FED-BATCH CELLULASE PRODUCTION BY TRICHODERMA AND PENICILLIUM STRAINS

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Fed-batch cellulase production by Trichoderma reesei *NRRL 11460,* Trichoderma viride *PxF, and* Penicillium *sp. 86 (a local isolate) was studied using corn cobs as carbon source with pH controlled at 5.0 in a 3.5-liter airlift fermenter. Of the three fungal strains tested,* T. reesei *11460 showed the highest volumetric activities, i.e., 1.34 I.U./mL on filter paper and 13.38 I.U./mL on CM-cellulose at a total substrate level of 80 g/L after 9.5 days of fermentation.*

Fed-batch cellulase production by T. reesei *11460 was further studied using "Bagras" (*Eucalyptus deglupta *Blume) and "Putian" (*Alangium meyeri *Merr) wood pulp as substrate. When the organism was cultured on "Putian" wood pulp, maximum volumetric activities were obtained after 10 days of fermentation, namely 3.53 I.U./mL on filter paper and 63.36 I.U./mL on CM-cellulose. Maximal specific activities of 0.84 I.U./mg and 12.10 I.U./mg were observed using filter paper and CM-cellulose as substrate, respectively, after 1-2 days fermentation.*

INTRODUCTION

Cellulose forms the bulk of the cell wall of higher plants. It can be fermented to products like alcohol and sugar. Wastes from farms, forests, and population centers provide an inexpensive and renewable source of food and energy.

Some microorganisms secrete enzymes which digest cellulose (1). The enzyme cellulase is a complex made up of at least two components with multiple activity like endoglucanase, exoglucanase, and β-glucosidase. Endoglucanase (1,4-β-4-glucan glucanohydrolase) forms free reducing ends by random cleavage of the internal β(1-4) bonds of cellulose. Exoglucanase (1,4-β-4-glucan cellobiohydrolase) forms mainly cellobiose by endwise hydrolysis of the free reducing ends of cellulose or cello-oligosaccharides. β-glucosidase (β-glucoside glucohydrolase) forms glucose by endwise cleavage of cello-oligosaccharides (2,3).

Some fungi synthesize and release into solution several cellulolytic enzymes (4,5,6,7,8). The present study evaluates three fungal strains for fed-batch cellulase production on corn cobs. The fungal strain yielding the most cellulase is further evaluated using two types of sulfate wood pulp as substrate.

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MATERIALS AND METHODS

Three fungal strains were used: *Trichoderma reesei* NRRL 11460 (9), *Trichoderma viride* PxF (10), and *Penicillium* sp. 86 (11). Cultures of each organism were maintained on potato dextrose agar (PDA) slants at room temperature, about 30°C.

Corn cobs and wood pulp were used as substrate. Corn cobs were ground first on a Hammer mill and then on a Wiley mill to a size of 40-mesh (12). Sulfate wood pulp, "Putian" (*Alangium meyeri* Merr) and "Bagras"(*Eucalyptus deglupta* Blume) were cut into small pieces and ground to pass a 40-mesh sieve on a Wiley mill (13).

For each organism, the medium used was similar to the standard salts medium for *T. reesei* (14) with increased levels of nutrients at high cellulose concentrations. The culture medium in g/L consisted of: $(NH_4)_2SO_4$, 1.4 g; urea, 0.3 g; KH_2PO_4, 2.0 $MgSO_4.7H_2O$, 0.3 g; $CaCl_2$, 0.3 g. The pH was adjusted to 4.0 with HCl. The carbon source was 1% unless otherwise stated.

A spore suspension was used in the inoculum buildup. Spores from 7-to 10-day-old cultures, which were grown on PDA slants at room temperature, were harvested by adding to the slants sterile distilled water adjusted to pH 5.0 and scraping the spores with a sterile wire loop. One mL of spore suspension was used for spore counting.

A sterilized culture medium was incubated for 1 to 2 days on a rotary shaker at room temperature. Five mL of inoculum suspension (concentration 10^8 spores/mL) was added to 45 mL of culture medium.

Fermentation was done at room temperature using a 3.5-liter airlift fermenter (Fig. 1). Aeration was at 1.0 volume of air per volume of medium per minute (vvm). The fermenter contained 300 mL inoculum. Culture medium was then introduced. Substrate at 20 g/L was introduced. More substrate was added after 48 h, and subsequently at intervals of 10-24 h whenever growth decreased as demonstrated by a decreasing rate of base addition. The total level of added substrate was 80 g/L. Ammonium hydroxide was added to the medium for control at pH 5.0, and for nitrogen supplementation. Samples were analyzed for enzyme activity and soluble protein content, and then frozen until further analysis.

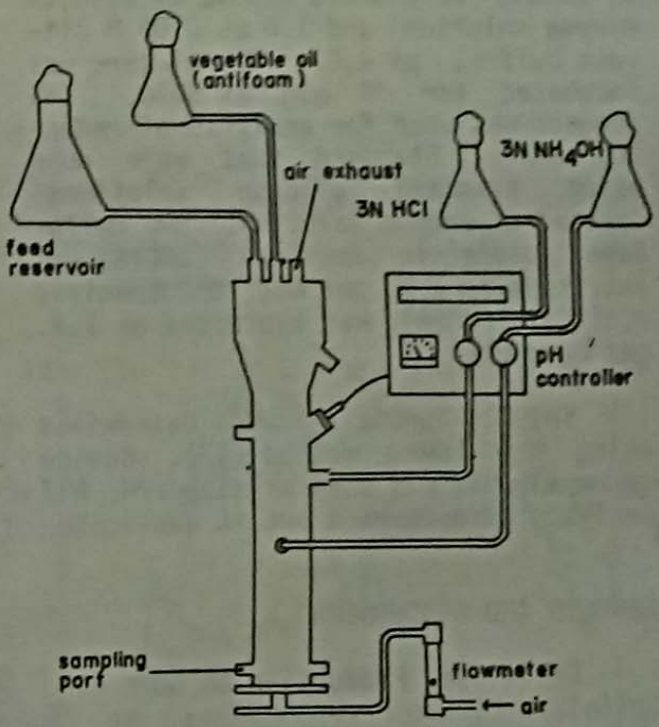

Figure 1. The fed - batch fermentation assembly.

Cellulase activity was assayed by the Mandels method (15) with filter paper as substrate. A 1 x 6 cm strip of Whatman No. 1 filter paper weighing 50 mg was incubated with 0.5 mL enzyme solution and 1.0 mL of 0.05 M citrate

buffer, pH 4.8. The reducing sugar produced by cellulase action was measured by using the dinitrosalicylate (DNS) method (16). All enzyme preparations were diluted to give the amount of glucose up to which glucose production was linear.

Filter paper volumetric activity (FPVA) was expressed in I.U. per mL; filter paper specific activity (FPSA) in I.U. per mg protein. The international unit (I.U.) of cellulase activity is defined as the number of micromoles of reducing sugar produced per minute under standard assay conditions.

Cellulose activity on soluble cellulose substrate was measured using carboxymethylcellulose (CMC). To 0.5 mL of 1% CMC were added 0.5 mL of diluted enzyme solution and 1.0 mL 0.05 M citrate buffer, pH 4.8. The mixture was incubated for 30 min at 50°C. DNS reagent was used for analysis of reducing sugar. Standard runs were done using standard glucose solutions instead of enzyme solution. CMC cellulase volumetric activity (CMCVA) was expressed as I.U. per mL; CMC specific activity (CMCSA) was expressed as I.U. per mg protein.

Soluble protein was determined using the Lowry method (17). Bovine serum albumin was used as standard. All analyses were carried out in duplicate.

RESULTS AND DISCUSSION

Cellulase Production on Corn Cob. Cellulase from *Trichoderma* sp. 86 showed a maximum of 0.44 FPVA and 6.25 CMCVA obtained after 9.5 days of fermentation (Fig. 2). CMCSA peaked on the second day. The initial rise of volumetric activities followed by wide fluctuations as values approached the maximum may be attributed to adsorption and immobilization of the enzyme on the cellulosic substrate parallel to additions of substrate. Adsorption removes the enzyme from the system (18,19).

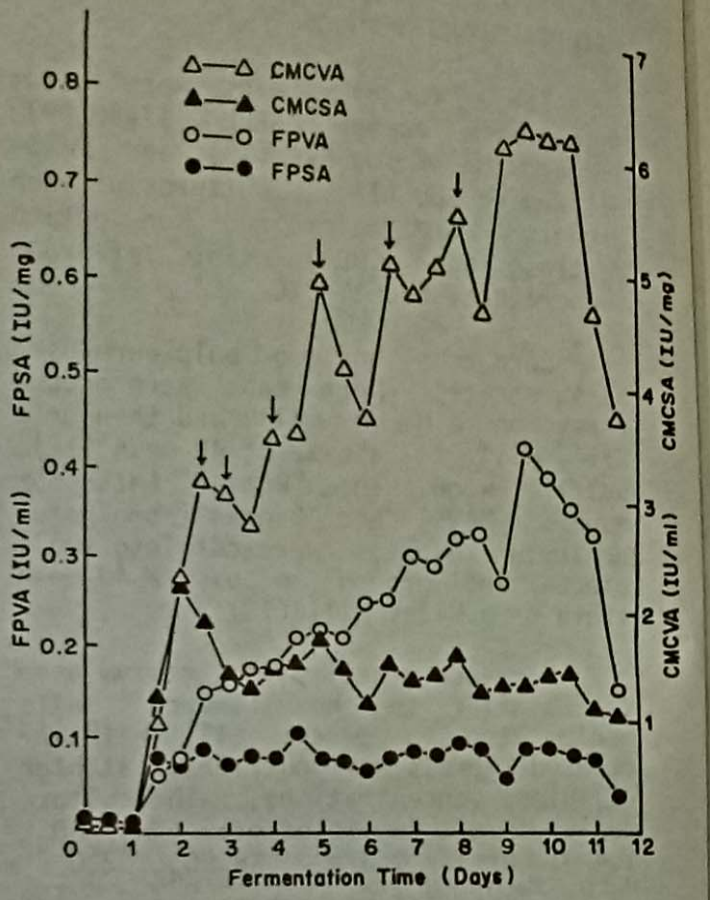

Figure 2. Plot of enzyme activities vs. fermentation time for Penicillium sp 86 grown in corn cobs. (time when substrate was added is indicated by arrows)

Cellulase from *T. viride* PxF showed maximal FPVA, CMCVA, CMCVA values after 9.5 days of fermentation (Fig. 3). A lag phase of 6 days indicates time needed by the organism to adjust to the medium, after which a sharp increase in activity is evident.

Cellulase from *T. reesei* 11460 showed maximal CMCVA and FPVA values at 9.0 and 9.5 days, respectively, while CMCSA and FPSA peaked at 1.5 and 3.0 days, respectively (Fig. 4).

Enzyme activities quickly or gradually increased on the second or third day parallel to the increase in substrate level, consistent with previous observations for *Aspergillu Niger* (20) and for *Penicillium* (21).

The three strains showed maximal protein secretion after 9.5 days of fermentation (Fig. 5). The *Trichoderma* species, especially *T. reesei*, produced

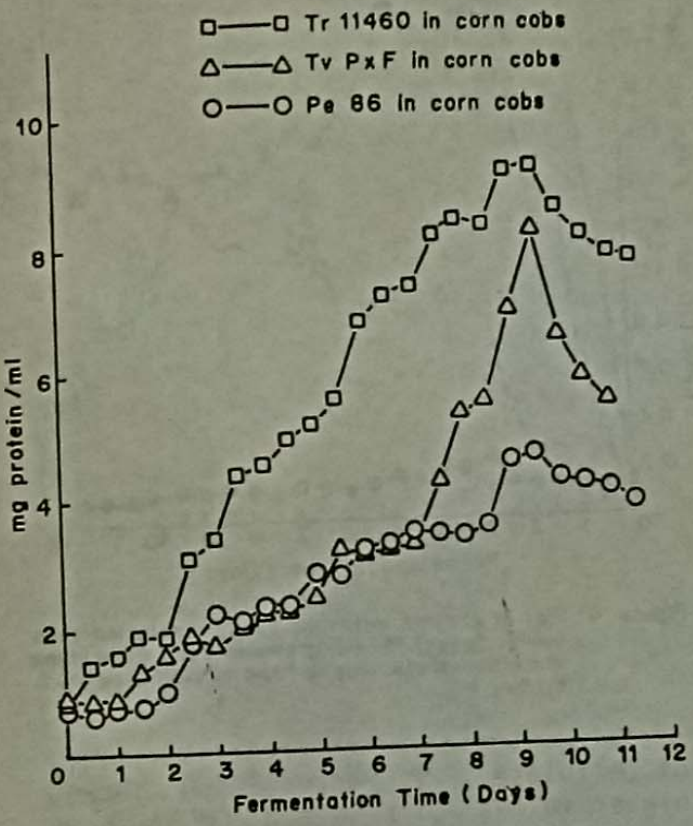

Figure 5. Plot of Lowry protein vs. fermentation time for the organisms tested.

a higher level of protein than *Penicillium*. Exo-β-1,4-glucanases (cellobiohydrolases EC 3-2-1-91) have been found to be associated with 70 percent of the *Trichoderma* proteins, and endo-β-1,4-glucanases (EC 3-2-1-4) with 30 percent (22).

Comparison of cellulolytic activities of the three fungi with corn cobs as carbon source indicates that the performance of *T. reesei* was highest in terms of volumetric activities (Table 1). The low specific activities (compared to volumetric activities) of *Trichoderma* cellulases may be due to the increasing resistance of residual substrate to hydrolysis, product inhibition, and enzyme inactivation as fermentation proceeds (23).

From the preceding results *T. reesei* 11460, a mutant strain from the parent QM6A, appears to be superior in cellulase production. The strain produces a complete extracellular cellulase with all the components required to hydrolyze crystalline cellulose. High yields of cellulase protein are attainable with this strain (24).

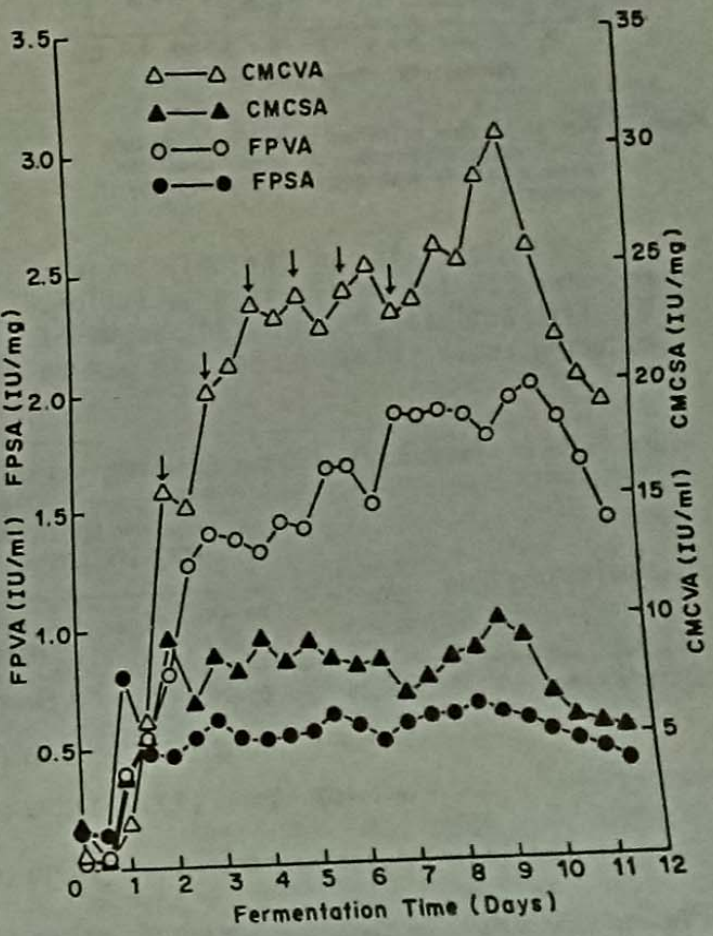

Figure 6. Plot of enzyme activities vs. fermentation time for T. reesei 11460 grown in Bagras' wood pulp. (time when substrate was added is indicated by arrows)

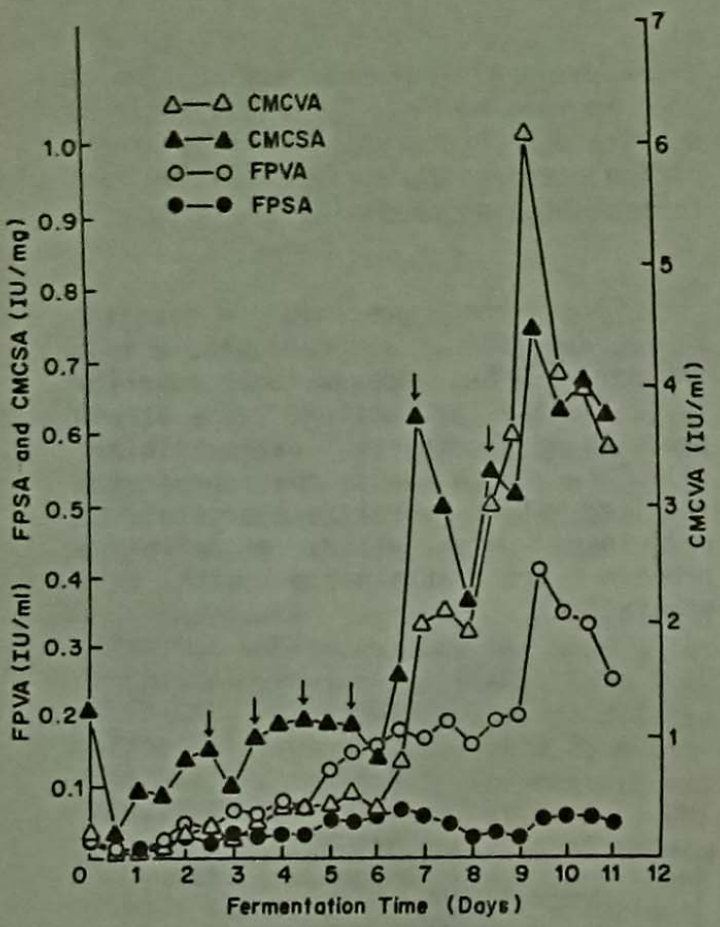

Figure 3. Plot of enzyme activities vs. fermentation time for T viride PxF grown in corn cobs (time when substrate was added is indicated by arrows).

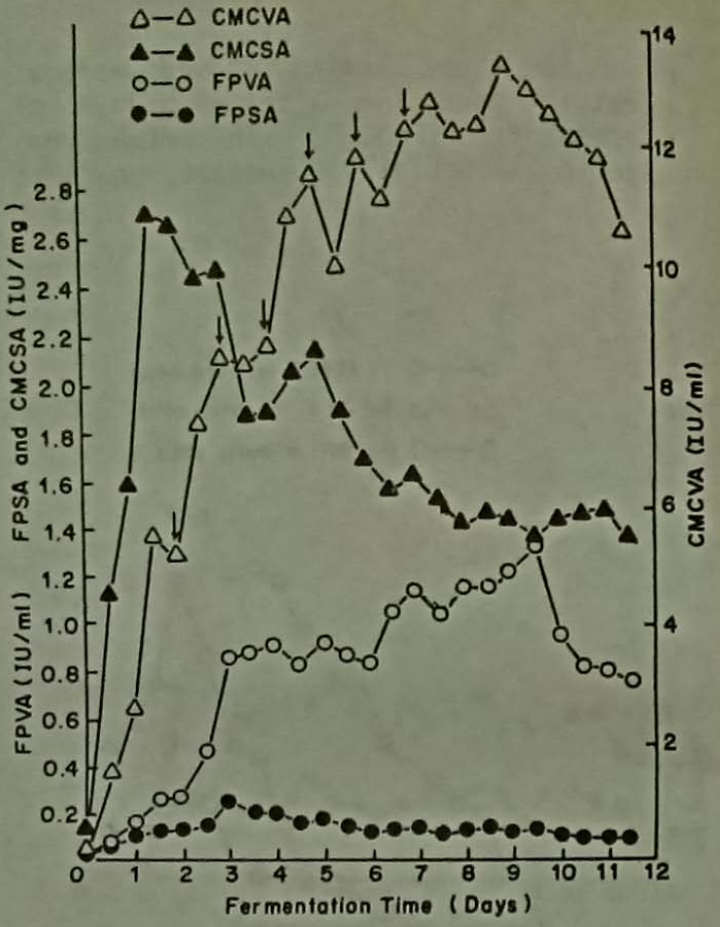

Figure 4. Plot of enzyme activities vs. fermentation time for T. reesei 11460 grown in corn cobs. (time when substrate was added is indicated by arrows)

Cellulase is not the only protein released by fungi into solution. Specific activity, expressed as μm of reducing sugar produced per min per mg protein, indicates the relative purity of cellulase. *T. reesei* 11460 showed higher values of FPSA and CMCSA compared to those of the other strains (Table 1).

Table 1. Maximum cellulolytic activities and soluble protein produced by three fungal strains.

SUBSTRATE	FUNGAL STRAIN	MAXIMUM VOLUMETRIC ACTIVITY (IU/mL)*		MAXIMUM SPECIFIC ACTIVITY (IU/mg protein)*		MAXIMUM LOWRY PROTEIN (No. of days)
		FP (No. of days)	CMC (No. of days)	FP (No. of days)	CMC (No. of days)	
Corn cobs	Pe 86	0.438 (9.5)	6.246 (9.5)	0.108 (4.5)	2.175 (2.0)	4.62 (9.5)
	Tv PxF	0.412 (9.5)	6.105 (9.5)	0.055 (6.5)	0.742 (9.5)	8.23 (9.5)
	Tr 11460	1.345 (9.5)	13.379 (9.0)	0.254 (3.0)	2.707 (1.5)	9.26 (9.5)
"Bagras"wood pulp	Tr 11460	2.003 (10.0)	30.710 (9.5)	0.817 (1.0)	10.006 (9.0)	3.740 (10.0)
"Putian"wood pulp	Tr 11460	3.529 (10.0)	63.363 (10.0)	0.836 (1.0)	12.096 (2.0)	6.50 (10.0)

*IU - International unit defined as the number of μmoles reducing sugar produced per minute under standard assay conditions.

Number of days of fermentation when activity value was determined is indicated in parentheses.

Cellulase Production on Wood Pulp and Corn Cob Using *Trichoderma reesei* 11460. Cellulases from *T. reesei* grown on "Bagras" wood pulp exhibited maximum activity at different times of the fermentation period (Fig. 6). CMCVA and FPVA peaked at 9.5 and 10 days, respectively, while FPSA and CMCSA peaked at 1.0 and 9.0 days, respectively. These results suggest accessibility of substrate to the enzyme. This discrepancy in the activities can again be attributed to enzyme adsorption on the substrate and inactivation with increasing level of substrate. Cellulase from *T. reesei* grown on "Putian" wood pulp showed both CMCVA and FPVA values at a maximum on the tenth day, while FPSA and CMCSA values were highest on the first and second days, respectively (Fig. 7). The enzymatic activities showed that there was a substantial increase in amounts of cellulose parallel to the increase in carbon substrate concentration.

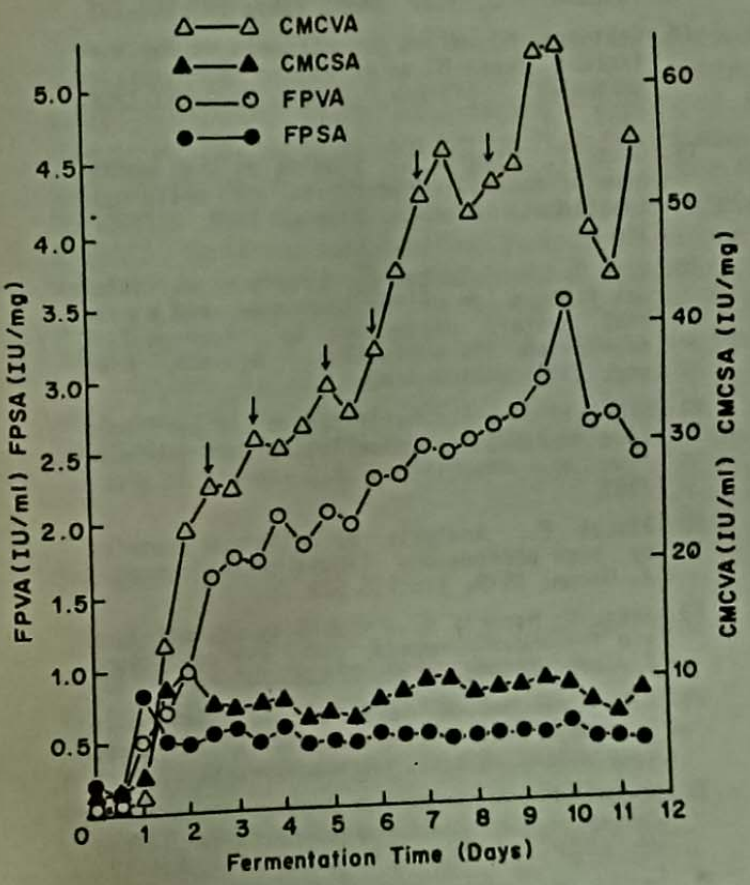

Figure 7. Plot of enzyme activities vs. fermentation time for T. reesei 11460 grown in 'Putian' wood pulp. (time when substrate was added is indicated by arrows)

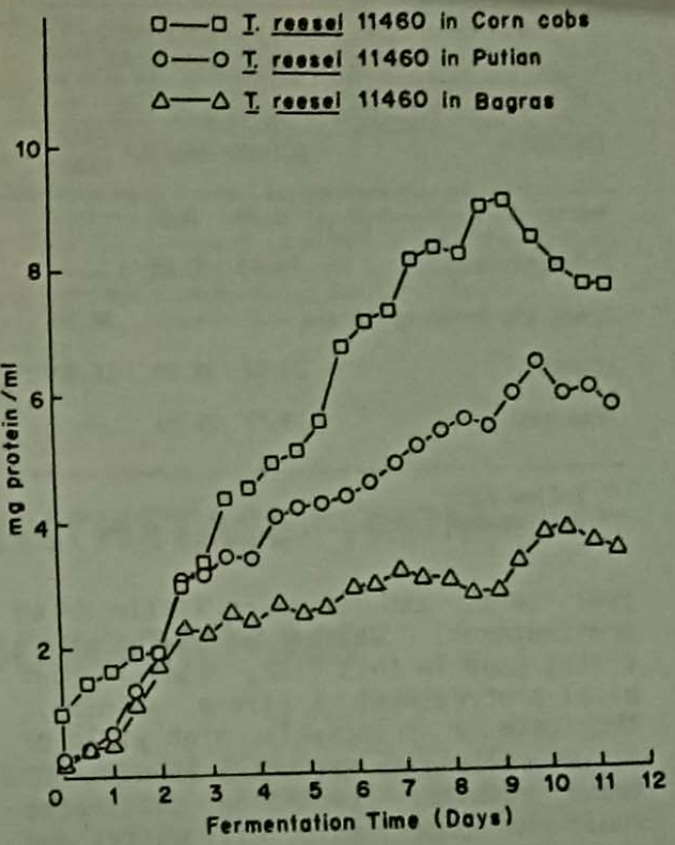

Figure 8. Plot of Lowry protein vs. fermentation time for T reesei 11460 grown on the substrate tested.

When protein production was compared among the substrates (corn cob, "Putian," "Bagras"), more protein was observed for corn cobs (Fig. 8). A maximum of 9.26 mg protein/mL was obtained at day 9.5 for corn cobs; a maximum of 6.5 mg protein/mL at day 10 for "Putian"; a maximum of 3.74 mg protein/mL at day 10.5 for "Bagras."

The proximate chemical composition of the corn cobs and wood pulp used in this study is shown in Table 2. Several studies on cellulose have been done on forest and agricultural wastes (25,26, 27). The ideal substrate should be cheap, available in large quantities

Table 2. Proximate chemical composition of some lignocellulosic materials.

CONTENT	PUTIAN*	BAGRAS*	CORN COBS**
Ash	1.09	0.66	4.58
Holocellulose	69.63	70.80	---
Cross and Bevan cellulose	---	---	36.20
Lignin	24.52	26.33	15.60
Pentosan	13.73	16.10	---

* Ballon (28)
** del Rosario *et al.* (29)

year-round, and requiring little or no pretreatment. While two of the substrates used in this study required chemical pretreatment (sulfate pulping), they gave a relatively high yield or active cellulase system. Based on these results, promising substrates would be paper pulp mill wastes and assorted wood wastes.

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12. The Hammer mill was provided by the Institute of Animal Science, College of Agriculture, UPLB. The Wiley mill was provided by the Biofuels Laboratory, National Institute of Biotechnology and Microbiology, UPLB.
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PROSTAGLANDIN SYNTHETASE FROM PHILIPPINE GORGONIANS

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Optimum extraction and assay conditions for prostaglandin (PG) synthetase from Echinogorgia sp., *a Philippine coelenterate, were studied. The co-oxidation of L-epinephrine with arachidonic acid to adenochrome ($Abs._{max}$ 480 nm) was used to assay PG synthetase activity. Optimum assay temperature was 22°C. Enzyme activity was associated with the microsomal-cytoplasmic fraction. Maximum activity was at pH 7.0. The enzyme was most stable in Tris buffer at pH 8.0 mixed with 0.1% Triton X-100. Optimum extraction temperature was 0-4°C. Purification was done by sequential Sepharose 4B gel filtration, DEAE-cellulose chromatography, and polyacrylamide gel electrofocusing. A 1920-fold purification was achieved. Isoelectric pH was higher than 7.5 but the upper limit is uncertain. Molecular weight was estimated at 160,000 by Sepharose 4B gel filtration. Kinetic studies showed K_m of arachidonic acid to be about 10^{-3}M; V_m about 10^3 µmol epinephrine oxidized per min per mg protein; turnover number about 10^5 per min.*

INTRODUCTION

Prostaglandins (PG) are synthesized from polyunsaturated fatty acids. The first stage of synthesis is mediated by PG endoperoxide synthetase. This enzyme is present in most mammals, other vertebrates, and marine coelenterates (1,2,3). The presence of PG in phylogenetically simple organisms suggests that PG may be involved in the regulation of such processes as ion and water transport, reproduction, and defense against predation or parasitism (1,4,5).

Mammalian PG synthetase has been purified from bovine and sheep vesicular gland microsomes. The bovine enzyme is a membrane-bound complex with

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