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CELLULASE AND HEMICELLULASE PRODUCTION BY CELLULOMONAS FLAVIGENA NIAB 441

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SUMMARY

Cellulomonas flavigena (strain NIAB 441) produced cellulase and hemicellulase activities when grown on *Leptochloa fusca* L. Kunth (Kallar grass), found to be the best inducer for enzyme production. The enzyme possessed the potential to saccharify bagasse, Kallar grass straw, wheat straw, carboxymethyl cellulose (CMC) and xylan to reducing sugars.

INTRODUCTION

The prevalent energy crisis has prompted research on the bioconversion of lignocellulosic materials into fermentable carbohydrates and chemical feedstocks. Enzyme preparations of fungal origin have been mostly used for saccharification purposes (Saddler *et al*., 1982; Robinson, 1984; Deschamps and Huet, 1984). The cellulolytic bacteria have, however, been mainly used to produce single cell proteins (Nakamura and Kitamura, 1982). Choudhury *et al*., (1980) earlier reported saccharification of alkali treated bagasse by *Cellulomonas* strains.

The present study deals with the production of cellulases and hemicellulases by *Cellulomonas flavigena* strain NIAB 441 when grown on different lignocellulosic and cellulosic substrates. Among lignocellulosic substrates, powdered plant material of *Leptochloa fusca* (Kallar grass) and *Sesbania aculeata* (Dhancha) was used. Both these plants are cultivated as primary and secondary colonizers of saline lands in Pakistan (Sandhu and Malik, 1975).

MATERIAL AND METHODS

Organism: The organism used in these studies was *Cellulomonas flavigena* strain NIAB 441. Stock cultures were maintained on CMC agar slants and plates which contained $NaNO_3$, 0.05 g; KCl, 0.1 g; $KH_2PO_4 \cdot 7H_2O$, 0.05 g; $FeSO_4 \cdot 7H_2O$, 0.001 g; CMC, 0.5 g; CMC, 0.5 g; yeast extract, 0.1 g and agar 2 g per 100 ml of water, pH 7.3.

Preparation of Substrates: Lignocellulosic substrates namely bagasse, Kallar grass straw, wheat straw, cotton stalks, *Sesbania aculeata* were dried separately in an air oven at 80°C. The dried substrates were made into 55 mesh powder by grinding in an electric grinder. These were soaked in 0.2 N NaOH and autoclaved for 15 minutes, washed to neutralize and again dried in the oven at 80°C.

Growth studies: In the fermentation studies, alkali treated lignocellulosic substrates were used at a rate to contain 1% (W/V) cellulose + hemicellulose Other cellulosic substrates (without pretreatment) were used at 1% level. The organism was grown as described by Choudhury et al. (1980) in 1 L conical flasks containing 200 ml of maintenance medium for specified time, the unused insoluble substrate was separated by passing through 2 layers of cheese cloth.

Studies of pH and temperature at which growth and enzyme production occurred were conducted using 1% cellobiose, glucose and Kallar grass as substrates in mineral salts medium supplemented with 0.1% yeast extract at initial pH 5-8 with an increment of 1 and at temperature from 20 to 60°C in 5 increments at pH7 in a reciprocal shaker.

Fractionation of enzyme preparation: After the specified time, the cultures were centrifuged at 15,000 x g for 15 minutes at 10°C. The supernatant was assayed for extracellular enzyme activity. The cells were suspended in McIlvaine's buffer (1:9) prepared as according to Diem (1962) and sonicated in Biosonik sonicator and centrifuged at 15,000 x g. The supernatant was collected and was assayed for intracellular activity.

Enzyme assays: Carboxymethyl cellulase (EC 3.2.1.4) activity was determined by measuring the reducing sugars produced from 1% CMC after incubation of 1 ml of appropriately diluted sample at 40°C in reciprocal shaker (Saddler et al., 1982). The buffer was used McIlvaine's citrate phosphate buffer, pH 7.0. Avicel activity (EC 3.2.1.19) and filter paper activity (FPase) were measured using the same conditions and 1% avicel or filter paper (cut into 6x2 cm size). Xylanase (EC 3.2.1.8) was determined using the same conditions and 1% xylan. All values are expressed in international units (μmole reducing sugar equivalent glucose or xylose) liberated/minute). Hemicellulase activity was assayed using the same conditions and 1% hemicellulose complex from bagasse. Reducing sugars were measured as μmoles of xylose equivalent/minute. β-glucosidase, (EC 3.2.1.21) and β-xylosidase, (EC 3.2.1.37) were assayed as described by Rickard et al., (1981) using corresponding glucoside. Units are international units (μmoles of nitrophenol produced/minute). Protein was determined by Lowry's method (1951) with bovine serum albumin as standard.

pH and temperature of enzyme assay were optimized by varying the assay pH from 5 to 8 at 40°C and by varying the assay temperature from 20 to 60°C at pH7.

Saccharification studies: Portions of 0.5 g of bagasse, kallar grass straw, wheat straw, CMC and xylan were dispensed into 50 ml conical flasks separately to which 3 ml of phosphate buffer was added. They were incubated at 40°C in shaking water bath incubator alongwith 7 ml of extracellular crude enzyme preparation obtained after growth of C.flavigena on 1% kallar grass straw. Duplicate flasks were removed periodically and properly diluted aliquots were used to determine reducing sugars measured as glucose (Miller et al., 1960) using dinitrosalicylic acid (DNS).

RESULTS AND DISCUSSIONS

Growth studies for C.flavigena strain NIAB 441 on three substrates namely glucose, cellobiose and kallar grass straw demonstrated that optimum growth occurred at 45°C and pH 7.3. No growth was observed above 55°C. Maximum enzyme production from kallar grass straw, Sesbania

TABLE 1: CMCase and xylanase activities of *C. flavigena* NIAB 441 after 3 days growth at 30°C and pH 7.3.

Growth substrate[a]	CMCase[b] IU/ml	Xylanase[b] IU/ml	Protein (mg/ml)	
			Extracellular	Intracellular
Bagasse	2.9	9.8	1.43	1.60
Cotton stalks	2.8	2.9	1.0	0.90
Kallar grass straw	3.8	16.0	1.56	1.68
Dhancha	2.8	5.6	1.3	1.48
Wheat straw	3.0	10.5	1.45	1.25
Cellobiose	1.0	2.6	1.02	1.00
CMC	1.9	2.8	1.00	0.92
Cotton wool	2.6	2.5	1.06	0.85
Filter paper	2.0	2.4	0.95	0.90
Xylan	1.2	13.0	1.25	1.20

a: See "MATERIAL AND METHODS" for physical and chemical treatments and for substrate concentration.

b: Enzyme activities were determined at pH 7 and temperature 40°C.

TABLE 2: Saccharification of different substrates using extracellular crude enzyme preparation from *C. flavigena* grown on 1% Kallar grass straw for 3 days.

Substrate*	mg reducing sugars made/ml at**				
	5 h	10 h	20 h	25 h	30 h
Bagasse	14	17	22	26	26
Kallar grass straw	12	15	18	20	20
Wheat straw	16	19	22	29	29
CMC	28	31	32	34	33
Xylan	30	35	36	35	35

* Bagasse, Kallar grass straw and wheat straw received physical and chemical treatments as in TABLE 1.

** For details, see "MATERIAL AND METHODS".

aculeata and filter paper occurred at 30°C and pH 7.3. At 45°C, xylanase and CMCase activities were suppressed to 50% of the maximum. Relative CMCase activity at 25°C, 30°, 35°, 40°, 45°, 50° and 55° were 70%, 100% 75%, 70%, 50%, 35% and 20%. The CMCase activity was also influenced by initial growth pH. Relative CMCase activity at pH, 5, 6, 7, 8, 9 was 50%, 60%, 100%, 60% and 30%. The optimum enzyme assay temperature was 40°C and pH 7.0.

For selection of substrate of choice, two types of lignocellulosic biomass raised from saline wastelands, namely Kallar grass and Sesbania aculeata, were compared with other lignocellulosic and cellulosic substrates for growth and production of endo-glucanase and xylanase activities when grown in liquid culture at 30°C and pH 7.3 in shake flask experiments (Table 1). The biosynthesis of cellulase and xylanase was maximum with kallar grass therefore it was found to be the best inducer for enzyme production. The extracellular protein produced by the organism when grown on kallar grass showed many cellulase and hemicellulase activities. The hemicellulases were better induced than cellulases. The production of exo-glucanase, xylanase and hemicellulase was extracellular while β-glucosidase and β-xylosidase were mainly cell bound. Intracellular protein though high in quantity, showed mild activity. Time course studies indicated that the optimum time for production of the activities for CMC, Avicel and filter paper was 3 days where as for xylan and hemicellulose it was 5 days. Production of β-glucosidase and β-xylosidase was greatly enhanced when cells were grown on xylan or cellobiose. The studies are in progress with an aim to obtain hyperproduction of these activities by changing the environmental conditions or by induced mutations.

Saccharification studies: CMC, xylan, Kallar grass straw, bagasse and wheat straw showed their susceptibility to the enzymatic saccharification from C.flavigena (TABLE 2). Presently efforts are being made to utilize the hydrolytic products from saccharification studies for production of ethanol using simultaneous saccharification and fermentation technique.

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