20
J (Flower bed)	04	05	25
A (Rice field)	06	10	67

Results are average of triplicate analyses

Qualitative Performance of the Mutagenised Strains

The cellulolytic performance of the mutagenised Aspergillus niger strains using hydroxylamine, in relation to the wild strains is presented in Table 1. The results are expressed as zone of clearing in millimeter (mm). Isolate 'A' gave a 67 % fold increase in activity (plate 1). The mutant obtained was from the third step of mutation. According to Inoue and Schroeder (1988), hydroxylamine modifies bases and causes base pair substitution just like nitrous acid. Hydroxylamine is a very useful mutagen for localized mutagenesis since it specifically modify (hydroxylate) cytosines (Miyoko and Henrik 2000).

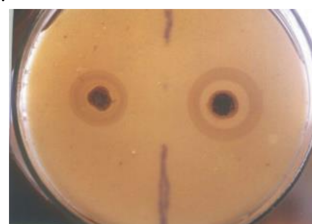

Plate 1: Zone of Clearing; i= wild, ii= mutant (for Isolate A)

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Cellulase Yield from Wild and Mutant Strains Using Different Cellulose Sources

The Enzyme production on the different untreated cellulosic substrates is illustrated in Figures 1 and 2 for the mutant and wild strains respectively. The substrates were supplemented with Mandels mineral salt medium and fermented at pH 4.8. Enzyme synthesis increased from day one and attained maximum by day five and thereafter their activities began to decline. The mutant showed elevated level of enzyme expression (0.04888 IU/ml/min) than the wild (0.04158 IU/ml/min) for bagasse. For the corn cob, activity of the mutant was 0.04192 IU/ml/min while that of the wild was 0.02936 IU/ml/min; whereas the least expression was observed in straw (0.03373IU/ml/min for the mutant and 0.02678 IU/ml/min for the wild). Both wild and mutant strains produced the cellulase independent of the cellulosic source. Highest cellulase expression was at day 5 for all the crystalline substrates irrespective of the strain. Further increase in incubation period may have resulted in the reduced secretion of the enzyme. The decrease in enzyme production observed may be explained on the premise that the susceptible portions of the cellulose molecules was rapidly digested and only the crystalline portion was left behind which could not be used for the production of enzyme by the organism. Enzyme expression in the amorphous cellulose used as the control was quite enormous (0.08381 IU/ml/min for the mutant and 0.06743 IU/ml/min for the wild) (Figure 3), and peak of expression was observed the 4th day. The cellulase standard gave an activity of 0.08968 IU/ml/min at 1mg/10ml concentration.

Conclusion

It is a known fact that Aspergillus niger are quite ubiquitous. Though Aspergillus niger was isolated from all the habitats surveyed, the isolate from rice field has high capacity to produce cellulase. Habitat of isolation is a strong influence on the performance of microorganisms in fermentation. The mutant generated from Aspergillus niger using hydroxylamine showed a remarkable increase (18%) in cellulase yield compared to the wild. This indicates that hydroxylamine is a good mutagen. Cellulase production from both amorphous and crystalline cellulose in shake flask showed that the enzyme expression was better in the amorphous substrate. Type of substrate is a factor influencing metabolite expression in microorganisms. The composition of the different cellulosic substrates may have influenced the performance of the Aspergillus niger AH3. Generally cellulase yield was in the order bagasse > corncob > corn straw. Corn straw has waxy cuticle and high silica content that may have proven hardy for degradation by the organism.

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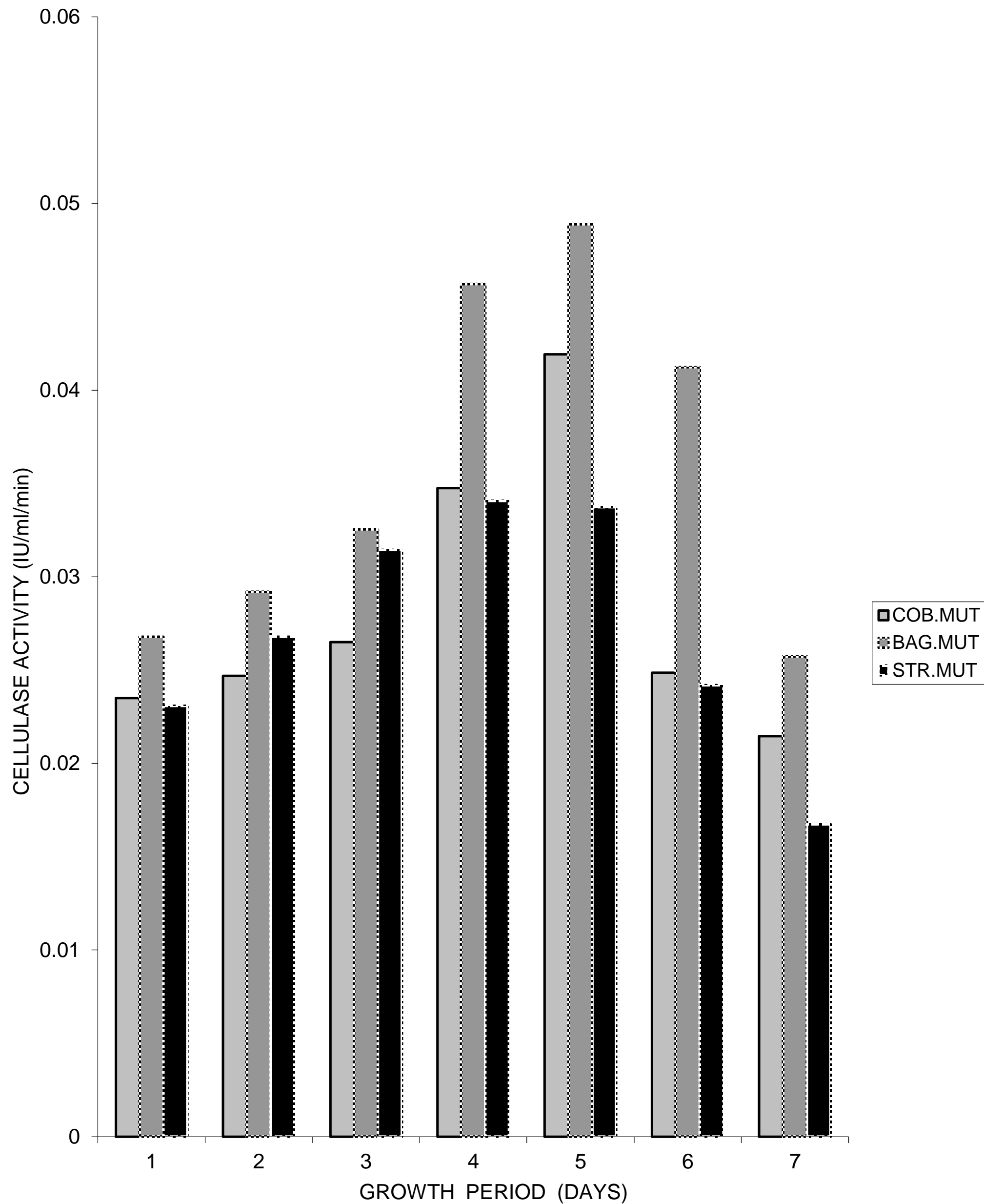


FIGURE 1: Cellulase Yield from Mutant Strain Using Different Cellulose Sources

Key: COB MUT= Corn Cob fermented with mutant
BAG MUT= Baggase fermented with mutant
STR MUT= Straw fermented with mutant

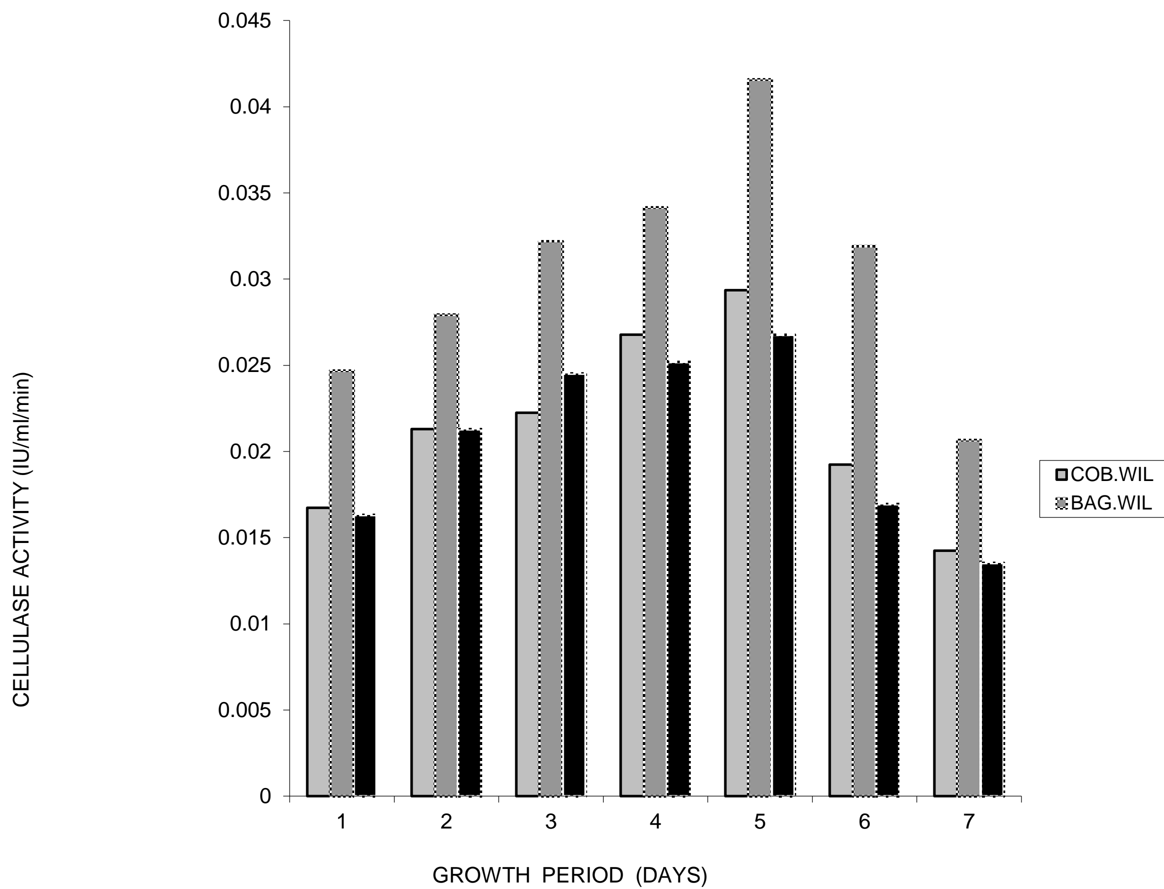
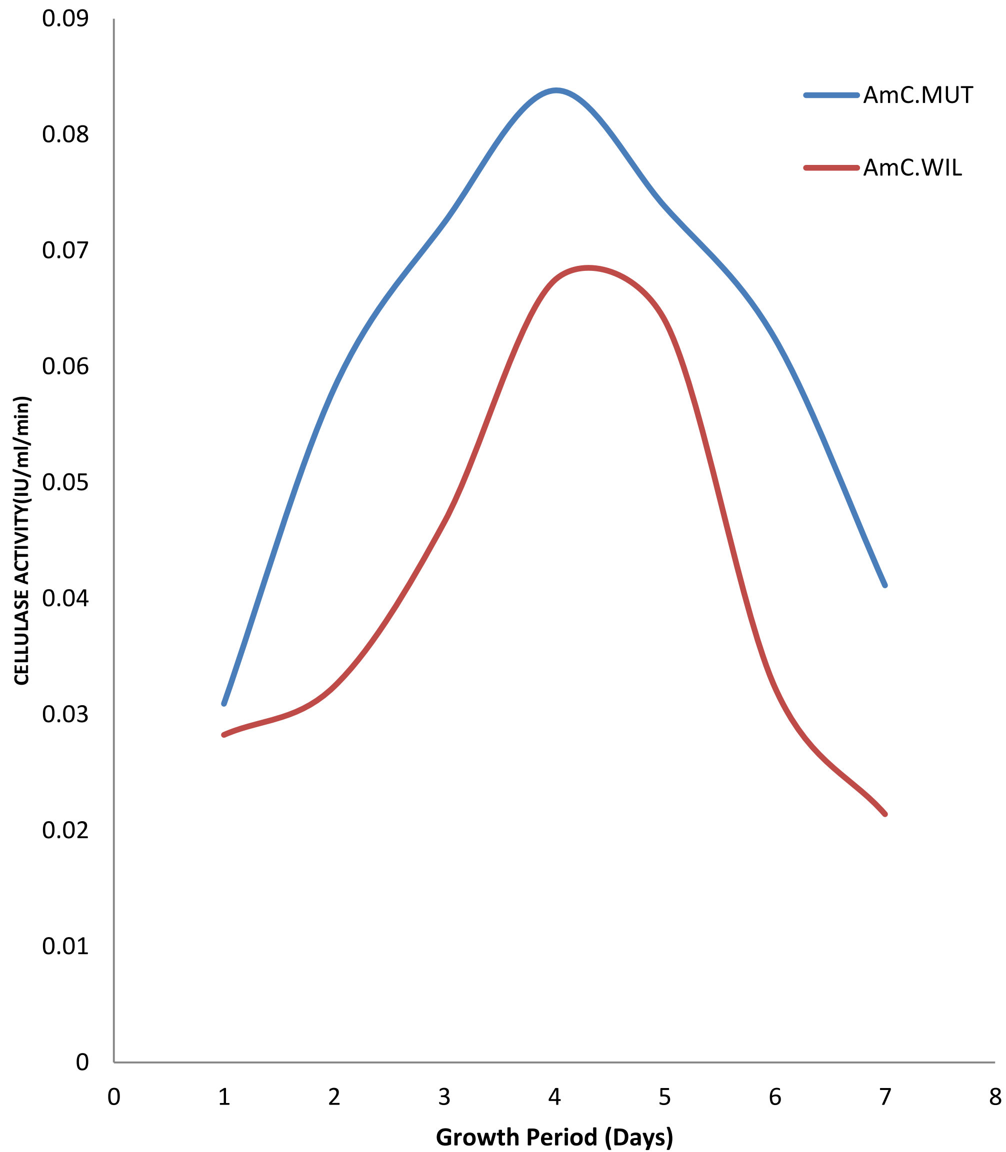


FIGURE 2: Cellulase Yield from Wild Strain Using Different Cellulose Sources

Key: COB WIL= Corn Cob fermented with wild strain
BAG WIL= Baggase fermented with wild strain
STR WIL= Straw fermented with wild strain

Figure 3: Cellulase Yield from Wild (WIL) and Mutant (MUT)Strain Using Carboxy Methyl Cellulose (AmC)



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