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Growth and Non-growth Associated Endo-xylanase and ß-χylosidase Production by a Mutant Derivative of *Cellulomonas biazotea*

M. Ibrahim Rajoka and Riaz Shahid
National Institute for Biotechnology and Genetic Engineering, P.O. Box 577, Faisalabad, Pakistan.

Abstract

Secretion of endo-xylanase and intracellular biosynthesis of ß-xylosidase by rifampin resistant mutant of *Cellulomonas biazotea* from xylan, carboxymethyl cellulose (CMC) or lignocellulosic (LC) substrates in shake flask culture studies followed logistic cum Luedeking-Piret equations. Values of growth (α, IU/g cells) and non-growth associated (ß, IU/g cells h) product parameters were fairly high which implied that the enzyme production was both growth as well as non-growth associated. In both cases, enzyme production was significantly ($p \leq 0.05$) altered in the mutant. Both enzyme production modes were regulated by substrate consumption parameters and changed on the expense of each other.

Introduction

Endo-ß-xylanases (EC 3.2.1.8.), and ß-1,4-D-xylosidases (EC 3.2.1.37) in the presence of debranching enzymes namely α-glucuronidases, esterases and glycosidases have potential applications in biopulping, food processing, nutritional improvement of lignocellulosic (LC) feedstock, production of ethanol, methane, and other products (Wong *et al.*, 1992; Li and Ljungdahl, 1994). Cellulase-free xylanases have important role in reducing consumption of chlorine and chlorine dioxide in paper and pulp industry (Bailey and Viikari, 1993) to release environmental friendly effluents. Due to these numerous novel applications, interest has increased in microbial production of xylanases in recent years.

Complete xylanase system can be produced by a large variety of micro-organisms including *Cellulomonas spp.* (Bailey and Viikari, 1993). Xylanases are produced when organisms are grown on xylan medium but may be exceptionally produced when they are cultured on cellulose medium (Bahkali, 1996; Rajoka, 1990). Enzyme production by both fungi and bacteria is normally considered to be growth-associated (Duenas *et al.*, 1995). Non-growth associated product formation has been reported in the case of lactic acid, polysaccharides, multiproducts, amino acids, acetic acid and kojic acid (Kösebalaban and Özilgen, 1992; Arif *et al.*, 1997). In the present investigation, application of Luedeking-Piret model combined with logistic growth equation (Bailey and Ollis, 1986; Arif *et al.*, 1997) to the kinetics of non-growth and growth associated production of xylanases was assessed by using a deoxy-glucose (DG') cum rifampin resistant (Rf') mutant derivative of *C. biazotea* isolated through gamma ray-induced mutagenesis (Rajoka *et al.*, 1998).

Materials and Methods

Chemicals and substrates: All chemicals were of analytical grade and were obtained from Sigma Chemical Co. (St. Louis, MO, USA). *Atriplex lentiformis* (a shrub) was collected from Biosaline Research Substation of Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, near Lahore where it is grown as an energy crop from saline waste lands (Latif *et al.*, 1988). Cotton stalks were farm-produce of NIAB. The latter is used as a domestic fuel but has very low heat energy content and should be exploited for alternate applications which may yield value-added biotechnological products. The dried powder of LC biomass was steam alkali-treated as described earlier (Latif *et al.*, 1988). *A. lentiformis* and cotton stalks contained 55 ± 1.2, and 65 ± 2.3 per cent cellulose ; 16 ± .23 and 23 ± 2.4per cent hemicellulose respectively as determined by methods described previously (Shirlaw, 1969).

Microorganisms: *Cellulomonas biazotea* NIAB 442 and its rifampin resistant mutant (Rajoka *et al.*, 1998) were grown on the medium containing (g l^{-1}): $K_2HPO_4.7\ H_2O$, 1.0; $NaNO_3$, 0.5; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.\ 7H_2O$, 0.01; yeast extract, 2.0 ; CMC, 10; agar 25 and rifampin 0.02.

Growth: The ability of the organisms to produce growth associated and non-growth associated enzymes was assessed by culturing them on *Atriplex lentiformis* and cotton stalks with reference to CMC and xylan in the Dubos salts aqueous culture medium containing 0.2per cent yeast extract as described earlier (Rajoka and Malik, 1997). In initial studies, substrates were used to contain 2per cent total carbohydrates. In further studies, all substrates were permitted to contain different concentrations of carbohydrates to support different values of specific growth rate (μ, h^{-1}) and enzyme syntheis. All media were adjusted to pH 7.3 with 1M NaOH or 1 M HCl and were dispensed in 200 ml aliquots into 1 l Erlenmeyer flasks in triplicate before autoclaving.

Enzyme production: Shake-flask batch cultures were carried out at 30°C under shaking at 100 rev/min in a gyratory shaking-incubator as described earlier (Rajoka and Malik, 1997). When the test organisms were grown on insoluble substrates, the whole culture medium after fermentation was centrifuged (4000 g, 15 min) to remove particulate substrate. The residue was shaken thrice with chilled water containing 1 per cent (V/V) Tween 80 for 30 min at 4 °C and solid material recovered by centrifugation (4000 g, 15 min). Clear supernatant was obtained by centrifugation

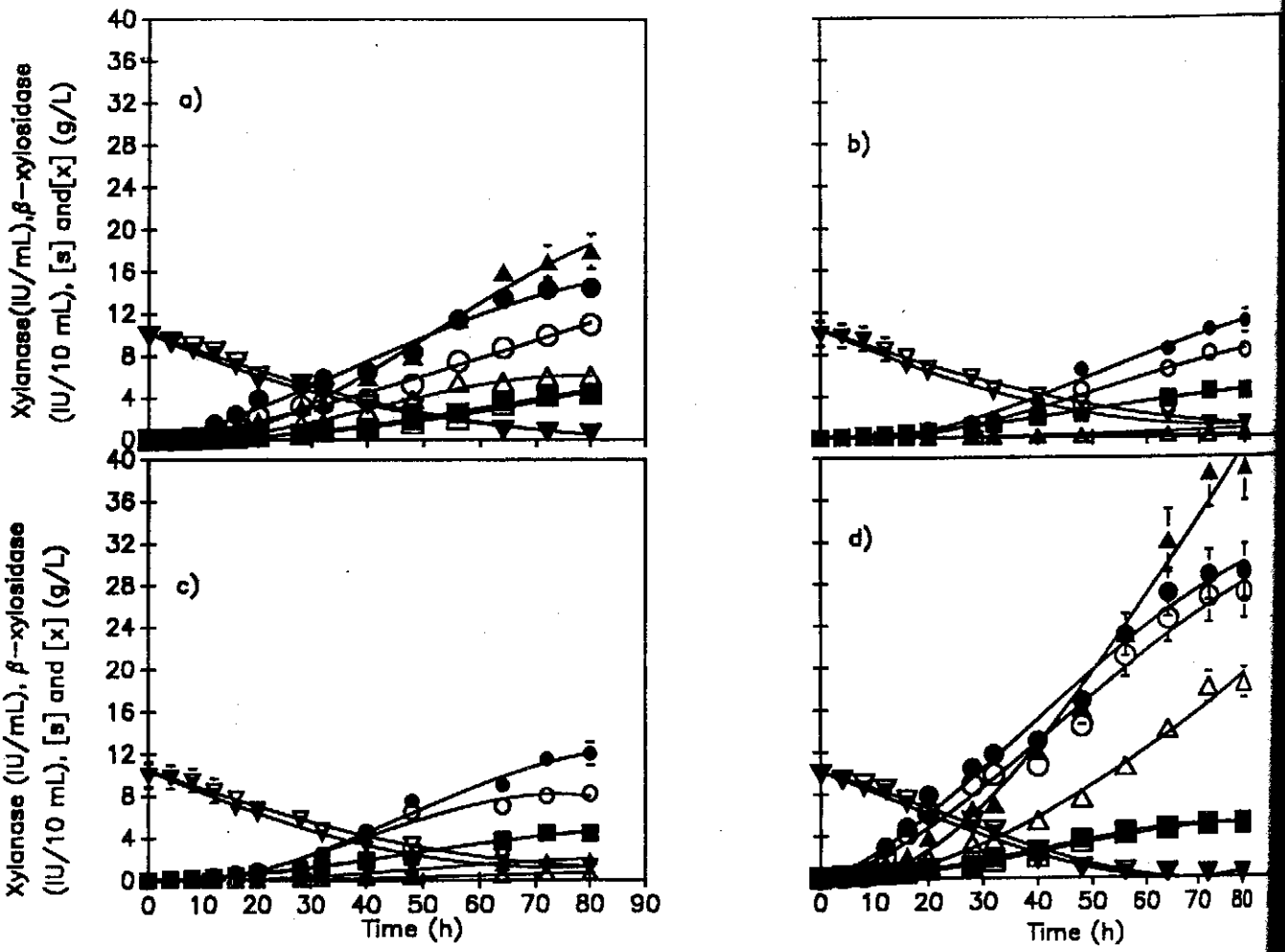

Fig.1: Xylanase, ß-xylosidase, cell mass production and substrate consumption kinetics of *Cellulomonas biazotea* and mutant, 7 Rf^r, cultures in fermentation of *Atriplex lentiformis* (a), carboxymethyl cellulose -Na salt (b), cotton sta (c), and xylan (d) in shake flask cultures: ○-xylanase by parent, ●-xylanase by mutant; Δ-ß-xylosidase by parent, xylosidease by mutant ; □-dry cell mass formation (g/l) by parent, ■-cell mass formation by mutant and ▽-substr consumption (g sugars in the substrate/l) by parent , and ▽-substrate consumption by mutant. The error bars repres standard deviation among replicates.

(15000 g, 30 min) and cell pellet maintained. All washings and cell pellets were pooled separately for determining extracellular and cellular enzyme activities respectively (see later) and correction was made for the adsorbed portion of xylanase and ß-xylosidase at the surface of the insoluble substrates. The cell pellet from 150 mL original culture was used to extract cellular fractions after Rickard *et al.* (1981) or Barron *et al.* (1986). The remaining 50 mL portion was also centrifuged (15000 g, 30 min). The cell-free supernatant was preserved for enzyme assays and solid material was washed twice with saline, suspended in 10 mL distilled water and dried at 70°C to constant mass.

Substrate-free culture samples were centrifuged (15,000 g at 4° C for 30 min) and the cell-free supernatant was used as extracellular fraction (cell-free extract). One portion of the cells (50 ml) was suspended in saline (0.9per c W/V) and used for gravimetric cell mass determination. cells in the second fraction (100 ml) were suspende chilled McIlvaine or Na-acetate buffer (pH 7) and c insoluble fraction. This fraction was suspended in 50 Tris/HCl (pH 7.5) and 10 mM EDTA. 0.2 g lysozyme added to the cell suspension and incubated at 37 °C for h with occasional stirring until the solution became visc and then disrupted by probe sonication in two bursts min with Braun sonicator 2000 on ice. The suspen was centrifuged at 25,000 g for 1 h to get the cell ext Supernatant from this disruption formed the third fra and called cytosolic fraction. The particulate (or associated) fraction was obtained as a pellet and suspended in buffer and formed the fourth fra

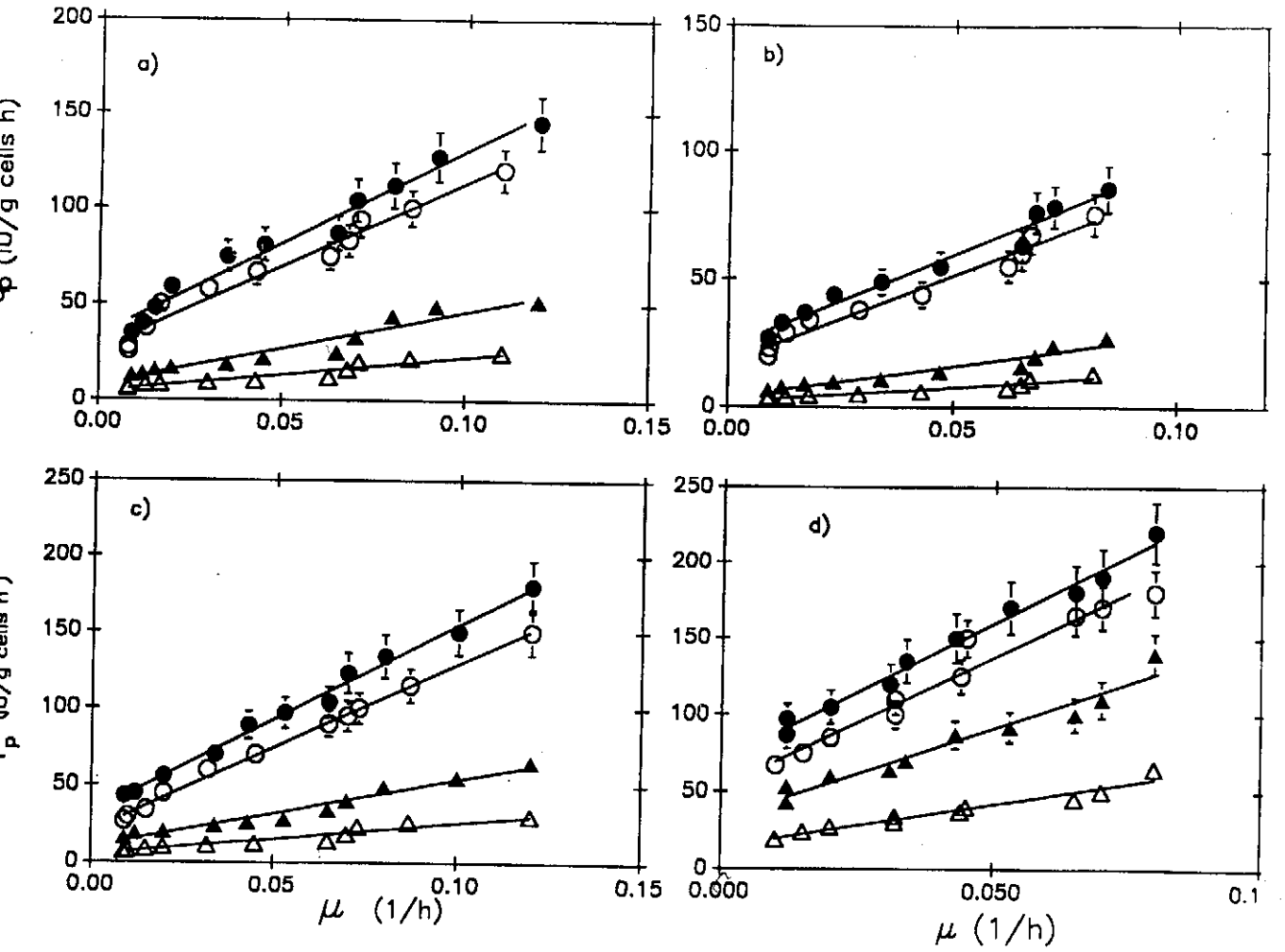

Fig.2: Luedeking-Piret plot of q_p (IU/g cells. h) of xylanase and ß-xylosidase produced by *Cellulomonas biazotea* (○, and Δ respectively) and its mutant derivative (● and ▲ respectively) virsus μ (h^{-1}) during growth on *Atriplex lentiformis* (a), carboxy methyl cellulose (b), cotton stalks (c) and xylan (d). Error bars represent standard deviations among the means of three readings.

Enzyme assays : Endo 1,4-ß-D-xylanase activity in the cell-free extract was assayed according to Bailey *et al.* (1993). ß-Xylosidase activity in the cell extract was determined using p-nitrophenyl ß-D-xylopyranoside (pNPX) as a substrate after Deshpande and Eriksson (1988). One IU each of endo-xylanase and ß-xylosidase has been defined as the amount of enzyme that releases 1 μmol xylose or p-nitrophenol equivalents per ml of enzyme per min of enzyme reaction.

Determination of kinetic parameters : All growth parameters conformed to first order batch kinetics in exponential growth phase. Basic kinetic parameters for batch fermentation process, namely, growth yield coefficient ($Y_{X/S}$), process product yield, $Y_{P/X}$ (IU/g cells), q_S (specific substrate uptake rate) were determined as described previously (Pirt, 1975; Rajoka *et al.*, 1998). Specific growth rate (μ, h^{-1}) was determined from a slope of ln (X) versus time of fermentation. Specific rate of enzyme production (q_P, IU/g cells h) was determined by $\mu \times Y_{P/X}$. The values of q_P have been plotted against μ (Fig. 2) after Bailey and Ollis (1988).

Statistical analysis : Treatment effects were compared by the protected least significant difference method (Snedecor and Cochran 1980) presented as two-factor factorial design in the form of probability (p) values.

Results and Discussion

Xylan and carboxy methyl cellulose (CMC) support maximum production of xylanases (Milagres *et al.*, 1993; Saxina *et al.*, 1995; Bahkali, 1996). The use of commercial xylan or CMC as substrates is uneconomical for large-scale potential of *C. biazotea* and its mutant derivative for production of xylanases in shake-flask batch culture studies was tested after growth in Dubos salts culture medium containing *A. lentiformis*, cotton stalks with reference to xylan, and CMC as 1per cent carbohydrate (cellulose +

hemicellulose content in the substrate) as sole carbon sources and kinetics of enzyme production has been presented in Fig. 1(a)-(d), respectively. These figures revealed that production of xylanase and ß-xylosidase was maximum on xylan. The yields of endo-xylanase were higher on all the substrates than were of ß-xylosidase in both wild parent and its mutant and that mutant was significantly (p 0.05) improved for specific activities of xylanases (Table 1). Maximum specific enzyme activities were several-fold improved over those from some other bacterial and fungal culture (Duenas *et al.*, 1995; Bahkali, 1996; Singh *et al.* ,1995; Milagres *et al.*, 1993; Sinner and Preslmayer 1992; Rickard *et al.*, 1981).These curves also indicated that production of xylanases was apparently growth-associated. In further studies, the enzyme production mode by the mutant derivative of *Cellulomonas biazotea* was assessed with reference to its parent by applying the Luedeking- Piret model:

$$dP/dt = \alpha . dX/dt + ß . X \quad \text{----------(1)}$$

where P is enzyme in IU/ l, X is cell mass in g/l

Table 1: Specific activities of xylanase and ß-xylosidase* produced by parental (P) strain of *Cellulomonas biazotea* and its mutant (M) derivative following growth on different substrates

Substrate/ organism		Xylanase	ß-Xylosidase
		IU/mg protein	
A.lentiformis			
	P	10.10c	0.59e
	M	13.43b	1.42d
CMC			
	P	07.63 d	0.66 e
	M	10.27 c	1.40 d
Cotton stalks			
	P	08.79 cd	1.41 d
	M	10.27 c	2.56 a
Xylan			
	P	24.64 a	1.84 c
	M	26.27 a	2.88 a
*Significance ($p<0.05$)		HS	HS

Values followed by same letters donot differ significantly at $p \leq 0.05$ according to multiple range test. HS stands for significantly different.

The growth rate (dX/dt) according to logistic model:

$$dX/dt = \mu . (X_m - X)/X_m \quad \text{----------(2)}$$

If X is smaller than X_m, the organism is in the exponential growth phase and product is growth associated. When values of X approach to X_m, $dX/dt = 0$, and product formation is non-growth associated.
Equation (1) can be solved to get:

$$q_p = \alpha . \mu + ß \quad \text{----------(3)}$$

where α and ß are growth-associated and non-growth associated enzyme production parameters respectively.

Table 2: Kinetic values of α (IU/g cells) and ß (IU/g cells. h) in Luedeking-Piret model for xylanase and ß-xylosidase produced by *Cellulomonas biazotea* (P) and its mutant derivative (M), 7Rf' , during growth on different substrates.

Substrate/ organism	α (IU/g cells)		ß (IU/g cells. h)	
	xylanase	ß-xylosidase	xylanase	ß-xylosidase
A. lentiformis				
P	918^{d}	193^{d}	28^{de}	4.2^{a}
M	967^{cd}	378^{c}	37^{c}	8.0^{c}
CMC				
P	682^{e}	122^{e}	19^{f}	2.4^{e}
M	720^{e}	246^{d}	24^{ef}	5.3^{d}
Cotton stalks				
P	1140^{bc}	219^{d}	22^{f}	4.8^{d}
M	1231^{b}	440^{c}	33^{cd}	8.9^{c}
Xylan				
P	1696^{a}	580^{b}	56^{b}	15^{a}
M	1797^{a}	1185	70^{a}	32^{b}
*Significance ($p<0.05$)	HS	HS	HS	HS

The results are average of three replicates. The values wi different superscripts within columns differ significantly ($p<0.05$. * HS stands for highly significant.

The values of μ, and q_p were obtained as described Methods by growing the organisms on *A. lentiform* cotton stalks, xylan and CMC. This was done by growi the organisms on varying concentrations of test substrat to support different values of μ. A linear relationship w obtained by plotting q_p of endo-xylanase and ß-xylosida produced on different substrates versus μ (Fig. 2). Fr equation 3, the slope values (α, $Y_{P/X}$) of xylanase and xylosidase for the parental and mutated enzymes are for growth-associated product formation (Table 2). T intercepts on the ordinates (ß) gave specific prod formation rate values (IU/g cells h) for the parental and mutated enzymes for non-growth associated parameter The parameters α & ß or both expectedly have sma values under genetic control which does not supp enzyme production; they are expected to have gre values when enzyme synthesis is derepressed. Equatio implies that greater the values of ß, the greater is the r growth associated enzyme production rate. A sim discussion is applicable to α, the growth associated enz production parameter.
All culture media supported product formation in growth associated and non-growth associated model maximum production of xylanase and ß-xylosidase b mutant was significantly ($p<0.05$) improved (Table 2 mentioned earlier, the substrate consumption param

mely Q_s, q_s ,$Y_{X/S}$, μ, and pH (Rajoka and Malik, 1997) gulated product formation; their values changed with ange in the above parameters. But change was at the pense of mode of product formation in these studies.

e values of α are significantly higher ($p \leq 0.05$) than those ported in growth-assocaited xylanase production by *sarium oxysporum* mutant NTG-19 (Singh *et al.*, 1995) *reptomyces lividans* recombinant, a mutant of *Sclerotium ffi UV 8*, *Cellulomonas* mutant CS1-17 (Sinner and eslmayer, 1992) and mutant *Trichoderma reesei* QM 414 (Milagres *et al.*, 1993). Values of ß for xylanase oduction are fairly higher than those reported for growth sociated parametrs reported by other workers (Duenas *et* , 1995, Bahkali, 1996).

e ß values of the operational kinetic parameters obtained r ß-xylosidase (Table 2) in mutant of *C. biazotea* is veral-fold higher than the growth associated values re- rted by other workers on *Cellulomonas sp.* and their utants (Rickard *et al.*, 1981) and for other bacteria axena *et al.*, 1995), *Penicillium wortmanni* (1.1 IU/g lls. h) , *A. awamori* mutant ANNTG 43 (2.7 IU/g cells. h), *niger* mutant (1.0 IU/g cells. h) and *T. reesei* mutant QM 414 (2.7 IU/g cells h; Smith and Wood, 1991) and mparable with mutant fungal strains (Milagres *et al.*, 993) in growth associated product formation mode. This plies that the enzyme syhthesis is non-growth associated well. The kinetics of xylanase production agree well with edeking-Piret model defined by logistic growth model rif *et al.*, 1997). The substrate consumption parameters anged the growth and non-growth associated product rmation parameters to each others expense.

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