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COMPARISON OF DIFFERENT STRAINS OF CELLULOMONAS FOR PRODUCTION OF CELLULOLYTIC AND XYLANOLYTIC ENZYMES FROM BIOMASS PRODUCED ON SALINE LANDS

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ABSTRACT: A comparison of the rate of carboxymethyl cellulase (CMCase), avicelase, xylanase, β-glucosidase and β-xylosidase production and rates of growth by 4 different strains of Cellulomonas revealed a wide range of behaviour; with some strains producing more CMCase, avicelase, xylanase, β-glucosidase and β-xylosidase from complex lignocellulosic (LC) biomass (from saline land) and CMC while some others producing small amounts of these enzymes. One strain, C. biazotea, was better with respect to enzyme production potential and growth behaviour than most of the other strains and has been chosen as a starting strain for genetic improvement for producing enzymes of the cellulase complex.

INTRODUCTION: Leptochloa fusca (L.) Kunth (kallar grass) and Sesbania aculeata (dhancha) are cultivated as primary and secondary colonizers of saline lands in Pakistan (Sandhu & Malik, 1975). The former alone can give upto 40 metric tons of biomass/ha. year (Malik & Zafar, 1985). In previous studies (Rajoka & Malik 1984), it has been found to be the best inducer among different lignocellulosic and cellulosic substances for the production of cellulases and hemicellulases in C. flavigena. The strains of Cellulomonas produce cellulolytic and hemicellulolytic enzymes under defined environmental conditions (Choi et al., 1978; Richard et al., 1981; Nakamura and Kitamura, 1982; Peiris and Rickard, 1982; Langsford et al., 1984; Rapp et al., 1984; Rodriquez et al., 1985; Rapp and Wagner, 1986). In this study, three strains of Cellulomonas have been compared with C. flavigena NIAB441 for their ability to synthesize cellulolytic and xylanolytic activities when grown on kallar grass, dhancha or CMC and for their growth behaviour on these carbon sources, celllobiose, glucose and xylose; the latter are produced as the end products of saccharification of biomass. Use of biomass, produced on saline lands, for enzyme production, can improve the economics of utilization of saline-lands for biomass production.

MATERIALS AND METHODS: Sigmacell 100 (avicel), CMC, xylan, p-nitrophenol-β-D-glucopyranoside (pNPG), p-nitrophenol β-D-xylopyranoside (pNPX) thiamine-HCl and riboflavin were from Sigma Chemical Co., USA. All other reagents were of analytical grade. Kallar grass and dhancha were obtained from Biosaline Research Substation of NIAB, near Lahore. The dried powder was steam alkali treated as described earlier (Rajoka & Malik, 1984).

Microorganisms: Four strains of Cellulomonas listed in Table 1 and Fig. 1 were maintained on avicel or CMC plates and slants which contained $NaNO_3$, 0.05 g; KCl, 0.1 g; $K_2HPO_4 . 7H_2O$, 0.05 g; $FeSO_4 . 7H_2O$, 0.01 g; CMC or avicel 0.5 g; yeast extract, 0.1 g and agar 2 g per 100 ml of water, pH 7.3.

Growth studies: The organisms were grown in 1 L conical flasks containing 200 ml of liquid maintenance medium with specified carbon source (Rajoka & Malik, 1984). Studies of pH and temperature, at which (i) growth and enzyme production occurred (ii) enzymes showed maximum activities, were performed as described previously (Rajoka & Malik, 1984). Growth rates were measured by following the increase in optical density (OD) at 610 nm in a spectrophotometer after growth in cellobiose, glucose and xylose (0.25 - 10%). For growth of kallar grass or dhancha, the matter was first removed by sieving through cheese cloth and OD measured. Growth in CMC was measured after removing CMC by centrifugation and resuspending in fresh minimal medium. The specific growth rate (μ) or doubling time was determined after Pirt (1975). The growth behaviour in solid culture was measured as described by Choi et al., (1978).

Enzyme assays: Enzyme production in all strains of Cellulomonas from CMC (0.25 - 1.5%), indicated that 1% (W/V) concentration was optimum for the biosynthesis of all enzyme activities observed. All further studies were performed with 1% CMC, 1% cellulose & hemicellulose in kallar grass or dhancha. After the specified time, the culture was filtered (for dhancha or kallar grass), centrifuged at 15,000 g for 15 minutes at 10°C. The supernatant was assayed for extracellular activities. The washed cells were suspended in 0.05 M citrate buffer, pH 7, sonicated in Labsonik 2000 sonicator (Braun) and assayed for cellular enzymes (Rickard et al., 1981). Enzyme activities for CMCase (EC 3.2.1.4), avicelase (EC 3.2.1.19), xylanase (EC 3.2.1.8) were determined as described earlier (Rajoka & Malik, 1984). One unit of enzyme liberates 1 μ mole of glucose or xylose equivalents per min. per millilitre of enzyme preparation. For determining activities for β-glucosidase (EC 3.2.1.21) and β-xylosidase (EC 3.2.1.37), the assay procedure was modified; 0.2 ml of appropriately diluted enzyme was mixed with 0.2 ml of pNPG or pNPX (5 mM) and 0.2 ml of 0.05 M citrate buffer pH 7.0, the mixture was incubated at 40°C for 15 min. The reaction was stopped by adding 2 ml of Na_2CO_3 (2%) and the amount of p-nitrophenol liberated was measured at 410 nm. One unit of activity liberates 1 μ mole p-nitrophenol from 5 mM respective glycoside per ml of enzyme preparation per min. under the assay conditions.

RESULTS AND DISCUSSION: Four different strains of Cellulomonas were grown at 30°C in shake flasks with salts media containing kallar grass, dhancha or CMC. In these studied, it was observed that the organism growing faster produced more CMCase, avicelase, xylanase, β-glucosidase and β-xylosidase (Fig. 1, Table 1). The former three activities were produced mainly extracellular in all the cultures whereas the latter two activities were exclusively cellular. C. biazotea was found to be the most potent organism and kallar grass the best enzyme inducer. C. flavigena produced maximum xylanase and hemicellulase activities from respective inducers after 5 days whereas from these substrates, all enzymes in all strains were produced at maximum level after three days. Strain C. biazotea had a shorter lag period and doubling time during growth on cellobiose, xylose and glucose (Table 1). All strains produced more cellmass from kallar grass followed by dhancha, cellobiose, glucose, xylose and CMC. C. fimi required thiamine and riboflavin for good growth and enzyme production and was processed in the presence of these growth factors.

Another consideration for strain selection for genetic improvement was its insensitivity to the presence of glucose, cellobiose and xylose in the medium. This is important for selecting the mutants with faster growth rate and end-product inhibition resistance. It was observed that C. biazotea could tolerate upto 10% (W/V) of these components in the growth medium e.g. specific growth rate (μ) on 1,3,5,8,10% (W/V) cellobiose was 0.274, 0.269, 0.255, 0.195 and 0.178 hr^{-1}.

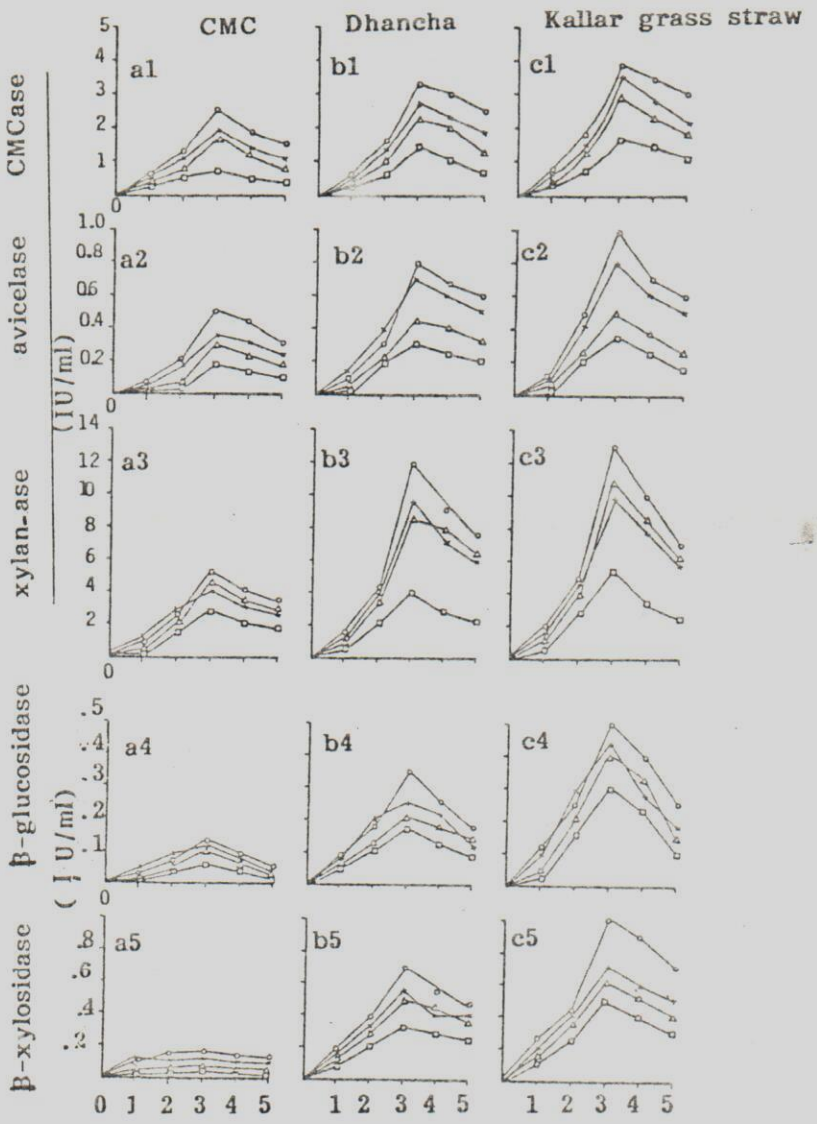

Fig.1.: Production of CMCase, avicelase, xylanase, β-glucosidase and β-xylosidase by C. biazotea (o—o), C. cellasea (Δ—Δ), C. fimi (□—□) and C. flavigena(x—x)after growth in CMC (a1-a5), dhancha (b1-b5) and kallar grass straw (c1-c5) liquid media. The cells were grown in shake flasks at 30°C in a reciprocal shaker in respective media, pH 7.3. The culture broth and cells were processed for determining enzyme activities (see Materials and Methods).

This was also confirmed by measuring the enzyme activites produced by C. biazotea when exposed to these components in the reaction mixture and remaining activities measured; it was found that these activities showed insensitivity to their presence in the reaction medium. When the four strains were grown at 25°C and 35°C, all were found to grow and produce enzymes in lower amounts than at 30°C except C. fimi which produced more enzymes at 25°C (10-15% higher than at 30°C). In these studies too, C. biazotea was found to be a better strain. The enzyme activities, endo-glucanase and xylanase from C. biazotea, showed storage stability at room temperature of 25°C for 7 days while β-glucosidase and β-xylosidase showed stability during storage in freezer.

When the rate of production of endo-glucanase, exo-glucanase, xylanase, β-glucosidase, β-xylosidase, growth rate in liquid and solid culture and end-product inhibition were considered together, it was found that C. biazotea was the most promising strain. It is also amenable to mutagenesis. Some stable and viable mutants have been isolated which gave 100-120 % improvement in production of β-glucosidase and β-xylosidase and can serve as a starting strain for further genetic improvement.

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Table 1: *Growth of different strains of Cellulomonas on cellobiose, glucose or xylose.

Strains	Lag period(h) after growth on			Doubling time(h) after growth on		
	[a]Cell.	[b]Glu.	[c]Xyl.	[a]Cell.	[b]Glu.	[c]Xyl.
C.biazotea NIAB442	1.5	1.0	1.5	2.9	4.0	3.3
C.cellasea NIAB443	3.0	4.0	2.0	3.6	4.7	5.5
C.flavigena NIAB441	4.0	3.0	2.0	3.0	4.7	6.0
C.fimi NIAB444	5.0	5.0	3.0	5.3	4.8	5.0

*The cultures at a cell density of 0.6 OD were inoculated at 10% (V/V) level in growth substrates used at the rate of 0.5% (W/V). cell absorbance was measured to prepare growth curves for calculating lag period and doubling time (Pirt, 1975).
a) Cell., Cellobiose b) Glu., Glucose, c) Xyl., Xylose.