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γ-Ray Induced Mutagenesis of *Cellulomonas biazotea* for Improved Production of Cellulases

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ABSTRACT. A rifampin-resistant mutant of *Cellulomonas biazotea* secreted elevated levels of cellulases *in vivo*. The cellulase production in the mutant was not inhibited in the presence of 5 % glucose, cellobiose or glycerol in the solid medium. The mutant exhibited approximately two- to three-fold enhanced product yields and productivity of cellular β-glucosidase over the wild parent in shake-flask culture studies when grown on either cellulosic or lignocellulosic substrates. Extracellular production of filter paper cellulase (FPase) and endo-glucanase (CMCase) were also significantly ($p \leq 0.05$) altered. During growth of the mutant on α-cellulose, the maximum volumetric productivities for CMCase, FPase and β-glucosidase were 52, 23.3, and 15.2 IU L^{-1} h^{-1}, *i.e.* 118, 121, and 229 % their respective values for the parental strain. Some enzyme properties of the mutant cellulases were altered. Mutant-derived cellulases produced higher yields of glucose arising by degradation of bagasse, wheat straw, and α-cellulose (1.53-, 1.57-, and 1.75-fold, respectively).

Under appropriate conditions, many cellulolytic organisms produce cellulases which perform cellulolysis necessary for cell growth and product formation (Beguin 1990). Cellulases, *e.g.* endo-glucanase (EC 3.2.1.4), exo-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21), which act in synergism, are used industrially to convert cellulose from abundantly available lignocellulosic (LC) biomass into glucose (Cusky *et al.* 1983). They should be present in a right proportion to cause effective saccharification (Warzywoda *et al.* 1992). Glucose can either be converted to fructose (sweeteners) by glucose isomerase, or to ethanol which can be used as a fuel. In this process, β-glucosidase catalyzes the conversion of cellobiose into glucose, removes the feed-back inhibition of other cellulases, provides an inducer for cellulase production and also increases saccharification of cellulosic substrates during the hydrolysis (Esen 1993). Similarly, it has been observed that β-glucosidase deficiency may reduce the saccharification rate of LC substrates (Latif *et al.* 1994).

The cellulase system of *Trichoderma reesei* has received the greatest attention as a tool for enzymic hydrolysis of cellulosic residues. Commercial extracellular cellulase preparations of *T. reesei* contain high amounts of exo- and endo-glucanases; many such preparations are deficient in β-glucosidases (Esen 1993; Gadgil *et al.* 1995) and may produce inhibitory amounts of cellobiose during saccharification of cellulosic biomass. Supplemental β-glucosidase from *Aspergillus* cultures improves saccharification (Breuil *et al.* 1992). Other sources of β-glucosidase are therefore being sought. Production of cellulases is regulated by repression and induction (Bahkali 1995; Gadgil *et al.* 1995) and the amount of cellulases produced per mg protein, very low compared with other hydrolases, needs improvement by mutagenesis. Hypersecretive mutants of different organisms have been obtained (Gadgil *et al.* 1995) but the amount of cellulases produced is still not sufficient for effective industrial saccharification.

Cultures of *C. biazotea* secrete all enzymes of the cellulase complex; however, the ratio of filter paper-cellulase (FPase) to β-glucosidase activities is 1.5 : 1.0 (Rajoka and Malik 1997), while the most appropriate ratio for effective saccharification is 1 : 1 (Breuil 1992). The approach used here has been used to improve the yield of β-glucosidase and other cellulases produced by *C. biazotea* by using a drug resistant and derepressed mutant with induction of drug and 2-deoxy-D-glucose resistance (DG^r) in the wild parent following γ-ray induced mutagenesis. Such mutation may yield strains capable of hyperproducing all components in the cellulase complex and thus enhancing the saccharification rate of pretreated LC biomass for low-cost large-scale exploitation (Latif *et al.* 1994).

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MATERIALS AND METHODS

Organisms. C. biazotea strain NIAB 442 (Rajoka and Malik 1997) and its mutant derivative were used. The strains were maintained on Dubos salts–agar medium containing (g/L) $K_2HPO_4 \cdot 7H_2O$ 0.5, KCl 1.0, $NaNO_3$ 0.5, $MgSO_4$ 0.5, $FeSO_4 \cdot 7H_2O$ 0.1, yeast extract 2, avicel 10 and agar 25 g (DYEA) plates and slants as described earlier (Rajoka and Malik 1997).

Chemicals. 4-Nitrophenol β-D-glucopyranoside (*p*NPG), Sigmacell-100 (avicel), α-cellulose, cellobiose, carboxymethyl cellulose sodium salt (low viscosity), 2-deoxy-D-glucose, ferric ammonium citrate, and rifampin were purchased from *Sigma* (USA). All other chemicals were of analytical grade.

Substrates. Among numerous lignocellulosic substrates, wheat straw and bagasse are comparable with Sigmacell-100 and filter paper, and better than *Sesbania aculeata* for cellulase production (Rajoka and Malik 1997). They were therefore included in these studies. Wheat straw was a farm produce of *Nuclear Institute for Agriculture and Biology*, Faisalabad. Bagasse was a gift from a local sugar factory. The dried bagasse and wheat straw powder was steam-and-alkali pretreated and contained 88 ± 1.5 and 82 ± 2 % total saccharides, respectively (Latif *et al.* 1994).

Isolation of mutants. C. biazotea cells were cultured in Dubos-yeast-extract–cellobiose (DYEC) culture medium at 30 °C for 20 h, centrifuged (15 000 ***g***, 15 min), and suspended in 50 mL of biological saline containing 0.01 % yeast extract. Cells (at a concentration equal to the absorbance of 0.6 at 610 nm) were dispensed in aliquots in 30 mL McCartney vials. The cells were exposed to different doses of γ-ray in a ^{60}Co irradiator. The exposure of cell suspension (2×10^9 cells per mL) to γ-irradiation of 400 and 500 Gy gave approximately a 3 log kill (Choi *et al.* 1978). Cells resistant to these γ-ray doses were grown to an absorbance of 0.9 in the presence of 20 μg/mL rifampicin (Rf) + 0.6 % DG medium. This treatment served us to isolate Rf^r and simultaneously derepressed mutants (cf. Montenecourt and Eveleigh 1979).

The resulting cells were serially diluted and plated on DYE–esculin–ferric ammonium citrate–DG–Rf, DYE–CMC–DG–Rf or DYE–Sigmacell 100–DG–Rf selection plates to obtain approximately 30 colonies per plate. Overall, 4200 different colonies were screened, larger ones systematically and smaller ones randomly for mutant selection. The isolated colonies were subsequently replica-plated on esculin + DG (0.6 %, *W/V*), esculin + Rf + DG, *p*NPG + Rf + DG, CMC + Rf + DG or Sigmacell 100 + DG + Rf agar plates. Each colony was tested for hyperproduction of β-glucosidase *in vivo* by measuring the diameter of the blackening zone. Colonies showing larger blackening zones were picked or *p*NPG plates were spread with carbonate buffer, 200 mmol/L (pH 8.5). The colonies surrounded by a yellow halo were picked and screened by measuring the diameter of the surrounding yellow halo. Each colony was also tested for endo-glucanase production in plate tests after Teather and Wood (1982) and for clearance on Sigmacell 100 plates. Among 3 mutants, one mutant designated $7Rf^r$ produced a larger zone of CMC hydrolysis, visualized as a yellow zone when stained with Congo red followed by NaCl treatment, a greater zone of Sigmacell clearance and a larger zone of blackening on esculin plates, or yellow halo on *p*NPG plates. The enzyme secretion was tested in the presence of increasing concentration (2, 4, 5, 6, 8, 10 %, *W/V*) of glucose, cellobiose or glycerol.

Batch-culture studies. The ability of the mutant to utilize bagasse, and wheat straw for improved production of cellulases relative to that achieved on α-cellulose and cotton wool was examined in basal Dubos salts medium containing 0.2 % yeast extract as described earlier (Rajoka and Malik 1997). Carbon sources were added individually to batches of the basal medium to give a saccharide level of 10 g/L. All media were adjusted to pH 7.3 with 1 mol/L NaOH or 1 mol/L HCl and were dispensed in 200 mL aliquots into 1-L Erlenmeyer flasks in triplicate.

The time course of cellulase production in shake-flask batch cultivations in above media was studied out at 30 °C on a gyratory shaker at a shaking frequency of 1.7 Hz. Sample flasks in triplicate were withdrawn at preset time intervals and processed. Cell growth, protein production and enzyme activities present in the extracellular or cellular fraction were assayed. When the test organism was grown on insoluble substrates, the culture medium after growth was centrifuged (10 min, 4000 ***g***) to remove the substrate. The residue was shaken with chilled water containing 1 % (*V/V*) Tween 80 for 30 min at 4 °C and clear supernatant was obtained by centrifugation. Further fractions were collected until no enzyme activity was observed in the supernatant. All washings were pooled for determining enzyme activities and compensated for the adsorbed portion of FPase and endo-glucanase. The washed substrate was oven-dried to constant mass for further processing.

The amount of growth was measured gravimetrically as dry cell mass. For this purpose, 50 mL portion of the cell suspension was also centrifuged (15 000 ***g***, 30 min). The cell-free supernatant was preserved for enzyme assays and cell pellet was washed twice with saline, suspended in 10 mL distilled

water and dried at 70 °C to constant mass. Clear supernatant from 100 mL original culture broth was obtained by centrifugation (15 000 *g*, 30 min). The cell pellet was used to extract cellular fractions as described earlier (Rajoka and Malik 1997). The enzyme activity present in the cell-free supernatant or cell extract was assayed as an indicator of improved enzyme production.

Enzyme assays. For cellulolytic enzyme assays, an appropriately diluted culture supernatant or cell extract was incubated with carboxymethyl cellulose (CMC), the substrate for endoglucanase (CMCase) activity, or Whatman no. 1 filter paper for combined exoglucanase and endoglucanase activity, in 0.2 mol/L acetate buffer (pH 7) at 40 °C for 30 min (Rajoka and Malik 1997). One unit of enzyme activity is defined as the amount of enzyme which releases 1 μmol reducing sugars (measured as glucose) per mL per min. β-Glucosidase activity was determined using *p*NPG as substrate (Rajoka and Malik 1997). One unit of enzyme activity is defined as the amount of enzyme which releases 1 μmol 4-nitrophenol per mL per min. The protein was determined according to Bradford (1976) using bovine serum albumin as standard.

Determination of kinetic parameters. The procedures given by Pirt (1975) were adopted for determining kinetic parameters for the batch fermentation process. Dry cell mass (g/L) of *C. biazotea* cultures after growth on different carbon sources was determined in triplicate samples as described earlier and residual saccharides (g/L) were determined on dry mass after Shirlaw (1969) after three washings of the substrate from low-speed centrifugation. Enzyme activities (products per L) were determined as described above. The "growth yield coefficient" ($Y_{X/S}$) was calculated as the dry cell mass per mass of saccharide utilized from the test substrates added to the medium. The volumetric rate of substrate utilization (Q_S) and enzyme productivity (Q_P) were determined from the maximum slope in the plot of the substrate and enzyme produced *vs.* time of fermentation. The values of μ were calculated from the slope of $\ln(x)$ *vs.* time of fermentation (Pirt 1975). Cell mass productivity (g dry cells per L per h), intracellular (P_i) or extracellular protein (P_e) productivity (mg protein per L per h) was determined from a plot of dry cell mass per L, intracellular or extracellular protein per L *vs.* time. Specific enzyme activity was obtained by dividing Q_P by P_i or P_e.

Kinetic data for enzyme properties were collected as described for endo-glucanase of *C. biazotea* and *Aspergillus niger* (Siddiqui *et al.* 1997).

Saccharification studies. Portions of 0.5 g of bagasse, wheat straw or α-cellulose were dispensed into 50 mL Erlenmeyer flasks. Ten mL enzyme preparation whose pH was brought to 7 with 1 mol/L HCl containing 0.1 mL of Tween 80 and 0.2 % sodium azide was added to each flask. They were incubated at 40 °C under shaking in a water bath incubator along with 10 mL of the crude enzyme obtained from *C. biazotea* strains growing in 1 % α-cellulose liquid culture and prepared as follows: The intracellular preparations, prepared in one half of the original volume of culture medium, were mixed with 2.5-fold concentrated (by Amicon ultrafiltration unit) extracellular fraction to a concentration of 2.5 mg/mL protein. Both preparations were mixed and the mixture assayed for the desired enzyme concentration in the enzyme systems of cellulases produced by non-mutated or mutated strains of *C. biazotea*. Duplicate flasks were harvested periodically and properly diluted aliquots were used to determine reducing sugars using the method with 3,5-dinitrosalicylic acid or glucose by Human glucose kit following the instructions of the suppliers.

RESULTS AND DISCUSSION

Production of glucose from cellulose requires a high concentration of the enzyme. Concentrated cellulases from *C. biazotea* saccharified 5 % bagasse and wheat straw and the liberated sugars were fermented to ethanol in 80 % theoretical yield using *Saccharomyces carlsbergensis* as the fermentative organism (Rajoka 1990). The yield was so high because the crude enzyme mixture possessed the desired ratio of 1 : 1 of filter paper-cellulase and β-glucosidase (Breuil *et al.* 1992). To improve this ratio in the unconcentrated crude extracts, the enzyme secretion by γ-ray mutagenesis was enhanced as described in *Materials and Methods*. These studies indicated that well-developed zones of clearance on Sigmacell 100–agar plates, bigger blackening zones on esculin–agar plates/bigger yellow zones on *p*NPG-agar plates, and larger hydrolysis zones on CMC–agar plates appeared around 3 colonies in the case of mutant strains. In semiquantitative plate studies after Montenecourt and Eveleigh (1979), one derivative capable of producing the largest amount of β-glucosidase, FP-ase and endo-glucanase was isolated and designated $7Rf^r$ for enzyme production studies *in vitro*. The organism was recovered from replica plate and its endo-glucanase, FP-ase and β-glucosidase activities were monitored in plate tests

in the presence of increasing concentrations of glucose, cellobiose and glycerol. The secretion of cellulases was the least affected in the presence of up to 5 % glucose, cellobiose or glycerol in the medium.

Initial studies of cellulase regulation in *C. biazotea* indicated that easily metabolizable substrates repressed the cellulase synthesis (Rajoka and Malik 1997). These studies indicated that addition of dimeric and polymeric saccharides stimulated cellulase secretion.

Time course of cellulase production and substrate utilization. The use of purified cellulosics as substrates is uneconomical for large-scale production of cellulases. Two natural agricultural residues were therefore evaluated for improved production of cellulases by the mutant of *C. biazotea*. For assessing the time course of cellulase production and substrate utilization in shaker flasks, *C. biazotea* and its mutant were grown in Dubos salts-culture media containing NaOH-pretreated ground plant material of two agricultural residues, *i.e.* bagasse, and wheat straw, and the data were compared with those obtained on α-cellulose and cotton wool. All enzymes were produced using 1 % saccharides present in the substrate or equivalent saccharides as described in *Materials and Methods*. Enzyme production, cell mass biosynthesis and substrate utilization kinetics under the conditions of shaker-flask cultures are shown in Figs 1 and 2. All carbon sources supported rich growth of the test organisms. Maximum cell mass was attained on α-cellulose and cotton wool. Overall, the growth yield coefficient in the mutant was insignificantly ($p \leq 0.05$) higher than the parental cultures from all substrates (Table I). Cellular and extracellular protein productivities on LC biomass were also reasonably high and were significantly ($p \leq 0.05$) different in the mutant during growth on different carbon sources (Table I). Bagasse and wheat straw as well as α-cellulose were easily degraded. Thus, the Q_S of bagasse was 1.045 g L^{-1} h^{-1} with cell mass productivity of 0.24 g cells per L per h, *i.e.* values comparable to those from an easily metabolizable substrate, cellobiose, which gave Q_S of 1.017 g L^{-1} h^{-1} and cell mass productivity of 0.23 g cells per L per h (Rajoka and Malik 1997). Enhanced substrate consumption and cell mass formation rates by *Cellulomonas* strains could lead to increased productivities in commercial cellulase production from cellulosic residues available in Pakistan.

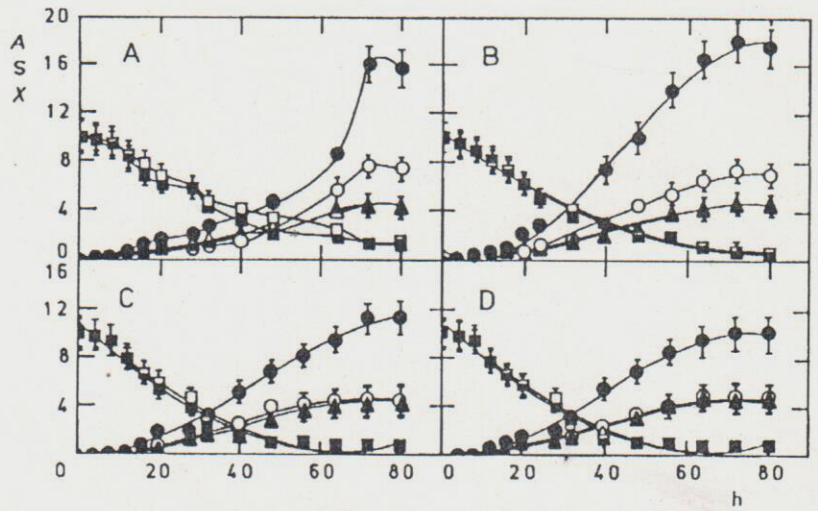

Fig. 1. β-Glucosidase and cell mass production kinetics of *C. biazotea* and its mutant 7Rf^r during fermentation of α-cellulose (**A**), bagasse (**B**), cotton wool (**C**), and wheat straw (**D**) in shaken-flask cultures; β-glucosidase production (*A*, IU per 0.1 g S) by parent (*open circles*) and mutant (*closed circles*); cell mass production (*X*, g/L) by parent (*open triangles*) and mutant (*closed triangles*); substrate consumption (S, g/L) by parent (*open squares*) and mutant (*closed squares*). The *error bars* show standard deviation among three replicates.

All three components of the cellulase complex of *Cellulomonas* were produced when 1 % carbon sources in the DYE-basal medium were added. Increased β-glucosidase (Fig. 1), FP-ase and endoglucanase production (Fig. 2) was observed in the case of the mutant. The enzyme production thus appeared to be growth- as well as non-growth-associated. In the present study, the wild parent and its mutant derivative grown on all substrates exhibited a lag phase of 8 h before cellulase activities increased appreciably up to a maximum after 64–72 h incubation (Fig. 1, 2). Since cellulases act synergistically, the presence of each enzyme component at optimal activities would give maximum saccharification (Warzywoda *et al.* 1992).

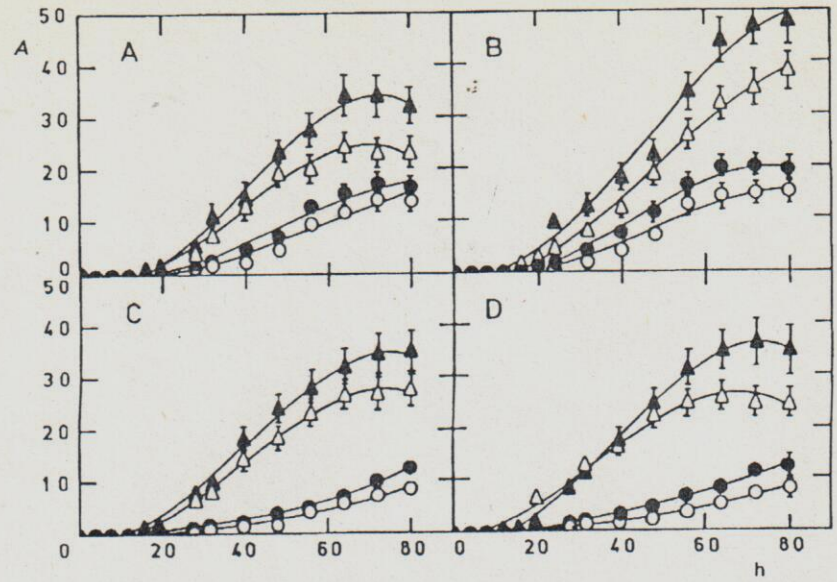

Fig. 2. FP-cellulase and carboxymethylcellulase (CMCase) production kinetics of *C. biazotea* and its mutant 7Rf^r during fermentation of α-cellulose (**A**), bagasse (**B**), cotton wool (**C**), and wheat straw (**D**) in shaken-flask cultures; FP-ase production (*A*, IU per 0.1 g S) by parent (*open circles*) and mutant (*closed circles*); CMCase production (*A*, IU per 0.1 g S) by parent (*open circles*) and mutant (*closed circles*); CMCase production (*A*, IU per 0.1 g S) by parent (*open triangles*), and mutant (*closed triangles*). The *error bars* show standard deviation among three replicates.

Table I. Comparison of substrate utilization parameters during growth of *Cellullomonas biazotea* (P) and its mutant (M) on different substrates*

Growth substrate		$Y_{x/s}$ g/g	Q_s g L^{-1} h^{-1}	P_c mg L^{-1} h^{-1}	P_i mg L^{-1} h^{-1}	RS mg/L
α-Cellulose	P	0.50^{a}	0.144^{ab}	128^{bc}	106^{b}	82^{bcd}
	M	0.54^{a}	0.163^{a}	109^{e}	134^{a}	78^{de}
Bagasse	P	0.49^{a}	0.148^{ab}	112^{de}	103^{b}	80^{cde}
	M	0.51^{a}	0.147^{ab}	120^{cd}	113^{b}	74^{e}
Cotton wool	P	0.48^{a}	0.143^{b}	129^{bc}	109^{b}	94^{a}
	M	0.49^{a}	0.143^{b}	109^{e}	134^{a}	89^{ab}
Wheat straw	P	0.48^{a}	0.143^{b}	132^{ab}	128^{a}	87^{abc}
	M	0.50^{a}	0.145^{b}	141^{a}	138^{a}	78^{de}
Significance ($p \leq 0.05$)		NS	S	HS	HS	HS

*Means of three sets of replicates. Within columns, values with different superscripts differ significantly ($p \leq 0.05$); RS, reducing sugars (mg/L); NS, nonsignificant; S, significant; HS, highly significant.

Production of β-glucosidase. Potential kinetic parameters for the production of β-glucosidase in the mutant of *C. biazotea* during growth in different culture media (Table II) indicated that α-cellulose supported 2.37-fold higher volumetric productivity than in the parental strain. During growth on different substrates, approximately 2.0−2.5-fold higher product yield coefficient ($Y_{p/x}$) was observed in the mutant compared to non-mutated cells. The effect of treatments such as mutagenesis and substrates on β-glucosidase production was highly significant (Table II). The values of the operational kinetic parameters obtained for β-glucosidase in *C. biazotea* mutant derivative is several-fold higher than the values reported by other workers on *Cellulomonas* spp. and their mutants (Rickard *et al.* 1981), other bacteria (Okeke and Paterson 1992) and fungi (Gadgil *et al.* 1995; Busto *et al.* 1996), and β-glucosidase from recombinant *E. coli* supplied by *Sigma* (USA). The ideal ratio of 1:1 between FP-ase and

β-glucosidase was also achieved in the mutant. In the mutant strain, β-glucosidase accumulated intracellularly on all substrates and was mainly located in the periplasmic fractions of the cells like in the parental strain (Rajoka and Malik 1997).

Table II. Potential of *C. biazotea* (P) and its mutant (M) for production of cellulases from different substrates measured as process product yield, $Y_{p/x}$ (IU/g cells), and enzyme productivity, Q_p (IU $L^{-1} h^{-1}$). Cells were grown on different substrates in Dubos culture medium at 30 °C*

Growth substrate		FP-cellulase		CMCase		β-Glucosidase	
		$Y_{p/x}$	Q_p	$Y_{p/x}$	Q_p	$Y_{p/x}$	Q_p
α-Cellulose	P	383^{b}	20.0^{b}	869.3^{bc}	37.8^{b}	150.7^{c}	6.4^{d}
	M	430^{a}	23.3^{a}	1026.0^{a}	52.1^{a}	346.3^{a}	15.2^{a}
Bagasse	P	200^{f}	8.93^{f}	654.0^{de}	24.4^{e}	111.0^{d}	3.6^{e}
	M	290^{d}	12.3^{e}	770.3^{cd}	27.3^{d}	281.0^{b}	8.5^{c}
Cotton wool	P	292^{d}	15.53^{d}	501.7^{f}	28.6^{d}	143.0^{c}	6.4^{d}
	M	360^{c}	18.77^{c}	906.2^{b}	31.2^{c}	331.3^{a}	13.0^{b}
Wheat straw	P	160^{e}	9.70^{f}	608.0^{ef}	21.4^{f}	105.3^{d}	3.6^{e}
	M	360^{c}	14.97^{d}	743.0^{d}	24.1^{e}	285.7^{b}	8.2^{c}
Significance ($p \leq 0.05$)		HS	HS	HS	HS	HS	HS

*Means of three sets of replicates. Within columns, values with different superscripts differ significantly ($p \leq 0.05$); HS, highly significant.

Production of FP-cellulase. The FP-cellulase activities (Table II) accumulated in the culture supernatants. During growth on α-cellulose culture medium, $Y_{p/x}$, and Q_p values for FP-ase were 1.18- and 1.20-fold higher in the mutant. The values of $Y_{p/s}$ (Fig. 2) were in the range of literature data for *T. reesei* mutants (40–168 FPU/g substrate; Persson *et al.* 1991). *C. biazotea* mutant derivative exhibited higher enzyme production parameters compared with all other *Cellulomonas* strains or their mutants (Choi *et al.* 1978), other bacteria (Okeke and Paterson 1992), and fungi such as *T. reesei* Rut C 30 (Reczey *et al.* 1996), and *T. reesei* mutant (Gadgil *et al.* 1995). The specific activity in the mutant was several-fold higher than that produced by *Aspergillus niger*, *T. reesei*, *T. viride* and *Penicillium funiculosum*, supplied by *Sigma* (USA).

The CMCase activities produced by the mutant cultures also accumulated in the culture media. Results on CMCase production (Fig. 2) revealed that *C. biazotea* and its mutant produced the maximum of the enzyme on α-cellulose. *C. biazotea* mutant CM-cellulases exhibited 1.2-fold improved Q_p, and $Y_{p/x}$ equal to that of the parental strain (Table II). The maximum Q_p of our *C. biazotea* mutant (Table II) was several-fold higher than the levels found in some other organisms (Okeke and Paterson 1992; Gadgil *et al.* 1995; Busto *et al.* 1996) and compared favorably with values for *Cellulomonas* CS1-1 and its mutants (Choi *et al.* 1978).

The amount of inducers produced in the culture medium during fermentation, and the extent of cellular and extracellular protein biosynthesis in the mutant were significantly ($p \leq 0.05$) different from those of the parent strain (Table I) and supported significantly ($p \leq 0.05$) greater amounts of β-glucosidase, FP-ase and CM-cellulase in the mutant. The 2- to 2.5-fold enhancement in β-glucosidase productivity was in agreement with the results of Nanmori *et al.* (1983) who observed a 4-fold improved production of β-amylase in a Rf^r mutant of *Bacillus* sp. Similarly, DG^r mutants of *T. reesei* have been found to hyperproduce cellulases (Montenecourt and Eveleigh 1979).

Enzyme properties of the mutant cellulases were compared with those obtained by ammonium sulfate precipitated endo-glucanases and β-glucosidases, purified by ion exchange and by gel filtration. The values of specific activities of endo-glucanase at the end increased from 2.25 to 30.4 in parent and from 8.12 to 27.4 for the mutant. Similarly, specific activity of β-glucosidase rose from 0.87 to 8.67 in parental cultures while in mutant it increased from 1.67 to 21.0. The native molar mass of endoglucanases from *C. biazotea* and its mutant, as determined by gel filtration column, was 61.66 and 27.54 kDa, respectively, while for β-glucosidase it was 81–230 kDa and remained unaltered in the

mutant. Subunit M was also the same (90–110 kDa). In the mutant, post-translational modification was altered for endo-glucanase production: The parent produced 3 active bands while the mutant produced 5 active bands of endo-glucanase in non-destructive polyacrylamide gel electrophoresis. At the incubation temperatures of 64 and 63 °C or 45.8 and 45.6 °C, endoglucanase and β-glucosidase from the parent strain and the mutant lost half of their activities respectively while optimum temperature for both endoglucanases and β-glucosidases was 40 °C.

The endoglucanases, FP-cellulases and β-glucosidases from the parent strain and the mutant showed broad pH optima ranging from 5.0 to 7.4. Using increasing amounts of carboxymethyl cellulose or *p*NPG as substrates, the K_m and V_{max} values of native and mutant endoglucanases and β-glucosidases from *C. biazotea* at 40 °C were 6.4 % and 8.56 μmol mL^{-1} min^{-1} (for the parent strain) and 16 % and 20 μmol mL^{-1} min^{-1} (for the mutant), respectively, for endo-glucanase, and 4.33 % 0.85 μmol mL^{-1} min^{-1} and 4.03 and 2.41 μmol mL^{-1} min^{-1}, respectively, for β-glucosidase. The influence of metal ions on endoglucanases and β-glucosidase from the *C. biazotea* mutant appeared to exhibit same behavior as observed for the enzyme from the parent strain (Siddiqui *et al.* 1997). The half-lives of the enzymes from mutant also remained unaltered.

In vitro saccharification studies showed that the enzyme systems produced by *C. biazotea* and its mutant derivative on α-cellulose saccharified wheat straw and bagasse more rapidly and to a greater extent but degraded α-cellulose to a lesser extent (Table III). Glucose was found to be a major component of hydrolyzate resulting from the action of the mutant cellulases. Since β-glucosidase level was higher in the cellulase system derived from the mutant, there was hyperproduction of glucose because the cellobiose produced was completely hydrolyzed to glucose. The yield of glucose from different substrates was higher than or favorably comparable with that reported by other workers (Latif *et al.* 1994; Warzywoda *et al.* 1992). Cellulases are prone to end-product inhibition by cellobiose and glucose. Cellulases from the mutant were not inhibited by free glucose or cellobiose up to 5 % in the assay or saccharification medium. At higher concentrations (10 %), although enzyme activities were inhibited in both cases to a certain extent, the inhibitory effect was less pronounced in the mutant-derived enzymes.

Table III. Reducing saccharides (RS) and glucose (% of total saccharides present in the substrate) released from different substrates using enzymes from *C. biazotea* (P) and its mutant (M)

Substrate		RS	Glucose
α-Cellulose	P	63 ± 9^{b}	40 ± 5^{c}
	M	78 ± 11^{ab}	69 ± 8^{ab}
Bagasse	P	79 ± 10^{ab}	49 ± 5^{bc}
	M	90 ± 9^{a}	73 ± 6^{a}
Wheat straw	P	82 ± 7^{ab}	53 ± 4^{abc}
	M	88 ± 7^{a}	76 ± 6^{a}
Significance ($p \leq 0.05$)		HS	HS

*Means of three sets of replicates ± SD. Within columns, values with different superscripts differ significantly ($p \leq 0.05$); HS, highly significant.

γ-Ray induced mutagenesis yielded a stable and viable mutant for hyperproduction of β-glucosidase, endo-glucanase and FP-ase *in vivo*, which has been confirmed by *in vitro* production from different carbon sources. The extent of the overall increase in β-glucosidase productivities and product yield coefficients was 2- to 2.45-fold compared with that in the wild-type parent strain from which this was derived. This enhancement may have been due either to an increase in the gene copy number or to improvement in gene expression, or both. The mechanism underlying this hypersecretion is of paramount significance and neeeds further study. Mutant-derived cellulases are less inhibited by cellobiose or glucose and release more glucose during saccharification.

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