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Original article

Time course for cellulase enzyme production by *Aspergillus Flavus* using different organic nitrogen sources

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ABSTRACT

The time course for the production of cellulase enzyme of *Aspergillus flavus* was carried out at 35oC in a shaken incubator using modified Mandel and Weber's medium containing in (g/l) (NH4)2S04 1.4, KH2P04 2.0, Urea 0.3, MgS047H20 0.3, CaCl3 0.3 FeS047H20 0.005, ZnS047H200.0014, MnS04H20 0.0016, CoCl3 0.002, Tween 80 2.0 ml, Carboxymethylcellulose 10.0 and pH 6.8. It indicated a period of 120 h (5days) as the optimum incubation period under the experimental condition using CMC as substrate. The mycelia growth pattern (biomass) indicated highest growth on the fifth day also corresponding to the time of maximum enzyme activity. The results from the studies also showed that Mandel and Weber's Medium was better than other substitutions with alternative nitrogen sources in all the assays, followed by yeast extract and the least being soya bean meal. The study therefore maintained that Mandel and Weber's Medium is the best at 5 days incubation for cellulase production by Aspergillus flavus.

1. Introduction

Industrial interest in cellulase is high due to its wide application in various industries such as animal feed production, starch processing, malting and brewing, grain alcohol fermentation, extraction of fruit and vegetable Juices, as well as manufacture of pulp, paper and textiles (Adsul et al., 2007; Kaur et al., 2007). Enzymatic hydrolysis of cellulosic materials is achieved by a sequence of reactions with the main components of cellulase complex enzymes, which include FPase, CMCase and β-glucosidase. The characteristics of all these three component of cellulase complex are the main factors that influence the application of enzyme-based bioconversion Technology. Therefore, research has been directed to discover new microorganisms that have capability to produce cellulolytic enzymes with high specific activity. Among the cellulolytic fungi, Trichoderma spp. and Aspergillus spp.have been widely studied for their ability to secrete high levels of cellulose-degrading enzymes (Zhou et al., 2008). Aspergillus spp. is the major agents of decomposition and decay and as such produce a broad range of enzymes, including cellulase. The production of cellulase is a key factor in the hydrolysis of cellulosic materials. Cellulase characteristics and production by Aspergillus spp. have been well documented in the literature (Lockington et al., 2002; Ong et al., 2004; Wang et al., 2006). Cellulase enzyme is composed of a complex mixture of proteins with different specificities to hydrolyze glycosidic bonds. They are divided into three major groups namely, Endoglucanases or endo β,1-4 glucanase Cm cellulase, Cx (EC3.2.1.4), exoglucanases or cellobiohydrolases C1(EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21). Endoglucanases are proposed to initiate attack randomly at multiple sites in the amorphose regions of the cellulose fibre, opening up sites for subsequent attack by the exoglucanases, which hydrolyze highly crystalline cellulose while β-glucosidases hydrolyze glucose dimmers and cellobiose to glucose. However the cost of substrate plays crucial role in the economics of the enzyme production. And the ability of fungal organisms to produce cellulase and subsequently degrade cellulose efficiently is thought to be associated with a mycelia growth habit which allows the fungus to transport scarce nutrients such as nitrogen over a distance into the nutrient poor lignocellulosic substrate that constitute its carbon source. Time course study is undertaken to avoid autolysis of the mycelia and / or loss of enzyme stability as a result of prolonged incubation

2. Materials and methods

2.1. Study area and materials used

The work is carried out at the Department of Microbiology, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria. Awka is the capital city of Anambra state, which lies within the southern part of Nigeria. The geographical coordinates of Awka corresponds to 6.22 North and 7.07 East and falls within the humid tropics of Nigeria. The town Awka was made after clearing much of the tropical grassland, and outskirts of the city are still covered with grassland. It has a moderate climate with a very high temperature during the dry season and average rainfall during the rainy season. Awka has the mean annual temperature and precipitation of 35oC and 1117mm, respectively (NIMET, 2006).

The materials used in this research include reagents, salts, solvents, resins, substrates media among others. Most of the materials were kindly provided by Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria, while others were either obtained from the Research Laboratory or purchased from FinLab. Ltd., Enugu.

2.2. Method

2.2.1. Experimental design

Generally, to ensure accuracy, most parameters were measured two times and the mean taken as the value of the parameter. Indices that were measured on graded levels were statistically analyzed using one way analysis of variance (ANOVA) and the differences between treatment means were separated using Duncan's New Multiple Range Test (DNMRT).

Also, data collected were presented in graphs and histograms to increase clarity. Other descriptive statistics such as range, intervals and the like were employed were necessary.

2.2.2. Determination of effect of organic nitrogen sources on enzyme production

Different organic nitrogen sources which include yeast extract, soya bean meal and peptone were used in substitution to the inorganic nitrogen (NH4SO4) in the culture medium for the cultivation of the process organism Aspergillus flavus isolated earlier by Okonkwo et al. (2012). The fermentation process was done using Mandel and Weber's medium in which the inorganic salt {(NH4)2S04} was replaced by each of the above listed organic nitrogen sources at 1.4 g/l, in separate 100 ml Erlemeyer flasks. The normal Mandel and Weber's medium was also inoculated to serve as control,and incubated at 35°C in a Stuart orbital shaker S-150 at 200rpm for 7 days. The saccharifying activity of the enzyme was later determined.

2.2.3. Determination of time course for enzyme production and mycelia growth rate

The time course for maximum enzyme production was studied using the Mandel and Weber's medium. One litre medium was prepared and distributed in 100 ml amount to seven 250 ml conical flasks. These were each inoculated with a loopful of conidia of the process organism and incubated at 35 0C in a Stuart orbital shaker S150 for 7 days at 200rpm. One flask was removed each day and checked for mycelia growth at 660nm using spectrophotometer JENWAY model 6405 .The content was then subjected to centrifugation at 4000 rpm for 20 min. using Centurion centrifuge to remove the mycelia and other insoluble materials. The supernatants were recovered and used for enzyme activities using CMC, filter paper and cotton wool.

2.2.4. Enzyme assays

2.2.4.1. Carboxymethyl cellulose (cmc) saccharifying activity

An appropriately diluted (1,2) enzyme sample (0.5 ml) was mixed with 0.5 ml of 1 % CMC dissolved in 0.2 M phosphate buffer (pH 6.8) and incubated for 30 min at 40 0C in a water bath (Memmert) The reducing sugar released was estimated by 3, 5- dinitrosalicylic acid method (Miller, 1959) as follows; at the end of incubation, the enzyme reaction was stopped by adding o.5 ml of 3, 5- dinitrosalicylic acid reagent (BDH). The mixture was placed in boiling water for 10 min, after which it was cooled, and 5 ml distilled water added. The absorbance was then read at 540 nm using the substrate solution treated in the same way as blank to zero the spectrophotometer (JENWAY), model 6405. One unit (IU) of CMCase activity was defined as the amount of enzyme required to liberate 1 µmol of glucose from the substrate under the assay condition.

2.2.4.2. Filter paper saccharifying activity

The reaction mixture containing 0.25 ml of diluted enzyme solution, 0.5 ml of 0.2 M phosphate buffer (pH 6.8) and 25mg of whatman No 1 filter paper strip was incubated at 40 0C for 1 h as described by Stephen et al. (2003). The reducing sugar liberated was determined by 3, 5- dinitrosalicylic acid method (Miller, 1959). One unit (1U) of filter paper activity is defined as the amount of enzyme required to liberate 1 µmole of glucose per 1 h.

2.2.4.3. Cotton wool saccharifying activity

To a mixture of 1.0 ml of diluted enzyme and 1.0 ml of 0.2 M phosphate buffer (pH 6.8) was added 50mg of absorbent cotton wool and incubated at 40 0C for 24 h. The reducing sugar liberated was determined by the 3, 5-dinitrosalicylic acid method described above. One unit of cotton saccharifying activity was taken to be mg of glucose liberated per 24 h.

2.2.4.4. Crystalline cellulose saccharifying activity

This was also done using the same method as in carboxymethyl cellulose assay. To a solution of 0.5 ml enzyme is added 0.5 ml of 1 % crystalline cellulose (Sigmacel 20) in 0.2 M phosphate buffer (pH 6.8) and incubated for 30 min. at 40 0C in a water bath (Memmert). The reducing sugar liberated was estimated by 3, 5-dinitrosalicylic acid method of Miller (1959). The mixture was placed in boiling water for 10 min, after which it was cooled, and 5 ml distilled water added. The absorbance was then read at 540 nm using the substrate solution treated in the same way as blank to zero the spectrophotometer (JENWAY). One unit of activity is defined as µmol of glucose liberated per minute. The values obtained were compared with the control.

2.2.5. Protein estimation

The protein content of the crude enzyme was determined by dye binding method as described by Bradford (1976). To this end, 450 mg of Coomassie Brilliant Blue (G. 250 Serva) were dissolved in 50 ml ethanol and 100 ml

of 85 % orthophospheric acid (BDH). The volume was made up to 1 litre with distilled water. The reagent was filtered twice. Bovine serum albumin (BSA) (5mg/ml) was used to prepare a standard curve. A 2.5 ml of 1,5 dilution of Bradford reagent was added to 0.05 ml of the standard samples. The mixture was shaken vigorously and the absorbance measured after 2 min of mixing at 595 nm using a spectrophotometer. The values were used to prepare a standard curve. The crude and the purified samples of the enzymes were treated the same way as the standard (BSA) and the values in milligramme extrapolated from the standard curve.

3. Results and Discussion

3.1. Effect of organic nitrogen sources on the growth of aspergillus flavus for cellulose production

The results from the studies showed that Mandel and Weber's Medium (1969) was significantly ($P<0.05$) better than other substitutions with alternative nitrogen sources in all the assays, followed by yeast extract and the least being soyabean meal (Table 1).

Table 1
Effect of Nitrogen sources on the growth of Aspergillus flavus.

Assay	Mandel & Weber	Soyabean	Yeast extract	Peptone
CMC	0.0409c	0.00013a	0.00045b	0.000391b
Filter Paper	0.00517c	0.00015a	0.00027b	0.000325b
Cotton Wool	0.00577c	0.00023a	0.00035b	0.000354b
Crystalline Cellulose	0.0325c	0.0037b	0.0023a	0.00285a

+Means bearing different superscripts on the same row are significantly different ($P<0.05$).

Time course for the enzyme production in the Mandel and Weber's medium indicated a period of 120 h (5 days) as the optimum incubation period under the experimental conditions using CMC as substrate (Fig. 1). The mycelia growth pattern (biomass) indicated highest growth also on the fifth day corresponding to the time of maximum enzyme activity.

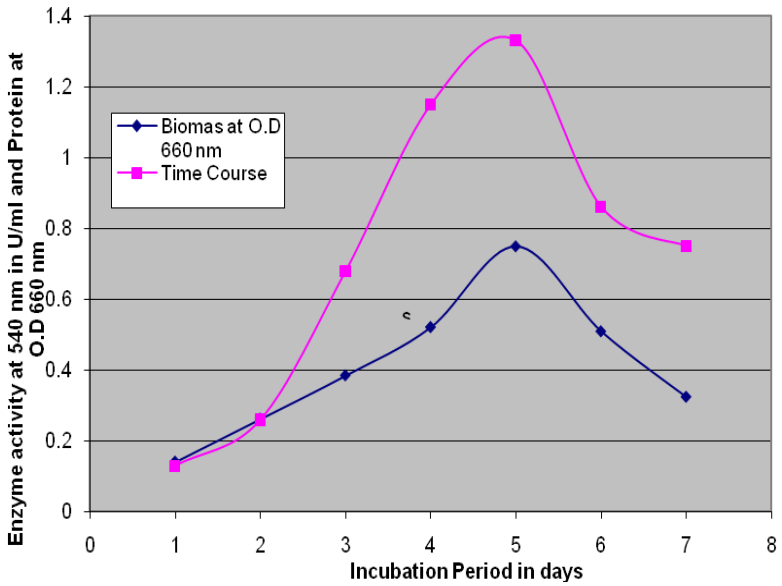

Fig. 1. Time course for cellulase enzyme production and mycelial growth using CMC Assay.

Time course studies indicate that the fungal culture may be effectively harvested in their highest cellulase production capacity in 120 h (5 days) under these conditions, above which the enzyme activity decreased Fig 1. The plausible reason for decreased enzyme activity after a prolonged incubation might be due to autolysis of the mycelia and / or loss of enzyme stability. The report is in accordance with earlier reports. Cellulase was produced from Aspergillus niger KH2 at 120 h incubation (Kang et al., 2004). Similarly, cellulolytic enzymes were produced by Aspergillus phoenix at 120 h incubation (Dedavid et al., 2008), likewise Kirchner et al. (2005) produced maximum β- glucosidase activity from Aspergillus niger C.6 after 96-120 h. But was at variance with the report of Ojumu et al. (2003), though, they used natural substrates. Maximum cellulase production may be affected by several factors including the presence of different ratios of amorphous to crystalline cellulose (Ogel et al., 2001).

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