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CELLULASE PRODUCTION BY *THERMOMONOSPORA FUSCA* YX, *STREPTOMYCES VIRIDOSPORUS* T7A AND *S. SETONII* 75 VI 2 GROWING ON LIGNOCELLULOSIC SUBSTRATES

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ABSTRACT

*The production of cellulases by **Thermomonospora fusca** YX, **Streptomyces viridosporus** T7A and **S. setonii** 75 Vi 2 grown differently on sugar cane **(Saccharum officinarum)** bagasse, Elephant grass **(Panicum maximum)** and sawdust lignocelluloses were studied. The organisms released cellulases into the media while growing in liquid cultures containing differently these delignified lignocelluloses. A maximum enzyme activity of 1.5 $FP^{U}ml^{-1}$ was obtained from **T. fusca** YX grown on a 0.75% **Saccharum officinarum** bagasse lignocellulose at 55°C and pH 6.0: while the maximum enzyme activities of 1.25 $FPUml^{-1}$ and 0.85$FPUml^{-1}$ were obtained from **S. viridosporus** T7A and **S. setonii** 75 Vi 2 grown on a 0.75% **Panicum maximum** lignocellublose respectively at 37°C and pH 6.0. The carboxymethylcellulase (Endo-1, 4-β-glucanase; EC. 3.2.1.4) activity of **T. fusca** YX grown on **Panicum maximum** and **Saccharum officinarum** bagasse lignocelluloses at 55°C and pH 6.0 was $5.2Uml^{-1}$ and $3.5Uml^{-1}$ respectively. The two organisms grown on sawdust lignocellulose showed no significant activity under the same culture conditions. The results obtained revealed that both **Panicum maximum** and **Saccharum officinarum** bagasse lignocelluloses are suitable substrates for cellulase production; and **S. viridosporus** T7A and T. **fusca** YX are suitable organisms for cellulase production. The two organisms therefore are as good as the typical fungus **(T. viride)** usually used for cellulase production studies.*

INTRODUCTION

Cellulosic materials are the most abundant natural resources available to man comprising about 95% of earth's land-based biomass (1,2 and 3). The great bulk of cellulose available in nature is derived from the stem of woody angiosperms (hardwoods), stems of woody gymnosperms (soft woods) and the stems of monocotyledons (grasses, such as palms, bamboo, wheat, rice, sugar cane, etc.). Other sources of cellulose in order of decreasing abundance include the non-lignified parenchyma cells of most leaves, certain non-lignified or only partially lignified fibres such as seed hairs of cotton and the best fibres of flax (4). The cellulose component of lignocellulose is a readily available raw material for the production of glucose from where various sources of food consumed by man and animals can be obtained. In addition, it can be used as feedstock to make solvents, plastics, and other chemicals now made from petroleum. It can also be used for the production of single-cell protein (SCP) and mushroom or it can be fermented into a clean burning fuel, such as ethanol (5). The first step in the utilization of cellulose component of lignocellulose materials is the hydrolysis of the cellulose using extracellular cellulases (2)

It is known that a variety of micro-organisms including fungi, bacteria and actinomycetes are cellulolytic (2 and 6). Although many of these grow quite rapidly, only a few product extracellular cellulases are capable of converting crystalline cellulose to glucose in--vitro(7). Among all the genera of cellulolytic organisms the fungal cellulase system has been widely studied. The *Trichoderma* has been found to be the best cellulase producers (8, 9 and 10). *T. viride* and *T. reesei* have been the initial micro-organisms of choice due to their high cellulolytic activities towards crystalline cellulose (11-18).

The purpose of the present investigation was to study the production of extracellular cellulases by *Thermomonospora fusca* YX, *S. viridosporus* T7A and *S.setonii* 75 vi2 as they grow on different lignocellulose substrates. The amounts of cellulases produced were compared to that of a well known cellulase producer, *T. viride*.

MATERIALS AND METHODS

Reagent: Dinitrosalicylic acid (DNS) reagent was used (16 and 19). To 800ml of 1.0% DNS. $(NO_2)_2\ C_6H_2\ (OH).\ COOH.H_2O$, (BDH, England) was added 300 ml of 4.5% NaOH and 255g of Rochelle salt (Sodium Potassium tartrate). This was the DNS solution. Crystalline phenol (Merck), 10.0g, was dissolved in 22 ml of 10% NaOH and finaly diluted to 100ml with distilled water. To 69 ml of this solution 6.9g of sodium disulphite was added to the previously prepared DNS solution. The resultant solution was well mixed until all the Rochelle salt was dissolved. This was stored in tightly stoppered brown bottle until needed.

Cultures

The *Streptomyces* strains: *Streptomvces viridosporus* T7A and *S. setonii* 75Vi2 were supplied by Dr. S.P. Antai, Department of Biological Sciences, University of Calabar, Calabar, Nigeria. The organisms were grown on yeast extract mineral salts agar (20). Stock cultures were kept as slants on tryptone yeast extract agar (21). *Thermomonospora fusca* YX was obtained from Professor David B. Wilson, Biochemsistry, Molecular and Cell Biology Section, Cornnell University Ithaca, New York, NY. 14853. The organism was grown up in Tryptone yeast extract Glucose (TYG) broth and the stock cultures were kept as slants on TYG agar at 4°C and transfers were made bimonthly (22).

Substrates Used

(i) *Grass lignocellulose:* Elephant grass (*Panicum maximum*) were cut from the field a little above the ground. They were cut into smaller pieces and dried in a hot air oven Gallenkamp at 70°C for 24 hours

(ii) *Sugar cane bagasse lignocellulose:* The sugarcane (*Saccharum officinarum)* bagasse were picked from the local markets cut into small pieces and dried in a hot air Oven (Gellenkamp) at 70°C for 24 hours.

(iii) *Sawdust lignocellulose:* The sawdust were obtained from the carpenter's shed at the Centre for Special Project (CSP) of the Rivers State University of Science and Technology, Port Harcourt, Nigeria.

(iv) Carboxymethylcellulose (CMC) type 7LF (Degree of substitution (DS) is 0.75). (Hercules Chemicals, Wilmington Delaware, U.S.A.) was a gift from Berger Paints (Nig) Ltd; Port Harcourt. Nigeria.

Lignocellulose Pretreatment

The different lignocellulose substrates were ground in a Glen Creston model grinder to pass a 600mm sieve. The substrates were then extracted according to the method of Crawford (1978). Generally, the substrates were extracted with hot water at 80°C until all water-soluble components were removed (four changes of water were used) and then in sequence with Benzene: Ethanol (1:1); 95% ethanol, and finally with hot water at 70°C for 24 hours. The lignocellulose substrates were then delignified (23).

Briefly, the extractive-free lignocellulose substrates above were autoclaved in 1.0% NaOH solution for lhr at 121°C. The autoclaved lignocellulosic substrated were washed with several changes of distilled water until there was no trace of NaOH: confirmed by no colour change when few drops of 1% phenolphthalein solution was added to the rinse water. The washed lignocellulosic substrates were then dried in a hot air oven at 70°C for 24 hours and used when needed. These were used as, delignified (pretreated) lignocellulosic substrates.

Cellulase Production

The *Streptomyces viridosporus* T7A and *S. setonii* 75vi2 showing good growth on Tryptone yeast extract agar slants were asceptically inoculated into 125ml of different mineral salt lignocellulose broth contained in 250ml flasks (21). This was done in triplicate for each organism and each lignocellulose substrate. The content of the flasks were mixed and incubated at 37°C in a Gallenkamp shaker incubator at 100rpm. One milliliter samples were taken periodically (every 24 hour) for 14 days and assayed for cellulase production. *T. fusca* Yx showing good growth on TYG agar was treated in the same manner as described aboved but the incubation was at 55°C for 12 days.

Enzyme assays

Carboxymethylcellulase (CMCase) activity.

All enzymatic assays were carried out on culture filtrates (Crude cellulase). Endo-1, 4-β- glucanase (CMCase, EC. 3. 2. 1.4) were assayed by measuring the amount of reducing groups liberated from CMC 7LF(24).

This was done by mixing 1.0 ml of the crude cellulase (culture filtrate) with 2.0ml of 2% (W/V) CMc 7LF solution and then monitoring the production of reducing sugar over time. Increasing sugar concentration versus time was used as a measure of cellulase activity.

Stepteomyces strains were grown for 14 days while *T. fusca* XY was grown for 12 days (the duration of incubation was determined during a preliminary test) in appropriate lignocellulose media. The crude cellulase was harvested by centrifugation of the lignocellulose culture at 300 rpm to sediment all insoluble materials (the supernatant obtained was used as the crude cellulase).

Seven test tubes previously preheated to 55°C were set up each containing 1.0ml of 0.067M phosphate buffer (pH 6.0); and 2.0ml of 2% (W/V) solution of CMC 7LF. One milliliter of distilled water and 3.0ml of DNS reagent was added to the first tube and then removed from the water bath. The crude enzyme preparation (1.0ml) was added to the remaining six tubes (this was regarded as the zero time addition of enzyme). The incubation of the six tubes was continued at 55°C. At 10 minutes intervals, the cellulase reaction in each tube was stopped by addition of 3.0ml of DNS reagent. At 60 minutes, the reaction in the last tube was stopped. All the tubes (including the blank) were heated in a boiling water bath for 15 minutes which resulted in the development of a brown colour in each tube, the intensity of the colour being proportional to reducing sugar concentration in the tube. After heating, 1.0ml of 40% Rochelle salt (Sodium Potassium tartrate) solution was added to each tube to stabilize the colour.

The tubes were cooled to approximately ambient temperature, thereafter the optical density (OD) of the solution in each tube was read in a WPA SlO5 spectrophotometer at 575nm. The control which did not contain the enzyme was used as the blank.

Filter Paper (FP) assay

The filter paper (FP) assay for cellulase activity was determined (25). Briefly a 1cm × 6cm strip of whatman No. 1 filter paper (50mg) was added to a test tube containing 1.0ml of crude enzyme. The tube was incubated for 1hr. at 50°C. DHS reagent (3.0ml) was added to stop the reaction. The tube was then placed in a boiling water bath for 15 minutes. A blank tube (without a filter paper strip) was included to correct for any

reducing sugar present in the enzyme preparation. The OD of the solution was read in a WPA SlO5 spectrophotometer at 550nm. The milligram of glucose produced in this test (read off from a prepared standard FP-activity curve) is the filter paper activity of the crude cellulase.

RESULTS

Cellulase Production

When the two *Streptomyces* strains; *S. viridosporus* T7A and *S. setonii* 75 Vi2 and the thermophilic organism, *T. fusca* Yx were grown in mineral salts cellulase activities were detected in the culture filtrates after 8 days incubation at 55°C for *T. fusca* Yx and 10 days incubation at 37°C for the *Streptomoyces* strains. The cellulase activities were found to increase with increase in incubation time and the highest enzyme activities of 1.5 $FPUml^{-1}$ was obtained after 12 days incubation of *T. fusca* Yx while 1.25 $FPUml^{-1}$ and 0.85 $FPUml^{-1}$ were obtained after 14 days incubation for *S. viridosporus* T7A and *S. setonii* 75Vi2 respectively (Figs. 1 and 2). It was further observed that there was a gradual decrease in enzyme activities with further increase in incubation time.

Enzyme assays

Carboxymethylacellulose (CMC) assay for cellulase activity

When the crude cellulase obtained after 14 days growth of the *Streptomyces* strains and 12 days culture of *T. fusca* Yx were assayed for cellulase activity the results obtained were as presented in Table 1 (19).

The highest CMCase (Endo-1, 4β-gluganase, EC.3.2.1.4) activity of $5.2Uml^{-1}$ was obtained for *T. fusca* Yx growing on grass lignocellulose. This organism growing on sugar cane bagasse lignocellulose produced $4.6Uml^{-1}$ of CMCase activity. The endo-1-4-β- glucanase activity was higher when *S. viridosporus* T7A was grown on grass lignocellulose than when it was grown on sugar cane baggase.

It was generally observed that endo-1, 4-β- glucanase activities were least when the three organisms were grown on sawdust lignocellulose as compared to the other two lignocellulose substrates (Table 1).

Filter paper assay for cellulase activities

When the filter (FP) assay was used to assay for the cellulase activities of the crude cellulase produced by growing the three organisms on different lignocellulose substrates, the results obtained were as presented in Table 2. (25).

The highest enzyme activity of 1.5 FPUml^{-1} was obtained for *T. fusca* YX growing on grass lignocellulose after a 12-day incubation at 55° C. *S. viridosporus* T7A growing on grass lignocellulose produced an enzyme activity of. 1.25 FPUml^{-1} for the same time period (Table 2). When *T. fusca* YX was grown on sawdust lignocellulose, 0.85 FPUml^{-1} enzyme activity (Table 2) was obtained, little or no enzyme activities were recorded when *S.viridosporus* T7A and *S.setonii* 75vi2 were on sawdust lignocellulose.

DISCUSSION

It has previously been recognised that for an efficient and economical utilization of the abundant renewable cellulosic materials, a cheap source of enzymes that can hydrolyze the cellulosic materials efficiently is required (2). It has also been known that different cellulase preparations vary widely in the rate and extent of hydrolysis of different cellulosic substrates depending on the proportions of the different components, source and growth conditions of the organism, and harvesting and handling procedure of the enzymes (1, 2 and 25). In this study, attempts were made to produce cellulase from *S. viridosporus* T7A and *S. setonii* 75vi2 and a thermophilic organism, *Thermomonospora fusca* YX. Under the conditions of growth, a lag time of 10 and 8 days were observed for the *Streptomyces* strains and *T. fusca* YX respectively prior to detection of cellulase activities in the culture filtrates (Figs. 1 and 2). This lag time can be reduced by supplementation of the culture filtrates with suitable carbon and nitrogen sources and surfactant (1).

Celluslase is a complex of enzymes containing chiefly endo- and exo-β-glucanases plus cellobiase (β-glucosidase). For complete hydrolysis of insoluble cellulose, synergistic action between the components is required. Reese (26) had proposed two enzymes to explain the hydrolysis of "native cellulose": C_1 enzyme, capable of degrading native cellulose to linear anhydroglucose chains, and C_X enzyme, capable of hydrolysing the chains to soluble low molecular weight products.

Since different cellulase preparations vary widely in the proportions of the different components, assay of the purified components requires a variety of fairly complicated procedure and sometimes it is not possible to distinguish the activities of these components. The 5.2 Uml^{-1} and 4.6 Uml^{-1} of carboxymethylcellulase (endo 1, 4-β-glucanase, EC.3.2.1.4) activities obtained for *T. fusca* YX growing on grass and sugarcane bagasse lignocelluloses respectively indicates that endo-β-glucanase is the chief component of the *T. fusca* YX cellulase (Table 1). Since measurement of endo-βglucanase (or of any other single component) is unsatisfactory, the filter paper (FP) assay was introduced (25). Cellulase activity of 1.5 FPU ml^{-1} was obtained when *T. fusca* YX was grown on 0.75 sugarcane bagasse lignocellulose in shake flask culture system. This peak enzyme activity was found to have decreased with further increase in incubation time (Figs. 1 and 2). This enzyme activity is higher than the 0.5 - 0.7 FPU ml-1 reported for *Trichoderma viride* Qm 6a (ATCC 26921) wild strain and Qm 9123 (ATCC 24449), the enhanced cellulase mutant derived from Qm 6a. It compares favourably with the 1.5 - 2.0 EPU ml^{-1} cellulase mutant derived from Qm 9123[15]. There was a significant difference in the observed FP-activities (Table II) but a a contingency chi-square analysis showed that there was no significant difference at 95% confidence limit (P=0.05). The low values of filter paper unit compared to CMC is an indication of low affinity of the enzyme to filter paper and is not characteristic of endo-glucanases.

The results of this study (Tables I and II) also indicated that the traditional assays for cellulolytic activity, such as FP and CMCase assays, do not necessarily reflect the overall hydrolytic activity of the cellulase preparation on all lignocellulose substrates. Although high FP and β-glucosidase activities generally indicate whether a cellulase preparation would have high hydrolytic activity, Saddler and Mes-Hartress (27) and Saddler *et al* (28) found that unless there was a substantial amount of endo-glucanase activity detected in the culture filtrate the overall hydrolytic activity remained low. It is obvious that *T.fusca* YX and the *Streptomyces* cellulases would be better hydrolytic enzymes for cellulosic materials because of their high endo-glucanase activities. This explains why substantial saccharification was previously reported when cellulase of a new isolate *Streptomyces* strains 21B was used to hydrolyze lignocellulase substrates (2). The results obtained in this

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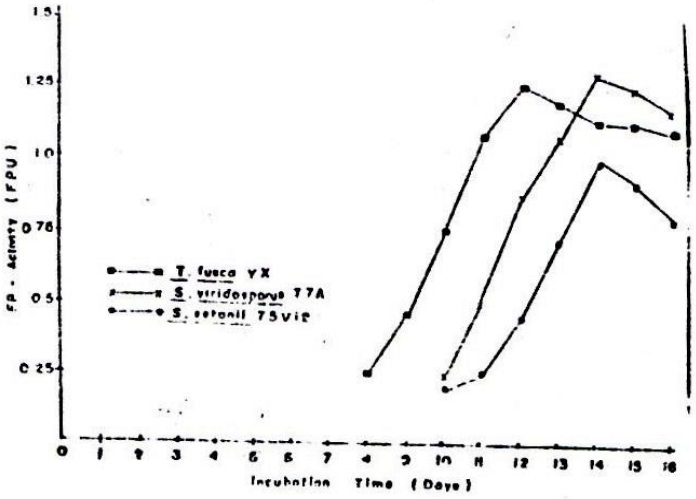

Fig. 1 Relationship between cellulase activity and incubation time of three organisms growing on pretreated grass lignocellulose

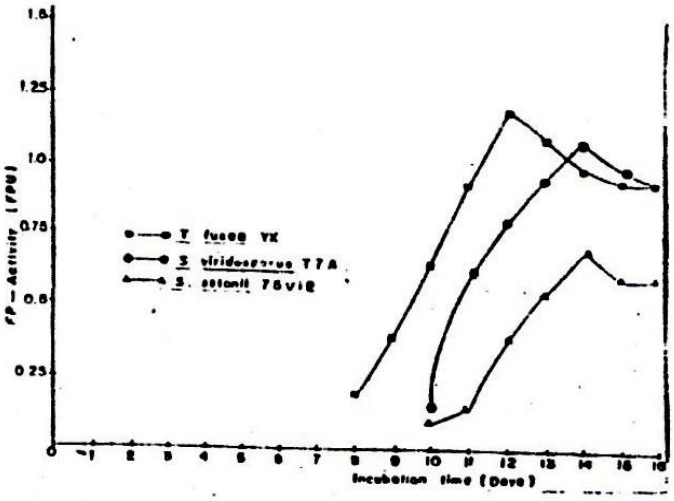

Fig. 2 Relationship between cellulase activity and incubation time of three organisms growing on pretreated sugarcane bagasse lignocellulose

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