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Effect of Substrate Inducers on Crude Cellulase Expression in *Aspergillus Niger*

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Abstract: *An optimized crude cellulase production fortified with carboxyl methyl cellulose (CMC) and avicel as substrate inducers respectively by Aspergillus niger using submerged fermentation technique was carried out. Microbial growth curves were determined using the production media to ascertain a correlation between optimal growth time against cellulase yield and activity. The quantitative and qualitative analysis were carried out using 3, 5 - dinitrosalicyclic acid protocol (DNSA) to obtain optimal functional conditions. From the investigation, it was observed that optimal growth of Aspergillus niger relating to optimal conditions at pH 5 and temperature 40^oC in CMC medium recorded activities of 2.63 U/ml/min and 2.80 U/ml/min respectively while in the case of optimal conditions in avicel at pH 6.0 and temperature of 50^oC, activities recorded were 2.78 U/ml/min and 2.75 U/ml/min respectively. Total crude cellulase activity was also determined with optimal activity of 2.03 U/ml/min recorded in avicel substrate medium while 1.16 U/ml/min was recorded in CMC substrate medium. The experiment thus showed that avicel was a better substrate inducer than CMC as a result of structural complexity and nutritional properties.*

Keywords*: Aspergillus niger, Avicel, Carboxyl Methyl Cellulose, Cellulase, Optimization, Assay*

I. Introduction

Cellulases are enzymes which hydrolyse the β-1,4- glycosidic linkage of cellulose and synthesized by microorganisms during their growth on cellulosic materials (Lee and Koo, 2001). Cellulases are comprised of independently folding, structurally and functionally discrete units called domains or modules, making cellulases modular (Henry et al., 1998). A typical free cellulase is composed of a carbohydrate binding domain (CBD) at the C-terminal joined by a short poly-linker region to the catalytic domain at the N-terminal. There are only two modes of action for the hydrolysis of cellulose by cellulases, either inversion or retention of the configuration of the anomeric carbon. At least two amino acids with carboxyl groups located within the active site catalyze the reaction by acid-base catalysis. Fungi are the main cellulase producing microorganisms, though a few bacteria and actinomycetes have also been reported to yield cellulase activity (Sakthi et al., 2011). Fungal genera like *Aspergillus niger* is known to be a high yield cellulase producer and crude enzyme produced are commercially available for relevant applications. The genus *Aspergillus niger* attack cellulose producing significant amount of cell free cellulase capable of hydrolyzing cellulose into fermentable soluble sugars such as glucose (Wainwright, 1992). Several studies have been carried out to produce cellulolytic enzymes from biological waste degradation process by many microorganisms including bacteria such as Bacillus species and fungi such as *Trichoderma*, *Penicillium* and *Aspergillus* species etc., (Sajith et al., 2016). Cellulases were initially investigated several decades back for the bioconversion of biomass which gave way to research in the industrial applications of the enzyme in animal feed, food, textiles and detergents and in the paper industry (Agger et al., 2001; Haq et al., 2005; Shafique et al., 2009). With the shortage of fossil fuels and the arising need to find alternative source for renewable energy and fuels, there is a renewal of interest in the bioconversion of lignocellulosic biomass using cellulases and other enzymes. In the other fields, however, the technologies and products using cellulases have reached the stage where these enzymes have become indispensable. Cellulase is used to modify the surface properties of cellulosic fibers and fabric in order to achieve a desired surface effect (Kotchoni et al., 2003). Maximum enzyme production is one of the most important goals in biotechnological processes. By optimizing the cultural conditions such as inoculum size, temperature, pH, agitation, aeration and dissolved oxygen etc. the enzyme production can be enhanced by many fold (Gigras et al., 2002). Enzyme production commences at a low rate during the logarithmic growth phase but reaches its maximum value during the stationary phase towards onset of sporulation. Time course study and agitation determines the efficacy of the batch process and subsequent product formation. The pattern of accumulated reducing sugar after specific incubation time is characteristic to the species (Matrai et al., 2000). The major obstacle to the exploitation of

cellulase is its high cost of production which includes other factors like complexity of cellulose structure, the type and source of cellulose employed for production and low amounts of cellulases production by cellulolytic organisms due to catabolite repression influence economics of cellulase production. One effective approach to reduce the cost of enzyme production is to replace lignocelluloses materials by relatively cheaper substrates such as substrate inducers (Oyeleke et al., 2012). Different culture conditions, including those during the inoculum preparation step, result in different fungal growth morphologies and consequently affect enzyme production (Domingues et al., 2000). The influence of pH and temperature (Krishna et al., 2000 and Sohail et al., 2009), type of nutrient medium (Domingues et al., 2000), mixed culture cultivations (Ahamed and Vermette, 2008), and bioreactor design (Ahamed and Vermette, 2010, Kim et al., 1997 and Wase et al., 1985) have been investigated. In several studies, cellulase inducers such as cellulose, lactose, or various lignocelluloses were added to the cultivation medium from the inoculum preparation step onwards for pre-induction of cellulase production (Aiello et al., 1996, Ahamed and Vermette, 2010,Ahamed and Vermette, 2008, Bailey and Tahtiharju, 2003, Gottschalk et al., 2010). The choice of inducer substrate, in both pre-culture and culture media, is one of the main factors influencing cellulase production and studies of enzymatic hydrolysis of biomass using different enzymatic cocktails indicated that the use of lignocellulosic substrates, in place of other commercial inducers such as carboxyl methyl cellulose or lactose, can contribute to the specificity of the enzymatic pool and improve hydrolysis yields (Castro et al., 2010a, Maeda et al., 2011 and Singh et al., 2009). The growth morphologies of *Aspergillus niger* in submerged fermentation under different culture conditions have a strong influence on fermentation performance (El Enshasy et al., 2006). This investigation was aimed at providing a definite relevance to the production of cellulase while studying the relevant microbial growth condition and comparing to influence of substrate inducers such as carboxyl methyl cellulose (CMC) and avicel as this case applies.

II. Materials and Methods

Microbial selection

Aspergillus niger sp was obtained from the department of biotechnology culture centre of the Federal Institute of Industrial Research, Lagos state, Nigeria, West Africa. Following standard microbiological practices under sterile condition the microorganism had been isolated from decayed wood sample using a 50µg/ml kanamycin fortified Potato Dextrose agar (PDA). The isolate was preserved on a PDA slant in 4^{o}C.

Microbial characteristics

Morphological Identification of *Aspergillus niger* sp. was carried out based on macroscopic and microscopic characteristics. Macroscopic characteristics such as colony diameter, exudates, colony reverse were observed but for microscopic characteristics including conidiophore, vesicle, metulae, phialides and conidia, slides were stained with cotton blue lactophenol and further observed under the microscope at magnification size X40. Photographs were taken with digital camera Canon Power Shot A550, 7.1 mega pixels. A morphological examination of specie was first made at low magnification power of microscope after that detailed examination were done according to (Hina et al., 2013, Raper and Fennell, 1965, Gams etal., (Gams et al., 1985).

DNA Isolation

Aspergillus niger genomic DNA was isolated using Plant/Fungal Sigma – Aldrich isolation kit (Cat. No. E5038). The genomic DNA was processed and run on a gel electrophoresis to determine the molecular weight. The DNA was further used for cloning and sequencing.

ITS amplification

`ITS1-5.8S- ITS2 rDNA region was amplified using the following primer pair; ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS-5 (5'- GGAAGTAAAAGTCGTAACAAGG-3'. Amplification protocol was carried out in 50 µl reaction mixture containing 34.6µl sterile water, 5µl of 10x PCR buffer, 3µl of 25mM MgCl2, 4µl of 10mM dNTPs, 1µl of each primer (20µM), 0.4µl (2 U) of Taq polymerase (TaKaRa Corp., China) and 1 µl (200ng) template DNA. Eppendorf DNA thermal cycles was used with the following PCR profile: an initial denaturation for 5 min at 95°C, 35 thermal cycles (1 min at 95° C, 1min at 54° C and 1 min at 72° C), and a final 10 min extension at 72°C. The amplified DNA was purified using Tiangen QUICK PCR purification kit following manufacturer's instructions (Tiangen, China). (Hřibová et al., 2011; Liu et al., 2009; White et al.,1990).

Cloning and sequencing

Purified DNA was ligated in pMD18®-T Easy vector (TaKaRa Corp., China) overnight at 16° C. The ligated DNA was transformed in DH5α competent cells. The recombinant clones were identified through blue/white color selection and the presence of insert in the recombinant clones (white colonies) was confirmed

following colony PCR. For sequencing, plasmid DNA was isolated following alkali lysis method (Sambrook, et al.,1989).

Preparation of inoculum

The inoculum was prepared by growing the organism in 250 ml Erlenmeyer flask with 50ml of Potato Dextrose broth at a final pH 5.0. The medium was inoculated with isolate from the Potato Dextrose Agar slant and incubated at 30 °C for 3 days in a shaker (200 rpm) before it was used for the fermentation process.

Cellulase production

Submerged fermentation was carried out in two (2) 250 ml Erlenmeyer flasks (A and B) containing 100 ml of fermentation medium separately. The composition of the medium contained the following: FeSO4 0.001g, NaNO3 1.5g, $NaHPO_4$ 0.5g, $MgSO_4$ 0.05g, KCl 0.25g, Tween 80 in the separate flasks. Each flask was fortified with 1g CMC and 1g avicel respectively. The separate media were sterilized by autoclaving at 121°C for 15 min. Each flask was inoculated with 1ml of the above prepared inoculum. The cultures were incubated using a rotary shaker (120 rpm) at 30°C for 120 h. At the end of the fermentation crude extract was centrifuged at 7000 rpm for 15 min and the supernatant was used for as a source of extracellular enzyme which was stored at 4^oC before further analysis.

Assay

Filter paper assay was used to estimate total cellulase activity and endoglucanase activity (CMCase activity) Soma and Rangasamy, (2011) in the crude enzyme preparation as given below. For filter paper activity Whatman no. 1 filter paper strip of dimension 1.0× 6 cm (50 mg) was placed into each assay tube. The filter paper strip was saturated with 1.0 ml of Na–citrate buffer (0.05 M, pH 4.8) and was temperate for 10 min at 50 °C Half milliliter of an appropriately diluted (in Na–citrate buffer, 0.05M; pH 4.8) enzyme was added to the tube and incubated at 50 °C for 60 min. And in case of endoglucanase activity half milliliter of 1 % carboxymethyl cellulose in 0.05M Na–citrate buffer, pH 4.8 was temperate for 10 min at 50 °C. After that half milliliter of an appropriately diluted enzyme was added to the tube and incubated at 50 °C for 30 min. Appropriate controls were also run along with the test. At the end of the incubation period, tubes were removed from the water bath, and the reaction was stopped by addition of 3 ml of 3, 5-dinitrosalicylic acid reagent per tube. The tubes were incubated for 5 min in a boiling water bath for color development and were cooled rapidly. The reaction mixture was diluted appropriately and was measured against a reagent blank at 540 nm in a UV-VIS spectrophotometer (Thermo Scientifc Spectramax M3, USA). The concentration of glucose released by enzyme was determined by comparing against a standard curve constructed similarly with known concentrations of glucose. One unit of enzyme activity was defined as the amount of enzyme required for liberating 1.0M of glucose per milliliter per minute.

Effect of Temperature on the Production of Cellulase

The optimum temperature of cellulase from *Aspergillus niger* was determined by incubating 1ml appropriately diluted enzyme with 2 ml 1% CMC in citrate buffer pH 4.8 (Bertrand et al., 2004) at different temperature (30 – 100ºC) for 2 hours. Reducing sugars were estimated by the 3, 5 - dinitrosalicyclic acid reagent method (Miller, 1959). This was carried for CMC and avicel cellulase samples respectively.

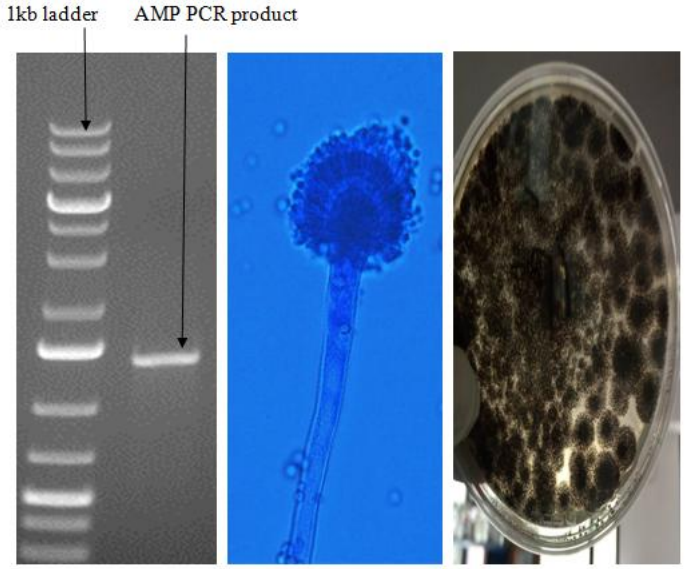
Effect of pH on the Production of Cellulase

The optimum pH was determined by incubating the 1 ml appropriately diluted enzyme mixed with 2 ml 3% CMC in 1 ml citrate buffer, pH 4.8 (Bertrand et al., 2004) buffer of different pH (3 – 11) for 2 hours at room temperature (40ºC). Reducing sugars thus released were estimated by the 3, 5 - dinitrosalicyclic acid reagent method (Miller, 1959). This was carried for CMC and avicel cellulase samples respectively

III. Results and Discusssion

(a) **(b)**

Figure 1: Microbial growth in carboxyl methyl cellulose (CMC) and avicel production media are shown in Figure 1a and 1b. The media were prepared and used to ascertain the correlation between optimal growth rate of *Aspergillus niger* against cellulase yeild and activity. Figure 1a shows cellulase production medium having 1% CMC as substrate inducer while Figure 1b shows cellulase production medium having 1% avicel as substrate inducer. Both media had been been inoculated with 1ml inoculum of *Aspergillus niger* and incubated for 120 hr at 30^{o}C.



(c) (d) (e)

Figure 2: The properties of *Aspergllus niger* are shown in different mode as described in Figure 2.

Figure 2c above is showing the amplified polymerization chain reaction product with molecular weight of about 1800bp. Figure 2d and 2e are illustrating the microscopic characteristics under microscope at X40 magnification. Figure 2e describes the front view of Aspergillus niger on potato dextrose agar medium fortified with 50µg/ml kanamycin to prevent contamination.

Note: Figure 2d is an kmage recently cited in article original published but it was also used here because of its relevance to information and avoidance of plagiarism.

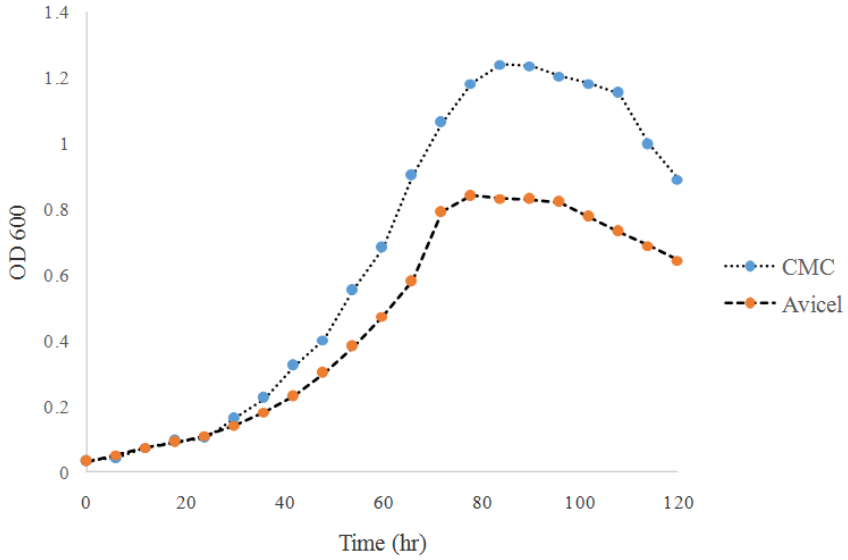


Figure 3: Growth curve comparison for *Aspergillus niger* between carboxyl methyl cellulose and avicel production media for cellulase. An illustration of *Aspergillus niger* growth curve obtained from two different cellulose production media is shown above. The optimal growth in time (hr) recorded from Carboxyl Methyl Cellulose fortified cellulase production medium was at 84th hour while optimal growth rate in time (hr) for avicel fortified cellulase production medium recorded at 78th hour respectively as described above.

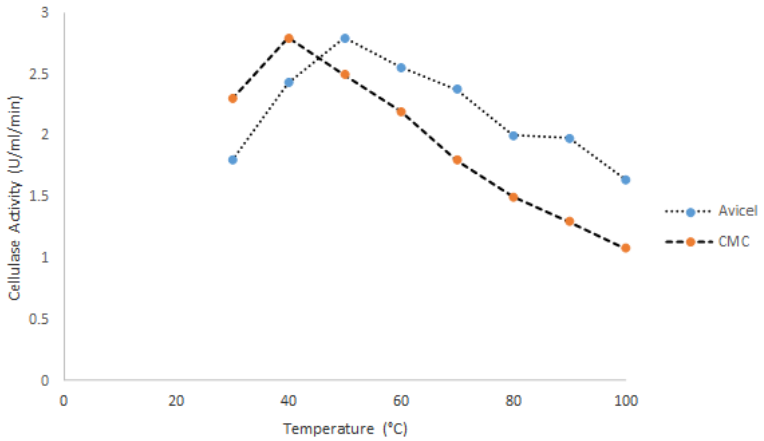


Figure 4: Effect of temeperature (^{o}C) on crude cellulase activity. The cellulolytic activity was deternined against optimial temeprature (^{o}C) and the graph above shows effect of optimal temeperature (^{o}C) on CMC and avicel crude cellulase samples respectively. From The graph it is observed that optimal temperature for CMC crude cellulase sample was 40^{o}C having recording an activity of 2.980 U/ml/min while similar result was obtained for avicel crude cellulase sample having optimal temperature at 50^{o}C recording activity of 2.75 U/ml/min.

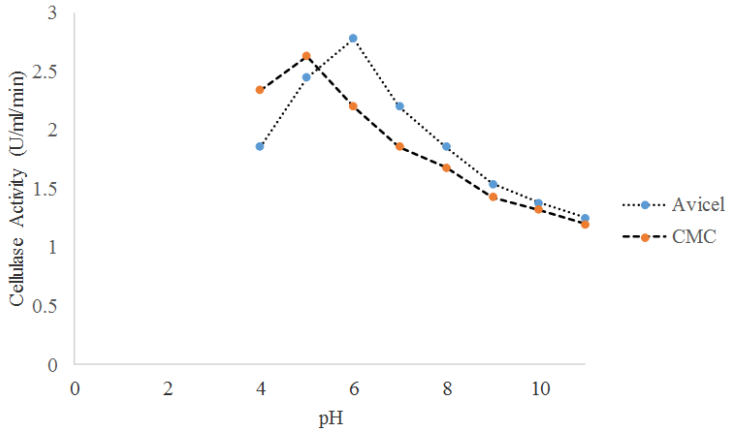


Figure 5: Effect of pH on crude cellulase activity. Effect of pH on CMC and avicel crude cellulase samples was also determined and the Figure above shows the optimal pH conditions for both samples respectively. From the graph it is observed that optimal pH for CMC crude cellulase sample was at 5.0 recording 2.63 U/ml/min activity while optimal pH for avicel crude cellulase sample was at 6.0 recording 2.78 U/ml/min activity.

Cellulase is an important enzyme for hydrolysis of agro wastes and other cellulosic materials. The present study was carried out to evaluate the relationship between growth conditions such as temperature (^{o}C) and pH for *Aspergillus niger* and further show the effect they have on cellulase activity while using carboxyl methyl cellulose (CMC) and avicel as substrate inducers in separate medium composition. To understand the biochemistry of cellulose degrading *Aspergillus niger*, it is necessary understudy its activities under various optimize under physical and chemical parameters as described in this invsetigation. Cellulase production by different organisms in submerged state fermentation has received more attention and is found to be cost-prohibitive because of high cost of process engineering. The results showed that the growth of the *Aspergillus niger* was slower in CMC cellulase production medium as compared to avicel cellulase. The production of cellulase enzyme was carried out under submerged fermentation conditions. Although both substrate inducers gave good activities comparatively, advanced research is required to identify other substrate inducers that can possible do better.

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