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MUTAGENESIS OF *CELLULOMONAS BIAZOTEA* FOR ENHANCED PRODUCTION OF XYLANASES

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Abstract

A rifampin-resistant mutant of Cellulomonas biazotea, *designated 7 Rf, secreted elevated levels of xylanases* in vivo. *The xylanase and β-xylosidase production in the mutant was not inhibited in the presence of 5% glucose, cellobiose or glycerol in the solid medium. This mutant showed approximately 2–3-fold enhanced product yield and productivity of cellular β-xylosidase over the wild type parent in shake flask culture studies when grown on either cellobiose, cellulosic or lignocellulosic (LC) substrates. Extracellular production of xylanase was also significantly ($P \leq 0.05$) altered in the mutant. During growth of* C. biazotea *mutant derivative on xylan, maximum volumetric productivities (Q_p) for xylanase and β-xylosidase were 493, and 30.7 IU/l/h, which were 1.21- and 2.29-fold improved over their respective values from the parental strain. The above values are statistically ($P \leq 0.05$) higher than the values reported in some other xylanolytic mutant organisms.* © *1997 Published by Elsevier Science Ltd.*

Key words: Xylanase, β-xylosidase, pretreatment, carbohydrates, protein, *Cellulomonas*, production, induction, lignocellulose, kallar grass straw.

INTRODUCTION

Hemicellulose is regarded as the second most abundant and biologically renewable resource for bioconversion into biofuels, as well as feedstock chemicals (Wise, 1984). Xylan is the major component of hemicellulose from monocots and is usually associated with cellulose and lignin from plant cell walls. Utilization of the hemicellulose component requires synergistic action of endo-β-xylanases (EC 3.2.1.8.), β-1,4-D-xylosidases (EC 3.2.1.37) along with debranching enzymes, namely α-glucuronidases, esterases and glycosidases (Rickard *et al.*, 1981; Thomson, 1993).

Xylanases are the key enzymes for breakdown of xylan since they depolymerize the backbone. They have potential applications in biopulping, nutritional improvement of LC feedstock, production of ethanol, methane, other products, and in the processing of food (Wong *et al.*, 1988; Wong and Saddler, 1992; Li and Ljungdahl, 1994). Cellulase-free xylanases have an important role in reducing consumption of chlorine and chlorine dioxide in the paper and pulp industry (Yang *et al.*, 1992; Bailey and Viikari, 1993). Owing to these numerous novel applications, interest has increased in microbial production of xylanases in recent years.

A functionally complete xylanase system can be produced by a large variety of micro-organisms grown on agro-industrial LC wastes (Mackenzie *et al.*, 1987; Thomson, 1993; Bailey and Viikari, 1993). In Pakistan, LC biomass can be produced from saline or saline–sodic soils using salt-tolerant plants, namely *Leptochloa fusca* (kallar grass), *Panicum maximum* (panic grass), *Atriplex* spp., and *Sesbania aculeata* (dhancha) (Malik *et al.*, 1986). Bagasse, wheat straw, cotton stalks, rice straw etc., also accumulate to a level of 50 million ton annually (Azad, 1986). These abundant but low-value resource materials contain up to 70% hydrolysable cellulose and hemicellulose that can be used to produce cellulases and hemicellulases inexpensively.

Synthesis of xylanases is regulated by induction and repression in both fungi (Milagres *et al.*, 1993; Bahkali, 1995) and bacteria (Okeke and Paterson, 1992). Simultaneous production of cellulases and xylanases has been reported in many organisms (Okeke and Paterson, 1992; Thomson, 1993). *Cellulomonas* spp. cause rapid degradation of plant LC biomass and produce cellulases and xylanases (Rickard *et al.*, 1981; Rajoka and Malik, 1986). Our studies on regulation of xylanase synthesis in *C. biazotea* have indicated that the formation of xylanases depended on growth rate, substrate uptake rates, rates of protein synthesis, and release of inducers from insoluble substrates (Rajoka, 1990). Disaccharides and oligosaccharides arise slowly during the hydrolysis of cellulose or LC biomass and are inducers of xylanases (Rajoka, 1990; Hrmova *et al.*, 1991; Thomson, 1993) and their quantity in the

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fermentation mash may influence the yield of enzymes. In *C. biazotea*, it appeared that β-xylosidase and xylanase synthesis varied under an induction mechanism and a growth-dependent repression mechanism that changed the rate of synthesis in induced over non-induced cultures. Addition of monomeric sugars to the basal medium containing xylan caused apparent catabolite repression of xylanase production in *C. biazotea* (Rajoka, 1990). This reflected a delay in the induction of xylanases by xylan until the alternative and easily metabolizable carbon source was completely exhausted and derepression occurred, resulting in larger β-xylosidase and xylanase production. Some improvements in enzyme production were expected through mutation and selection programs, as have been achieved in fungal systems including the *T. reesei* system (Cusky *et al.*, 1983), hyperxylanolytic strain of *Aspergillus* sp. (Bisawa *et al.*, 1990) and *Fusarium oxysporum* (Singh *et al.*, 1995). In the present investigation, a deoxy-D-glucose resistant (DG^r) cum rifampin-resistant (Rf^r) mutant derivative of *C. biazotea* was isolated through gamma ray-induced mutagenesis. The DG^r phenotype is related to mutation of a specific genetic structure closely related to membrane transport, hydrolase regulation and its hyperproduction. DG^r mutants of different fungi have been found to hyperproduce hydrolases (Montenecourt and Eveleigh, 1979; Ghosh *et al.*, 1991; Antier *et al.*, 1993). The enhanced production of xylanases by the mutant derivative was studied to establish a relationship of substrate and production of endo-β-xylanase and β-xylosidase.

METHODS

Chemicals and substrates

α-Cellulose, oatspelt xylan, *p*-nitrophenyl β-D-xylopyranoside (pNPX), glucose, glycerol, lactose and cellobiose were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade. Kallar grass, dhancha and panic grass were collected from the Biosaline Research Substation of the Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, near Lahore where these are grown as energy crops from saline waste lands in Pakistan (Malik *et al.*, 1986). Wheat straw was a farm-produce of NIAB. Bagasse was collected from a local sugar factory. The dried powder of LC biomass was steam alkali treated as described earlier (Rajoka and Malik, 1984a; Latif *et al.*, 1988). Kallar grass, panic grass, bagasse, wheat straw, dhancha straw and cotton stalks contained 55 ± 1.2, 65 ± 2.3, 68 ± 2.0, 61 ± 1.0, 61.5 ± 1.1, 64 ± 1.8% cellulose; 23 ± 2.4, 16 ± 1.23, 20.0 ± 2.1, 23 ± 2.6, 20.5 ± 2.1, 19 ± 1.4% determined by methods described

Micro-organisms

A strain of *C. biazotea* isolated from a bagasse heap (Rajoka and Malik, 1984b) and its mutant derivative were maintained on Dubos salt–yeast extract–avicel (DYEA) and DYEA + rifampin plates, respectively. The medium contained (g/l): $K_2HPO_4.7H_2O$, 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; agar 25 and rifampin 20 mg.

Isolation of mutants

Cellulomonas biazotea cells were cultured in Dubos–yeast extract–cellobiose (DYEC) culture medium at 30°C for 20 h, centrifuged ($15000 \times g$, 15 min), and suspended in 50 ml of biological saline containing 0.01% yeast extract. The cells at an optical density of 0.6 at 610 nm were dispensed equally in 30 ml McCartney vials and exposed to different challenging doses of gamma rays in a Co-60 irradiator. The exposure of cell suspension (2×10^9 cells/ml) to Γ-irradiation of 40 and 50 krad gave approximately a 3 logarithmic reduction (Haggett *et al.*, 1978). The resistant cells from these doses were allowed to express to an absorbance of 0.9 in the presence of 20 μg/ml rifampicin (Rf)+0.6% DG in DYEC medium to isolate Rf^r and simultaneously derepressed mutants (Montenecourt and Eveleigh, 1979; Smith and Wood, 1991).

A serial dilution was applied to the expressed cells and plated on DYE-xylan-DG-Rf, DYE-β-pNPX-DG-Rf selection plates to yield approximately 30 colonies per plate. Overall, 2000 different colonies were screened, larger ones specifically and smaller ones randomly for mutant selection. The isolated colonies were subsequently replica-plated on xylan + DG + Rf, and pNPX + Rf + DG agar plates. Each colony was studied for hyperproduction of β-xylosidase *in vivo* by measuring the diameter of the yellow zone as visualized with carbonate buffer, 200 mM (pH 8.5) spread on the agar plates (Smith and Wood, 1991). Similarly each colony was also tested for endoxylanase production by measuring the diameter of clearance zones on oatspelt xylan plates. Among three mutants, one mutant designated $7Rf^r$ produced a larger zone of xylan clearance and a larger yellow-coloured zone on pNPX plates. Enzyme secretion was tested in the presence of increasing concentrations (2, 4, 5, 6, 8, 10%, w/v) of glucose, cellobiose or glycerol.

Nutrition

The ability of the organisms to utilize bagasse, wheat straw, kallar grass straw, *P. maximum*, and *S. aculeata* with reference to cellobiose, xylan or α-cellulose as sole carbon source was examined in basal Dubos salts medium containing 0.2% yeast extract as previously described (Rajoka and Malik, 1986). All LC substrates used contained 1% cellulose plus a hemicellulose component and were

a sugar level of 10 g/l. Cellobiose was used at rate of 10 g/l (w/v) and was added to autoclaved lium after filter-sterilization. All media were usted to pH 7.3 with 1 M NaOH or 1 M HCl and e dispensed in 200 ml aliquots into 1-l Erlen-yer flasks in triplicate before autoclaving.

zyme production

ake-flask batch cultures were carried out at 30°C der shaking at 100 rev/min in a gyratory shaking-cubator. Cultivations were for up to 80 h after oculation of 2 ml of an overnight inoculum of 0.6 D at 610 nm. The amount of growth was measured ravimetrically as dry cell weight. Enzyme activity as assayed as a function of the carbon sources. The nzyme activity present in the extracellular (cell-free upernatant) or cellular fraction (cell extract) was assayed as the induction or repression indicator. When the test organism was grown on insoluble substrates, the culture medium after the specified fermentation period was centrifuged (4000 × *g* for 15 min) to remove particulate substrate.

Cell fractionation

Insoluble substrate-free culture samples were centrifuged (15 000 × *g* at 4°C for 30 min) and the cell-free supernatant used as the extracellular fraction (Rickard *et al.*, 1981). A portion of the cells (50 ml) was suspended in biological saline and used for gravimetric cell mass determination. The cells in the second fraction (100 ml) were fractionally isolated after Barron *et al.* (1986).

Enzyme assays

Endo 1,4-β-D-xylanase activity was assayed according to Bailey *et al.* (1992) by incubating the diluted enzyme solution at pH 7.0 and a temperature of 40°C for 5 min using a substrate solution of 1% (w/v) crystalline oatspelt xylan in 0.05 M acetate buffer (pH 7.0). The reducing sugars were assayed by adding 3 ml of 3,5-dinitro salicylic acid reagent, boiling for 5 min, cooling, and measuring the absorbance at 540 nm (Miller, 1959) against xylose as standard. β-Xylosidase activity was determined using pNPX as substrate after Deshpande and Eriksson (1988) by replacing pNPG by pNPX. One IU of endoxylanase, and β-xylosidase has been defined as the amount of enzyme that releases 1 μmol xylose or para-nitrophenol equivalents per millilitre per minute.

Protein determination

The proteins were determined by adding 5 ml of alkaline copper sulphate reagent to 2 ml of extracellular or sodium hydroxide-extracted intracellular protein sample, standing for 10 min at room temperature, adding 0.5 ml Folin phenol reagent, standing for 30 min at room temperature, and measuring the absorbance at 750 nm, as described by Lowry *et al.* (1951) using bovine serum albumin as the standard.

Carbohydrate determination

Cellulose and hemicellulose in lignocellulosic materials were determined after Shirlaw (1969). Carbohydrates released from lignocellulosic substrates in the fermentation medium during growth of the organisms were assayed by adding 3 ml of 3,5-dinitro salicylic acid reagent to 1 ml of sample plus 2 ml of water, boiling for 5 min, cooling, and measuring the absorbance at 540 nm (Miller, 1959) against xylose or glucose as standards.

Determination of kinetic parameters

Potential kinetic parameters for a batch fermentation process, namely, growth yield coefficient ($Y_{x/s}$), volumetric rate of substrate utilization (Q_s) and enzyme production (Q_p), specific substrate uptake rate (q_s), process product yields namely, $Y_{p/x}$ and $Y_{p/s}$, were determined as described previously (Pirt, 1975; Lawford and Rousseau, 1993). Cell mass productivity (B) expressed as g dry cells/l/h, intracellular (P_i) or extracellular protein (P_e) productivity (mg protein/l/h) was determined from a plot of g dry cells/l, intracellular or extracellular protein (mg/l) versus time. Specific enzyme activity was obtained by dividing Q_p by P_i or P_e.

Statistical analysis

Treatment effects were compared by the protected least significant difference method (Snedecor and Cochran, 1980). Significance of difference has been presented as two-factor factorial design in the form of probability (P) values.

RESULTS AND DISCUSSION

Improvement in enzyme secretion by Γ-ray mutagenesis was sought to isolate catabolite resistant and simultaneously xylanase hyper-producer mutant derivatives of *C. biazotea* after Smith and Wood (1991). These studies indicated that well-developed zones of clearance on xylan plates, and larger yellow zones on pNPX–agar plates appeared around three colonies in the case of mutant strains. Semi-quantitative plate studies revealed that one derivative capable of producing the largest amount of β-xylosidase, and endoxylanase could be isolated and designated 7Rf^r, for enzyme production studies *in vitro*. The organism was recovered using replica plating and its endoxylanase, and β-xylosidase activities were monitored in plate tests in the presence of increasing concentrations of glucose, cellobiose and glycerol. It was found that the secretion of xylanases was least affected in the presence of up to 5.0–7.5% (w/v) glucose, cellobiose (w/v) or glycerol (v/v). Initial studies indicated that

addition of dimeric compounds in the basal salt liquid medium stimulated xylanase secretion in the mutant, but maximum activities of xylanases were produced during growth on xylan medium in both wild as well as mutated cultures.

Production of xylanases

The use of commercial xylan as a substrate is uneconomical for large-scale production of xylanases, therefore, several renewable substrates were included in these studies. The 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 α-cellulose, xylan, dhancha, kallar grass, bagasse, wheat straw, *P. maximum* and cellobiose as 1% carbohydrate (cellulose + hemicellulose content or cellobiose alone, w/v) in the substrate as sole carbon sources and the kinetics of enzyme production are presented in Fig. 1(a)–(h), respectively. These figures reveal that production of xylanase and β-xylosidase was maximum on xylan. The yields of endoxylanase were higher on all substrates than those of β-xylosidase in both wild parent and its mutant, and that mutant was significantly ($P \leq 0.05$) improved for production of xylanases. These curves also indicated that production of xylanases was apparently growth-associated. However, application of the Leudeking and Piret model, $q_p = \alpha.\mu + \beta$ (Bailey and Ollis, 1986) by plotting q_p of endox-

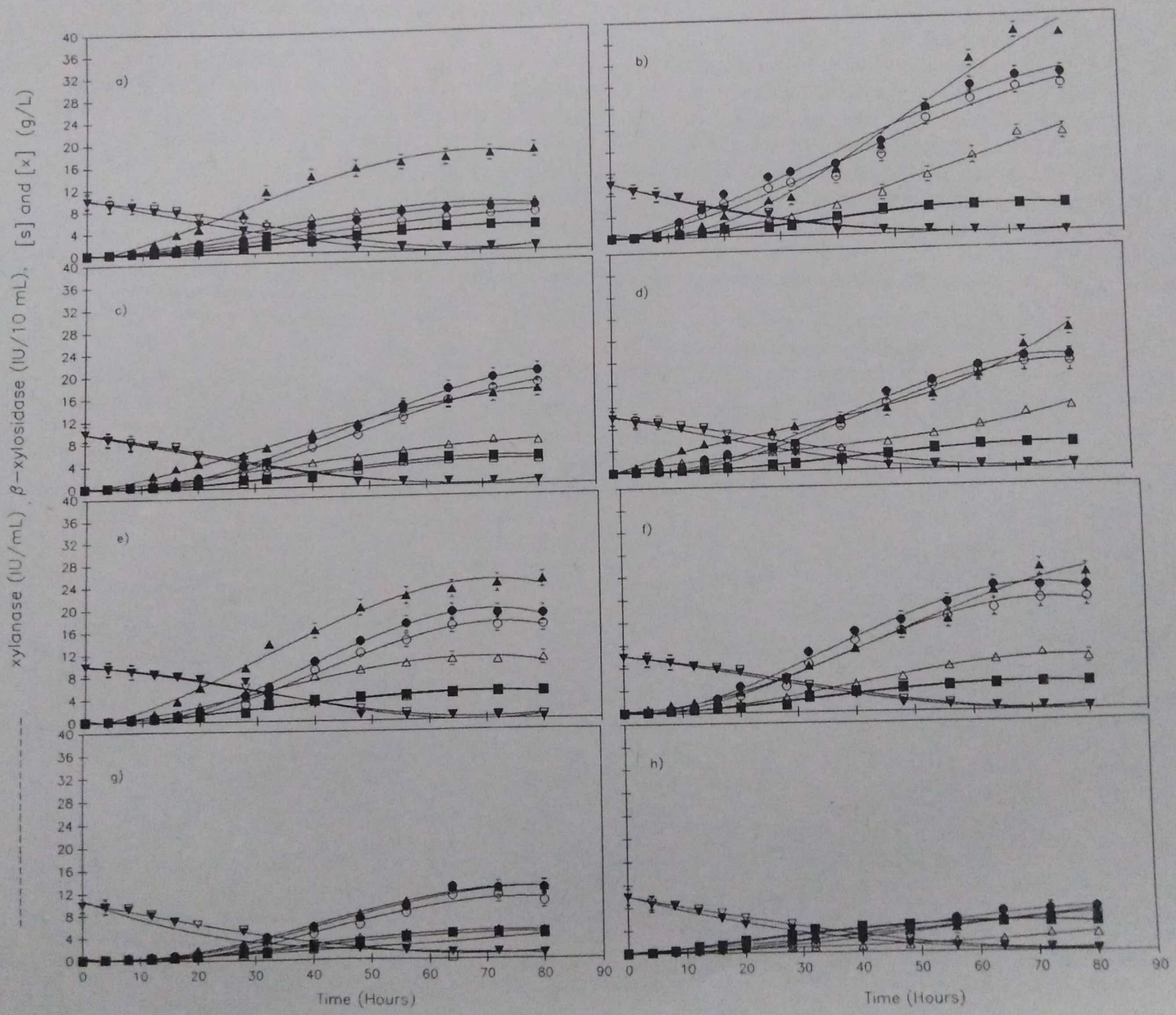

Fig. 1. Xylanase, β-xylosidase, and cell mass production kinetics of *Cellulomonas biazotea* and its mutant, 7 Rf^r, cultures in fermentation of α-cellulose (a), xylan (b), dhancha straw (c), kallar grass straw (d), bagasse (e) wheat straw (f), *Panicum maximum* and cellobiose in shake-flask cultures. Xylanase production by parent (open circle), mutant derivative (closed circle); β-xylosidase production by parent(open triangle), mutant derivative (closed triangle); cell mass biosynthesis by parent (open square), mutant derivative (closed square); substrate consumption by parent (open inverse triangle) and mutant derivative (closed inverse triangle). Error bars show standard deviations among three replicates; some values are too small to be visible.

ylanase and β-xylosidase produced on different substrates versus μ, gave slope values of 3349 ± 1127 and 728 ± 165 IU/g cells of xylanase and β-xylosidase, respectively for the parental enzymes, and 4052 ± 751 and 1409 ± 488 IU/g cells for the mutated enzymes for the growth-associated product formation. The intercepts on the ordinates gave 17.5 ± 5.6 and 8.2 ± 4.8 IU/g cells/h for the parental enzymes and 17.2 ± 6.1 and 6.0 ± 2.2 IU/g cells/h for the mutated enzymes, respectively for non-growth-associated parameters, which implied that product formation was both growth-associated and non-growth-associated.

On the basis of the dry weight of the residual substrate, the maximum utilization of cellobiose, xylan and kallar grass took place (Fig. 1). Maximum growth in terms of dry cell weight was non-significant ($P \leq 0.05$) during growth of *C. biazotea* and its mutant on cellobiose, xylan, kallar grass, wheat straw and dhancha straw and least on *P. maximum* (Table 1). Mean protein formation rates by *C. biazotea* mutant derivative were significantly higher ($P \leq 0.05$) than the values from the parental strain. When cultures grown on various substrates were monitored for $Y_{x/s}$, B, and Q_s, there was insignificant enhancement ($P \leq 0.05$) in the values of B, Q_s, and $Y_{x/s}$ in mutant cultures over those obtained in wild cultures of *C. biazotea* on all substrates.

Production of xylanase in different media containing various insoluble substrates

Xylanase activities accumulated in the culture media. The results on xylanase production (Fig. 1, Table 2) revealed that *C. biazotea*, and its mutant produced maximum levels of enzyme from xylan and minimum levels on cellobiose medium. Among LC substrates, kallar grass-, and wheat straw-culture media supported maximum production of xylanase and xylanase synthesis in the mutant was significantly ($P \leq 0.05$) improved. For comparison of data for extrapolation, IU/g cells, IU/g substrate consumed, volumetric productivities or specific productivities of the enzyme have been presented (Table 2).

C. biazotea mutant xylanases exhibited 1.22-fold improved Q_p, $Y_{p/s}$ and $Y_{p/x}$ over the parental strain. The lowest values of operational kinetic parameters were obtained from the cellobiose culture medium (Table 2) followed by α-cellulose medium. Maximum $Y_{p/x}$, $Y_{p/s}$ or Q_p of *C. biazotea* and its mutant (Table 2) was several-fold improved over those from some other *Cellulomonas* strains (Rapp and Wagner, 1986), other bacterial cultures (Tabassum *et al.*, 1990; Okeke and Paterson, 1992) and fungal strains (Duenas *et al.*, 1995; Milagres *et al.*, 1993) and compared favourably with values from *Cellulomonas CS1-1* and its mutant derivatives (Choi *et al.*, 1978).

Table 1. Comparative study of substrate utilization parameters during growth of *Cellulomonas biazotea* (P) and its mutant (M) on different substrates

Growth substrate and organism	Substrate utilization parameters				
	$Y_{x/s}$ (g/g)	Q_s (g/l/h)	Ext. P (mg/l/h)	I n.P (g/l/h)	R.S[a] (mg/l)
Bagasse					
P	0.49^{a}	0.144^{a}	112 ± 5^{fg}	107 ± 4.6^{ef}	80 ± 3.4^{de}
M	0.51^{a}	0.145^{a}	120 ± 5^{ef}	113 ± 4.4^{ef}	74 ± 2.8^{ef}
K. grass straw					
P	0.50^{a}	0.146^{a}	130 ± 6^{abcde}	116 ± 4.7^{de}	78 ± 2.9^{def}
M	0.52^{a}	0.176^{a}	134 ± 6.1^{abc}	129 ± 4.3^{bc}	73 ± 2.5^{f}
Dhancha straw					
P	0.49^{a}	0.147^{a}	132 ± 5.7^{abcd}	103 ± 2.4^{f}	81 ± 2.6^{cd}
M	0.51^{a}	0.150^{a}	140 ± 6.0^{ab}	143 ± 3.2^{a}	78 ± 2.5^{def}
Wheat straw					
P	0.48^{a}	0.143^{a}	132 ± 5.5^{abcd}	128 ± 3.2^{bc}	87 ± 2.3^{gh}
M	0.50^{a}	0.145^{a}	141 ± 5.7^{a}	138 ± 3.2^{ab}	78 ± 2.2^{def}
Cellobiose					
P	0.50^{a}	0.174^{a}	112 ± 4.9^{fg}	125 ± 4.5^{cd}	40 ± 1.9^{g}
M	0.50^{a}	0.179^{a}	133 ± 5.7^{abcd}	131 ± 5.0^{bc}	34 ± 1.2^{gh}
Xylan					
P	0.51^{a}	0.174^{a}	112 ± 4.9^{fg}	143 ± 6.0^{a}	40 ± 1.8^{g}
M	0.52^{a}	0.196^{a}	122 ± 5.2^{def}	138 ± 6.2^{ab}	33 ± 1.5^{h}
α-Cellulose					
P	0.50^{a}	0.144^{a}	128 ± 4.2^{cde}	109 ± 4.1^{ef}	82 ± 2.6^{cd}
M	0.54^{a}	0.166^{a}	109 ± 4.0^{g}	134 ± 4.8^{abc}	78 ± 1.7^{def}
P. maximum					
P	0.48^{a}	0.143^{a}	129 ± 3.5^{bcde}	109 ± 4.3^{ef}	95 ± 2.3^{a}
M	0.49^{a}	0.145^{a}	109 ± 2.7^{g}	134 ± 4.2^{abc}	89 ± 2.1^{b}
Significance ($P = 0.05$)	N.S.	N.S.x	H.S.x	H.S.	H.S.

Values are means of three sets of replicates. Within columns, values with different superscript letters differ significantly ($P = 0.05$).

[a]R.S., reducing sugars, mg/L.x; N.S., not significant; H.S., highly significant.

C. biazotea and its mutant produced 5744 and 6662 IU xylanase/g cells, with maximum volumetric productivity of 435 and 491 IU/L/h, respectively on xylan medium. These productivity levels are significantly higher ($P \leq 0.05$) than those reported in *Streptomyces roseiscleroticus* (337 IU/l/h) (Grabski and Jeffries, 1991), *Fusarium oxysporum* mutant NTG-19 (296.7 IU/l/h) (Singh *et al.*, 1995) *Aspergillus sojae* (223 IU/l/h) (Kimura *et al.*, 1995), *Streptomyces lividans* recombinant (333 IU/l/h), *Sclerotium rolffi UV 8*, a mutant derivative (327 IU/l/h (all grown on xylan), *Cellulomonas* mutant CS1-17 (347 IU/l/h), *Chainia* NCL 82-5-1 (390 IU/l/h) and *Penicillium funiculosum* (70 IU/l/h) grown on different substrates as reported by Sinner and Preselmayer (1992). The productivity of xylanase from *C. biazotea* and its mutant compares favourably with that from a hyperxylanase-producing mutant *Trichoderma reesei* QM 9414 (436 IU/l/h) and *Penicillium janthinellum* (467.7 IU/l/h) grown on xylan (Milagres *et al.*, 1993). The productivity of xylanase from the mutant organism is appreciably high because it is about half of that produced from *T. reesei* RUT C-30 (930 IU/l/h), the best xylanase producer (Milagres *et al.*, 1993). The q_p values of xylanase production from mutant derivatives of *C. biazotea* on xylan medium were 929 IU/g cells/h and were 1.53–1.75-, 22.7-fold improved over *A. awamori* mutant ANNTG 43, *Aureobasium pullulans* and *Trichoderma reesei* mutant CL847 (Smith and Wood, 1991).

The possibility of using locally available substrates (Table 1) for enzyme production was promising in that induction on kallar grass and dhancha medium yielded xylanase at a level greater than 0.9 and 0.77 of that induced by xylan; other LC substrates were also significantly better than cellulosic substrates.

It was noteworthy that xylanase was also produced by cultures during growth on pure cellulosic substrates, as noted in *Thermomonospora fusca* (Ghanghas *et al.*, 1989; Irwin *et al.*, 1994), *Trichoderma* strains (Wong and Saddler, 1992), actinomycetes (Okeke and Paterson, 1992) and in different fungi (Milagres *et al.*, 1993). Its production from the cellulosic substrates may be due to the presence of a small amount of xylan residues (Irwin *et al.*, 1994). Alternatively, a common mechanism of induction for cellulases and hemicellulases may also induce xylanases, but its production from cellobiose (Table 1) indicates that it is produced constitutively as well.

Production of β-xylosidase from different media

β-Xylosidase is an important enzyme in xylanolysis as it releases xylose which acts as an inducer of

Table 2. Potential of *C. biazotea* (P) and its mutant derivative (M) for xylanase production from different substrates measured as process product yields [$Y_{p/x}$(IU/g cells) and $Y_{p/s}$ (IU/g substrate)], enzyme productivity, Q_p (IU/l/h) and q_p (IU g cells/h) by growing on different substrates in Dubos culture medium at 30°C

Growth substrate and organism	Xylanase production profiles of *C. biazotea* and its mutant derivative measured as			
	$Y_{p/x}$	$Y_{p/s}$	Q_p	q_p
Bagasse				
P	3773 ± 151^{g}	1660 ± 75^{g}	259 ± 12^{h}	468 ± 19^{f}
M	4341 ± 217^{cd}	1861 ± 65^{f}	284 ± 13^{g}	511 ± 23^{e}
K. grass straw				
P	4356 ± 174^{cd}	2440 ± 96^{d}	387 ± 16^{de}	545 ± 23^{d}
M	4412 ± 154^{c}	2733 ± 110^{b}	418 ± 18^{c}	600 ± 25^{c}
Dhancha straw				
P	4000 ± 180^{f}	2350 ± 101^{d}	367 ± 16^{e}	512 ± 25^{e}
M	4127 ± 206^{ef}	2585 ± 103^{c}	403 ± 14^{cd}	549 ± 23^{d}
Wheat straw				
P	4222 ± 211^{de}	1960 ± 84^{f}	306 ± 20^{g}	538 ± 23^{de}
M	4348 ± 152^{cd}	2178 ± 87^{e}	335 ± 15^{f}	592 ± 23^{c}
Cellobiose				
P	1280 ± 53^{l}	640 ± 29^{k}	100 ± 5^{ijk}	154 ± 8^{i}
M	1312 ± 54^{l}	718 ± 33^{ijk}	121 ± 5^{j}	170 ± 8^{i}
Xylan				
P	5744 ± 270^{b}	2700 ± 92^{bc}	453 ± 23^{b}	745 ± 23^{b}
M	6234 ± 218^{a}	3032 ± 105^{a}	491 ± 26^{a}	820 ± 34^{a}
α-Cellulose				
P	1500 ± 64^{k}	660 ± 33^{k}	92 ± 5^{k}	186 ± 8^{hi}
M	1739 ± 73^{j}	792 ± 45^{j}	114 ± 4^{ijk}	203 ± 8^{h}
P. maximum				
P	2325 ± 93^{i}	1023 ± 45^{i}	212 ± 10^{f}	288 ± 14^{g}
M	2667 ± 112^{h}	1150 ± 52^{h}	238 ± 9^{h}	317 ± 13^{g}
LSD ($P = 0.05$)	H.S.	H.S.	H.S.	H.S.

Values are means of three sets of replicates. Within columns, values with different superscript letters differ significantly ($P = 0.05$). The effect of treatments on all kinetic parameters is highly significant (H.S.), as determined by DMRT using two-factor factorial statistical analysis.

hemicellulases (Saxena *et al.*, 1995). Studies performed to measure the potential of *C. biazotea* and its mutant derivative, to produce β-xylosidase during growth in culture media containing different substrates (Table 3) indicated that xylan was a significantly ($P \leq 0.05$) better carbon source to support maximum β-xylosidase in both wild parent and its mutant derivative. The medium containing an LC substrate was a better stimulator of this enzyme than either cellobiose, or purified cellulosic substrate. During growth on different substrates, approximately 2–2.5-fold higher levels of product yield coefficients ($Y_{p/s}$ and $Y_{p/x}$) were observed in the mutant compared with non-mutated cells (Table 3). The values of the operational kinetic parameters obtained for β-xylosidase in *C. biazotea* and its mutant are several-fold higher than the calculated values reported by other workers on *Cellulomonas* spp. and their mutants (Rodriguez *et al.*, 1985; Rapp and Wagner, 1986) and for other bacteria (Saxena *et al.*, 1995; Nanmori *et al.*, 1990). *C. biazotea* produced 417 IU/g cells with maximum Q_p of 30.8 IU/l/h and the mutant was 2-fold improved for production of this enzyme. These levels of productivity and product yield coefficients are several-fold higher than those displayed by a well-documented mutant of *Cellulomonas* CS1-17 (Rickard *et al.*, 1981), *Clostridium cellulolyticum* (Saxena *et al.*, 1995), *Bacillus stearothermophilus* (Nanmori *et al.*, 1990) and comparable with that produced by wild fungal strains (Milagres *et al.*, 1993). The q_p values of parent and its mutant derivative on xylan medium were 62 and 140 IU/g cells/h, respectively and were several-fold improved over *Penicillium wortmanni* (1.1 IU/g cells/h), *A. awamori* mutant ANNTG43 (2.7 IU/g cells/h), *A. niger* mutant (1.0 IU/g cells/h) and *T. reesei* mutant QM 9414 (2.7 IU/g cells/h) (Smith and Wood, 1991).

During growth of the organisms on different cellulosic and LC substrates, reducing sugars accumulated slowly in the growth medium as unmetabolized substances and induced xylanases. *C. biazotea* released relatively less reducing sugars (Table 1) from some LC substrates and secreted the highest amount of xylanases, while from some other substrates it released the highest amount of sugars, and synthesized the lowest amount of xylanases (Table 2 and Table 3), e.g., *P. maximum*. The mutant derivative accumulated significantly different amounts of reducing sugars compared with wild parent but synthesized higher amounts of xylanases. It was concluded that higher levels of sugars in the fermentation mash might have repressed biosynthesis of xylanases in the wild parent. A mutant strain of *Cellulomonas* accumulating more reducing sugars compared with the parent and producing more enzymes has been also reported by Haggett *et al.* (1978).

Table 3. Potential of *C. biazotea* (P) and its mutant derivative (M) for β-xylosidase production from different substrates measured as process product yield [$Y_{p/x}$ (IU/g cells) and $Y_{p/s}$ (IU/g substrate)], enzyme productivity, Q_p (IU/l/h) and q_p (IU/g cells/h) by growing on different substrates in Dubos culture medium at 30°C

Growth substrate and organism	β-Xylosidase production profiles of *C. biazotea* and its mutant derivative measured as			
	$Y_{p/x}$	$Y_{p/s}$	Q_p	q_p
Bagasse				
P	240 ± 10^{h}	106 ± 5^{g}	16.6 ± 1^{g}	29.8 ± 3^{h}
M	492 ± 20^{c}	245 ± 10^{c}	34.4 ± 3^{d}	61.6 ± 4^{c}
K. grass straw				
P	266 ± 13^{g}	122 ± 7^{f}	19.0 ± 1^{g}	34.3 ± 2.7^{g}
M	559 ± 28^{b}	296 ± 13^{b}	54.4 ± 2.7^{b}	72.3 ± 3.2^{b}
Dhancha straw				
P	174 ± 81^{j}	82 ± 4^{h}	13.0 ± 0.7^{hi}	22.2 ± 2.1^{i}
M	365 ± 17^{f}	190 ± 9^{de}	32.0 ± 2^{e}	47.1 ± 3.4^{e}
Wheat straw				
P	160 ± 6^{j}	97 ± 3^{g}	11.3 ± 0.6^{ij}	21.0 ± 1.4^{i}
M	360 ± 12^{f}	253 ± 11^{c}	30.4 ± 2^{e}	48.3 ± 2.3^{de}
Cellobiose				
P	6.60 ± 0.3^{l}	3.3 ± 0.2^{j}	0.5 ± 0.1^{k}	1.7 ± 0.2^{k}
M	16.3 ± 1^{l}	8.5 ± 0.3^{j}	1.4 ± 0.3^{k}	4.1 ± 0.4^{k}
Xylan				
P	417 ± 18^{d}	195 ± 8^{d}	30.5 ± 2.5^{e}	62.0 ± 3.1^{c}
M	842 ± 29^{a}	437 ± 14^{a}	83.0 ± 3.2^{a}	140.0 ± 5.1^{a}
α-Cellulose				
P	190 ± 8^{i}	87 ± 4^{h}	14.0 ± 1^{k}	23.6 ± 2.1^{i}
M	389 ± 18^{e}	183 ± 7^{e}	37.0 ± 2.1^{c}	51.1 ± 3.1^{d}
P. maximum				
P	140 ± 6^{k}	60 ± 3^{i}	9.3 ± 1^{j}	17.4 ± 1.1^{j}
M	158 ± 6^{jk}	78 ± 4^{j}	25.4 ± 2.1^{f}	40.0 ± 3.1^{f}
Significance ($P = 0.05$)	H.S.	H.S.	H.S.	H.S.

Values are means of three sets of replicates. Within columns, values with different superscript letters differ significantly ($P = 0.05$). H.S., Highly significant.

Alteration in either cell wall synthesis, protein synthesis, or cell membrane permeability is a common mechanism of resistance to antibiotics. Such permeability changes may sometimes lead to increased production, presumably through an increased rate of product export from the cell (Nanmori *et al*., 1983) in case of extracellular enzymes. Nanmori *et al*. (1983) isolated a rifampin-resistant mutant of *B. cereus* which was capable of producing 4-fold more β-amylase.

It is conceivable that like the parental strain, the mutant derivative can effectively utilize cellulosic and LC substrates, continue to grow, accumulate β-xylosidase and secrete endoxylanase in the medium. Furthermore, the extent of growth of the parent and the mutant derivative was almost identical from different substrates (Fig. 1, Table 1). The amount of inducers produced in the culture medium during fermentation, and the productivity values of cellular and extracellular proteins biosynthesis were significantly ($P \leq 0.05$) different from the wild parent and supported greater amounts of β-xylosidase and endoxylanase in the mutant, as reported by Gadgil *et al*. (1995). There was less enhancement in xylanase activities but greater enhancement in β-xylosidase activity. This was in contrast to the work of Gadgil *et al*. (1995), who observed enhanced endoglucanase and FPase but no enhancement in β-glucosidase activity. These results are, however, in good agreement with Allen and Roche (1989) that in the case of xylanase, mutation may not affect the xylanase genes directly but is likely to affect control over synthesis and secretion of the protein. However, there was greater enhancement in β-xylosidase productivity (2–2.5-fold enhancement) which in this respect was in greater agreement with the work of Nanmori *et al*. (1983), who observed 4-fold improved production of β-amylase in Rf^r mutants of a *Bacillus* sp. Therefore, it is conceivable that higher levels of β-glycosidases in the periplasmic fraction may be due either to the increase in copy number of the genes for β-xylosidase or the efficiency of enzyme synthesis from this, or both have been altered in the mutant derivative, while in the case of xylanase, only the gene expression in the mutant has been altered.

In conclusion, a strain of *Cellulomonas* with clearly improved xylanase and β-xylosidase enzyme profiles has been isolated through mutation. Further studies on optimizing the overproduction of extracellular xylanase and cell-associated β-xylosidase are underway. *C. biazotea* mutant strain also hyperproduced hemicellulase A (1.5-fold) and hemicellulase B (1.26-fold), arabinase (1.35-fold) and α-arabinofuranosidase (1.8-fold), α- and β-galactosidases (2.35 and 2.15-fold, respectively), α- and β-mannosidases (1.5- and 2.0-fold respectively), α- and β-glucosidases (2.24- and 2.13-fold). Xylanases in the presence of all these enzyme activities can help in biobleaching of pulps. *C. biazotea* strains also produced cellulases and for application in this process cellulase-free xylanases are desired. Mutation and selection programs may give the desired organism with inactivated cellulase genes and hyper-producing xylanase genes. Production of xylanase from a xylanase gene cloned in a non-cellulolytic host is another means of achieving cellulase-free xylanase preparations, as has been recently achieved by cloning a xylanase gene in a cellulase-less strain of *Streptomyces halstedii* (Ruiz-Arribas *et al*., 1995).

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