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CELLULASE PRODUCTION BY *CELLULOMONAS BIAZOTEA* CULTURED IN MEDIA CONTAINING DIFFERENT CELLULOSIC SUBSTRATES

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Abstract

Production of filter paper cellulase (FPase), endo-β-glucanase and β-glucosidase by Cellulomonas biazotea *was investigated during growth on different substrates. The organism utilized four different cellulosics, NaOH-pretreated ground plant material of four lignocellulosic (LC) substrates grown on saline lands, three agricultural wastes, carboxymethyl cellulose (CMC), cellobiose and xylan as carbon sources in Dubos salts liquid medium and produced the enzymes. The highest level of volumetric productivity (Q_p) of FPase occurred in the cell-free supernatants of* C. biazotea *during growth on α-cellulose followed by* Leptochloa fusca *(kallar grass), while that of endo-β-glucanase occurred on kallar grass followed by α-cellulose. Maximum β-glucosidase was produced in culture media containing cellobiose and kallar grass as carbon sources. Thus the production of these enzymes is influenced by the carbon source used. β-Glucosidase was produced mainly periplasmic and was several fold greater in quantity than that reported in other strains of* Cellulomonas, *as well as other bacteria. Kallar grass culture medium, during growth of* C. biazotea, *supported maximum Q_p levels of 37.5, 17.5 and 6.1 IU/l/h for CMCase, FPase and β-glucosidase, respectively, with cell mass productivity of 0.235 g/l/h and was selected as a preferred substrate for cellulase production.* © *1997 Elsevier Science Ltd. All rights reserved.*

Key words: Endoglucanase, β-glucosidase, FP-cellulase, *Cellulomonas*, cellulose, lignocellulose, kallar grass straw.

INTRODUCTION

Photosynthesis is the most efficient method of harnessing solar energy. The global production of LC material from the land is 120–150 billion tons dry matter/annum, some 30 billion tons oil equivalent (TOE), or more than four times the world's yearly total energy consumption. Therefore, cellulose can be regarded as the most abundant and biologically renewable resource for bioconversion. Utility of cellulosics can be tremendously increased if these are first hydrolysed chemically or enzymatically to glucose and other soluble sugars which can be subsequently used for making sweeteners, single-cell protein (SCP), energy materials (alcohols) or other fermentation products. Increasing knowledge on the mode of action of cellulases and their recent applications (Kubicek *et al.*, 1993) has greatly increased the prospects of enzymatic hydrolysis over chemical processes, because of its potentially high saccharification efficiency (Latif *et al.*, 1988, 1994; Cochet, 1991) and avoidance of pollution (Kubicek *et al.*, 1993).

For utilization of cellulosic substances as carbon and energy sources, many fungi and bacteria secrete enzymes of the cellulase complex, namely carboxymethyl cellulases (CMCase, endo-β-1,4-glucanases, EC 3.2.1.4), cellobiohydrolases (exo-β-1,4-glucanases, EC 3.2.1.91) and β-glucosidases (cellobiases, EC 3.2.1.21) (Beguin, 1990; Bayer & Lamed, 1992; Kubicek *et al.*, 1993). Among fungi, *Trichoderma reesei* has been most extensively studied (Persson *et al.*, 1991; Goyal *et al.*, 1991) but some of its preparations have low activity of β-glucosidase and need its supplementation from other sources to increase the rate and extent of cellulose hydrolysis (Duenas *et al.*, 1995). Among bacterial species reported for cellulolytic activity, strains of *Cellulomonas* have been most widely studied for cellulase production (Choi *et al.*, 1978; Kim & Wimpenny, 1981; Stoppok *et al.*, 1982; Rajoka & Malik, 1984a; Langsford *et al.*, 1984; Sami *et al.*, 1988; Nakamura & Kitamura, 1988; Gilkes *et al.*, 1991). Strains of *C. biazotea* have received less attention, though these may secrete considerable amounts of FPase and β-glucosidase (Rajoka, 1990).

The biosynthesis of the cellulase complex is markedly dependent upon the type of inducer used (Mandels, 1982; Nochure *et al.*, 1993). Therefore, by

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conducting systematic screening studies, highly efficient and cheaper inducers for hyper-production of cellulases can be selected. It must be recognized that major cost factors in producing enzymes for commercial use are the carbon source and its pretreatment (Ryu & Mandels, 1980; Marsden & Gray, 1986). These costs are relatively low in the developing countries. Although some authors have used sugarcane bagasse and wheat straw for production of these enzymes (Kim & Wimpenny, 1981; Acebal *et al.*, 1988; Duenas *et al.*, 1995), most investigators have used expensive cellulosic substrates for production of cellulases (Nakamura & Kitamura, 1982; Haggett *et al.*, 1978; Sami *et al.*, 1988).

The amount and the availability of cheap and high activity cellulases are essential elements in the successful enzymatic conversion of cellulosic substrates (Ryu & Mandels, 1980). In the present study, the relationship of substrate and cellulase production was investigated to select the best substrate for active cellulases. For this purpose, cellulase production by *C. biazotea* was studied in media containing different LC substrates grown on saline soils and agricultural residues with reference to pure cellulosics and soluble inducers.

METHODS

Chemicals and substrates

Sigmacell-100 (avicel), CMC, α-cellulose, cellobiose, and p-nitrophenyl-β-D-glucopyranoside (pNPG) were from Sigma Chemical Co., USA. Xylan was from BDH, Australia. All other chemicals were of analytical grade. Kallar grass, *Sesbania aculeata* (dhancha), *Atriplex lentiformis* and *Panicum maximum* were from Biosaline Research substation of Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, near Lahore, where these are grown as energy crops for bioconversion (Latif *et al.*, 1988, 1994). Cotton stalks and wheat straw were collected from NIAB farm. Bagasse was provided by a local sugar factory. The dried powder of LC biomass was pretreated by alkali as described earlier (Rajoka & Malik, 1984a; Latif *et al.*, 1988). The pretreated kallar grass, *P. maximum*, *A. lentiformis*, bagasse, wheat straw, dhancha straw and cotton stalks had 78 ± 2, 81 ± 1.5, 59 ± 1.3, 88 ± 1.5, 80 ± 2, 84 ± 1.2, $83 \pm 1.0\%$ total carbohydrates, respectively, determined using a standard method (Shirlaw, 1969).

Micro-organism

The strain of *C. biazotea*, isolated from a bagasse heap (Rajoka & Malik, 1984b), was maintained on Dubos salt–yeast-extract–avicel or CMC plates and slants after Rajoka & Malik (1984a).

Enzyme production

The ability of *C. biazotea* to utilize the various substrates (Table 1) as sole carbon sources and produce cellulases was studied as follows. The basal liquid medium was optimized Dubos salts medium (Rajoka, 1990). The carbon sources were added individually to batches of culture medium to give a carbohydrate level of 10 g/l. All media were adjusted to pH 7.3 with either N–NaOH or N–HCl, dispensed in 200-ml aliquots into 1-l Erlenmyer flasks and autoclaved. Flasks were then inoculated with an overnight inoculum of 0.6 optical density adjusted at 610 nm. For this purpose, cells were grown on cellobiose in basal medium, washed twice with 0.89% NaCl solution, and used at a concentration of 1% (v/v). The flasks were incubated at 30°C on a gyratory shaker at 100 rpm. Sample flasks in triplicate were removed after predetermined time intervals and processed for assaying growth, protein

Table 1. Comparative study of substrate utilization kinetic parameters during growth of *C. biazotea* on different substrates

Growth substrate	Substrate utilization parameters				
	$Y_{x/s}$ (g/g)	B (g/l/h)	Q_s (g/l/h)	P_e (mg/l/h)	P_i (mg/l/h)
Bagasse	0.49	0.21	0.144	14.6	14.0
Cotton stalks	0.48	0.20	0.142	14.2	14.9
Kallar grass straw	0.50	0.24	0.146	14.0	14.7
Dhancha straw	0.49	0.24	0.147	21.0	14.3
Wheat straw	0.50	0.23	0.143	17.6	14.3
Cellobiose	0.50	0.24	0.147	15.7	18.1
CMC	0.40	0.20	0.129	12.0	11.5
Cotton wool	0.50	0.21	0.142	13.2	12.9
Filter paper	0.50	0.22	0.142	14.2	17.4
Xylan	0.52	0.23	0.144	14.4	15.3
α-Cellulose	0.50	0.22	0.144	14.2	15.3
Sigmacell-100	0.50	0.22	0.142	15.1	15.4
P. maximum	0.49	0.23	0.143	14.4	12.4
A. lentiformis	0.48	0.22	0.141	14.6	15.3

$Y_{x/s}$, cell mass/g sugar utilized from the substrate; Q_s, g substrate consumed/l/h; P_e, extracellular protein productivity mg/l/h; P_i, intracellular protein, mg/l/h. The values of the variable attributes among replicates varied very little hence no standard deviations have been shown.

and enzyme activities present in the cultures. When the test organism was grown on insoluble substrates, the culture medium after fermentation was centrifuged at 4000 g for 15 min at 4°C to remove the substrate (cells settled at 15000 g). The residue was washed twice and oven-dried to constant weight for further processing.

Cell fractionation

Culture samples (prepared after removal of insoluble substrates) were centrifuged at 15000 g for 15 min at 4°C and the cell-free supernatant was used as the extracellular fraction. Cells were divided into two portions; the 50 ml portion was suspended in biological saline, washed twice in saline and resuspended in saline for determining cell mass gravimetrically while the second portion (150 ml) was suspended in chilled McIlvaine or Na-acetate buffer (pH 7) and called the insoluble fraction. This fraction was disrupted by probe sonication in two bursts of 1 min with a Braun sonicator 2000 on ice. Supernatant from this disruption formed the third fraction called the cytosolic fraction. The particulate (or cell associated) fraction was obtained as a pellet by centrifugation at 15000 *g* for 15 min (4°C). The pellet was suspended in buffer and formed the fourth fraction.

Enzyme assays

For cellulolytic enzyme assays, the appropriately diluted culture supernatant or cell extract was incubated with carboxymethyl cellulose (CMC), the substrate for endo-glucanase (CMCase) activity, or Whatman No. 1 filter paper, the substrate for FPase, a measure of complete cellulase (Duenas *et al.*, 1995), in 0.2 M acetate buffer (pH 7) at 40°C after Nakamura and Kitamura (1988). In these tests, reducing sugars were estimated colorimetrically with dinitrosalicylic reagent method, using glucose as standard (Miller, 1959). One unit of enzyme activity in each case is defined as the amount of enzyme which releases 1 μM of glucose equivalent/ml/min. β-Glucosidase activity was determined using pNPG as the substrate (Deshpande & Eriksson, 1988). One unit of enzyme activity is defined as the amount of enzyme which releases 1 μM para-nitrophenol/ml/min.

Protein determination

The proteins were determined by Lowry's method (Lowry *et al.*, 1951) using bovine serum albumin as standard.

Determination of kinetic parameters

For determining kinetic parameters of batch fermentation process, the procedures of Lawford & Rousseau (1993) or Pirt (1975) were adopted. Dry cell mass (g/l) of *C. biazotea* after growth on different carbon sources in a time-course study was determined on triplicate samples as described earlier and residual carbohydrates (g/l) were determined on dry substrate after Shirlaw (1969). Enzyme activities (products/l) were determined as mentioned earlier. The 'growth yield coefficient' ($Y_{x/s}$) was calculated as the dry cell mass per mass of carbohydrates utilized. The volumetric rate of substrate utilization (Q_s) and enzyme productivity (Q_p) were determined from the maximum slope in a plot of substrate and enzyme produced versus time of fermentation and q_p was calculated by multiplying $Y_{p/x}$ by μ. The values of μ were calculated from the slope of ln (x) versus time of fermentation and $Y_{p/x}$ was calculated from product (IU/l) and dry cell mass (g/l) formed. Cell mass productivity (g dry cells/l/h), intracellular (P_i) or extracellular protein (P_e) productivity (mg protein/l/h) was determined from a plot of 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.

RESULTS AND DISCUSSION

Time-course cellulase production and carbon source utilization

Studies on production and secretion of cellulases are important to develop enzyme systems which could be directly used for converting LC biomass into safe alternative energy sources. The use of purified cellulosics as substrates is uneconomical for large-scale production of cellulases, therefore, several natural agricultural residues were used as carbon sources. For time-course cellulase production and substrate utilization in shake-flask cultures, *C. biazotea* was cultivated in Dubos salts–liquid media containing pretreated ground plant material of four LC substrates grown on saline lands, namely kallar grass straw, dhancha straw, *P. maximum*, *A. lentiformis* (Latif *et al.*, 1994; Malik *et al.*, 1986); three agricultural residues, namely bagasse, cotton stalks, and wheat straw, with reference to four different cellulosics, and soluble substrates, namely CMC and cellobiose. All enzymes were produced using 1% carbohydrates present in the substrate or equivalent carbohydrate as described in the Methods. All substrates supported rich growth and production of endo-β-glucanase, FPase and β-glucosidase. Representative enzyme production and substrate utilization kinetics from CMC, kallar grass straw, wheat straw, and α-cellulose under the conditions of shake-flask cultures are shown in Fig. 1(a)–(d), respectively. These results suggested that enzymes of cellulose complex appeared to reach maximum activities after 64–72 h. Of these components, the comparative yield of CMCase was higher on all substrates than were yields of FPase and β-glucosidase. This might be due to the fact that these components show different responses to different substrates for their production. From these Figures and others (not shown), apparently, the enzyme production

ppeared to be growth-associated. However, a plot f Q_p of FPase, CMCase, and β-glucosidase from epresentative substrates versus μ (Leudeking–Piret nodel, data not shown) gave slope values of 70±31, 576±179, 145±44 units/g cells for growth-associated parameters. Application of $\beta = (dp/dt)_{stationn}/x_{max}$ gave values of 4.1±0.9, 10.8±1.5 and 1.8±0.7 units/g cells/h for non-growth associated parameters respectively which suggested that the product formation was both growth associated and non-growth associated.

The operational fermentation parameters with respect to substrate utilization, namely maximum biomass and protein productivities, Q_x and $Y_{x/s}$ from different substrates, have been presented in Table 1. Maximum growth in terms of dry cell mass was significantly obtained during growth of *C. biazotea* on cellobiose and xylan, while minimum growth occurred on CMC. The $Y_{x/s}$ on cellobiose, kallar grass, dhancha straw, wheat straw and cellulosics was 0.50 g/g carbohydrate utilized, 88.5% of the maximum theoretical $Y_{x/s}$ of 0.565 from cellulose (Duenas *et al.*, 1995). Overall, values of B, Q_x, P_i and P_c on cellobiose, xylan, cellulosics, and LC substrates were reasonably high. In the case of LC substrates, this may be due to a degree of swelling (Saddler *et al.*, 1987) and dissociation of lignin in LC substrates by NaOH pretreatment (Latif *et al.*, 1988). *Cellulomonas biazotea* was an active degrader of cotton wool, α-cellulose, filter paper and Sigmacell 100 and gave B and Q_x comparable with their corresponding amounts from cellobiose (Table 1). Similarly, growth parameters on kallar grass were favourably comparable to their corresponding values from cellobiose utilization.

Production of FPase in different media

Among the enzyme activities, FPase was significantly higher on α-cellulose and kallar grass culture media than on media containing filter paper, cotton wool, Sigmacell 100, CMC, other LC substrates, and xylan. Cellobiose, though recognized as a strong inducer of cellulases (Marsden & Gray, 1986; Ryu & Mandels, 1980), induced only 17% FPase of maximum activity (Table 2). The FPase activities from all substrates accumulated in the culture supernatants. The major difference in FPase production by *C. biazotea* compared with all other strains of *Cellulomonas* reported elsewhere appears to be in the increased secretary levels of extracellular FPase. *Cellulomonas biazotea* NIAB442 produced a remarkably high amount of FPase on α-cellulose and kallar grass media (Table 2) which is significantly higher than the values reported on *C. flavigena* (Kim & Wimpenny, 1981; Nakamura & Kitamura, 1982; Sami *et al.*,

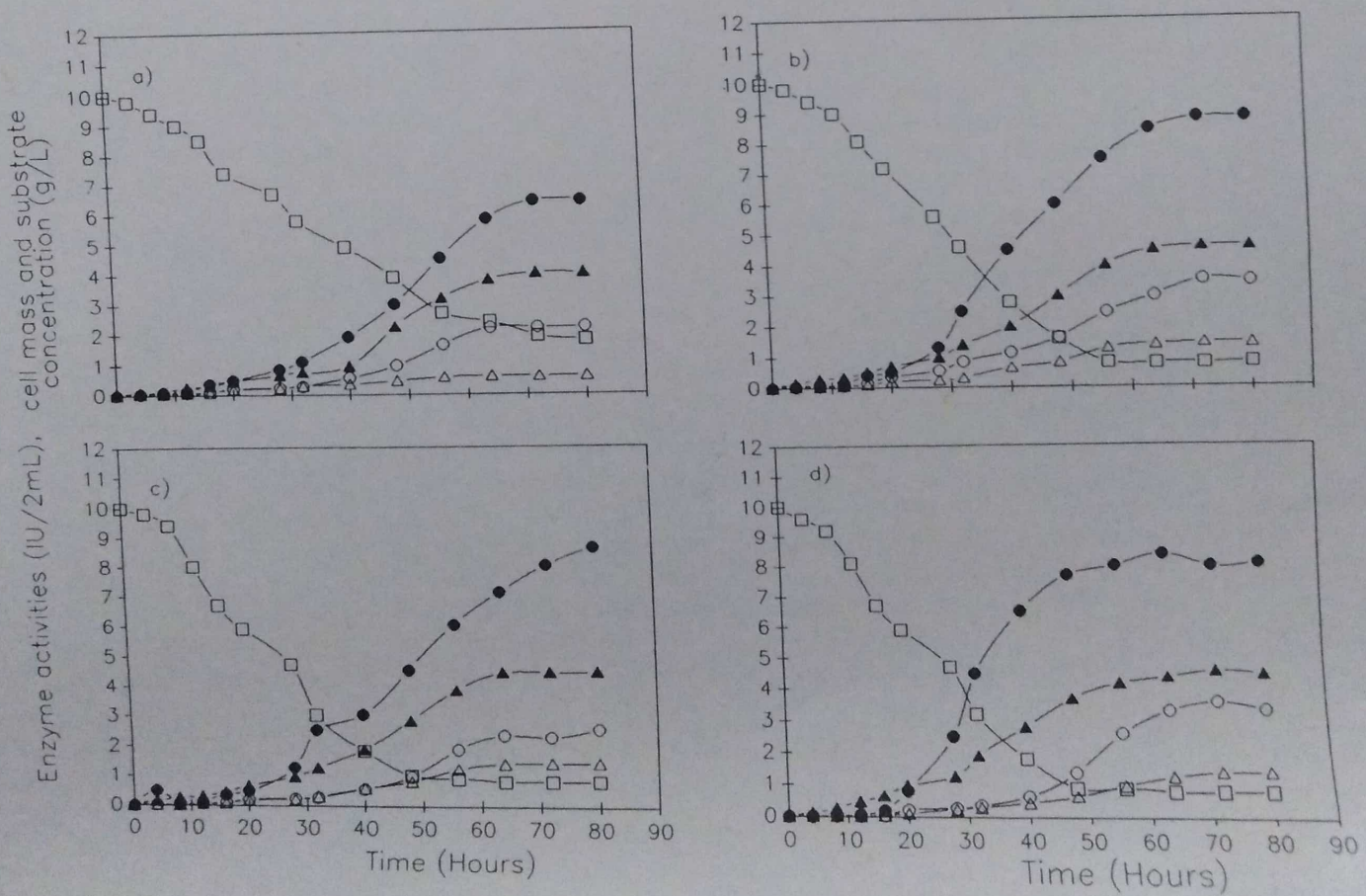

Fig. 1. Enzyme and cell mass production kinetics of *Cellulomonas biazotea* cultures in fermentation of (a) CMC, (b) kallar grass straw, (c) wheat straw and (d) α-cellulose in shake-flask cultures: ○ FP-cellulase, ● CMCase, △ β-glucosidase, ▲ cell mass, □ sugar concentration in the substrate. The results are means of three replicates with a standard deviation of 3.0–4.5% which has not been presented

Table 2. Potential of *C. biazotea* for cellulase production from different substrates measured as Q_p (units/l/h) after growth on different substrates in Dubos culture medium at 30°C

Growth substrate	Cellulase production profiles of *C. biazotea*		Q_p (units/l/h)
	FPase	CMCase	β-Glucosidase
Bagasse	08.2	22.3	3.6
Cotton stalks	08.8	26.4	2.5
Kallar grass straw	17.5	37.5	6.1
Dhancha straw	11.6	19.6	3.8
Wheat straw	08.9	19.6	3.6
Cellobiose	03.4	10.2	6.2
CMC	11.0	29.7	2.8
Cotton wool	14.0	30.0	5.7
Filter paper	14.5	20.1	5.8
Xylan	04.6	20.9	0.3
α-Cellulose	18.0	33.3	5.6
Sigmacell-100	12.0	26.7	5.7
P. maximum	09.9	24.9	4.1
A. lentiformis	07.0	27.7	3.5

The values of Q_p among replicates varied very little hence no standard deviations have been shown.

1988). This yield is a several-fold improvement over that formed by *Cellulomonas* CS 1-1 (Choi *et al.*, 1978), and *C. uda* CB 4 (Nakamura & Kitamura, 1982). The Q_p levels of FPase from *C. biazotea* on cellulose (18 IU/l/h) and kallar grass media (17.5 IU/l/h) are greater than those reported in mixed fungal liquid culture studies using *T. reesei* and *Aspergillus phoenicis* (12.5 IU/l/h) (Duff, 1985), *T. reesei* and *A. wentii* (9.7 IU/l/h) (Panda *et al.*, 1983) and *T. reesei* (9.7 IU/l/h) grown on bagasse (Duenas *et al.*, 1995). Moreover, the organism is superior to cellulolytic fungi as the maximum enzyme productivities in this organism are achieved after 3 days, while in fungi, maximum yields occur normally after 6–12 days of fermentation (Iqbal *et al.*, 1991; Duenas *et al.*, 1995).

Production of CMCase in different media

Comparative results on endo-β-glucanase production in media containing different substrates (Table 2) revealed that *C. biazotea* produced the highest enzyme level on kallar grass medium followed by α-cellulose, cotton wool, and *A. lentiformis* media. These media supported 90.7, 81.6, and 75.5% Q_p of kallar grass medium (Table 2). CMC medium produced 73% Q_p of kallar grass medium and was not recorded as the best inducer of CMCase, as observed in other organisms. Cellobiose proved to be the poorest inducer of CMCase. *Cellulomonas biazotea* produced 37.5 IU/l/h CMCase, which was double that produced by *C. flavigena* NIAB 441 (Rajoka & Malik, 1984a), and 10-fold improved over that by *C. flavigena* ATCC 482 (Nakamura & Kitamura, 1982). The enzyme productivities reported here compare favourably with values from *Cellulomonas* CS1-1 (Choi *et al.*, 1978; Haggett *et al.*, 1978). *Cellulomonas uda* CB 4 (Nakamura & Kitamura, 1982), *C. flavigena* (Sami *et al.*, 1988), and *C. fimi* (Langsford *et al.*, 1984), however, outproduced *C. biazotea* with respect to production of CMCase.

Production of β-glucosidase in different media

The highest β-glucosidase was recorded on cellobiose medium, followed by kallar grass, filter paper, Sigmacell 100, cotton wool and α-cellulose media, which gave 98, 94, 92, 92, and 90% respective productivities of cellobiose as a carbon source. These poroductivities were significantly higher than those obtained in media containing other LC substrates and CMC. β-Glucosidase was the lowest on xylan medium, giving only 4.8% of peak productivity on cellobiose. β-Glucosidase accumulated intra-cellularly and was mainly located in the periplasmic fractions of the cells. The highest Q_p value (6.2 IU/l/h) of β-glucosidase is quite respectable and is several fold higher than the values reported earlier on *Cellulomonas* spp. (Lee & Lee, 1985; Choi *et al.*, 1978; Haggett *et al.*, 1978; Nakamura & Kitamura, 1982) as well as other bacteria (Lin & Wilson, 1987; Waldron *et al.*, 1986).

β-Glucosidase produced by *C. biazotea* is sufficiently active to produce glucose from cellulosic substrates for subsequent conversion. For cellulose hydrolysis, the ratio of 1:1 of FPase:β-glucosidase is considered an optimum ratio (Cochet, 1991; Duenas *et al.*, 1995). To improve β-glucosidase production to get a desired ratio of FPase and β-glucosidase in the unconcentrated cell extracts, we intend to isolate the drug resistant and deoxy-D-glucose resistant mutant in which the yields of β-glucosidase are a two-fold improvement over the parent. Further strain breeding studies (using mutagenesis or gene cloning) may yield hyper-producers of other cellulase components as well.

CMC was utilized significantly slower than cellobiose, cellulosics or LC substrates and supported 61, 71 and 45% productivities of FPase, endo-β-gluca-

nase and β-glucosidase, respectively, from the best inducers. Although CMC is not regarded to be an inducer of cellulases, slowly utilizable substrates may be more effective inducers as glucose is liberated slowly and cannot cause catabolite repression of cellulase synthesis (Ryu & Mandels, 1980; Reese & Mandels, 1984). Cellobiose, the primary product of cellulose hydrolysis, is believed to be a strong inducer of the cellulase complex (Ryu & Mandels, 1980) but in these studies it proved to be the poorest inducer of both FPase and CMCase, although it was the strongest inducer of β-glucosidase (Table 2). However, disaccharides and oligosaccharides liberated during the hydrolysis of cellulose or LC biomass (Rajoka, 1990) are strong inducers of cellulases (Hrmova *et al*., 1991; Thomson, 1993).

CONCLUSIONS

In Pakistan, fuel reserves are depleted. Moreover, agriculture-based economy is badly effected because of the loss of one-third of cultivable land to salinity. The use of such lands for growing perrenial grasses, like kallar grass, followed by dhancha or *P. maximum*, have shown promise for reclamation of these soils and production of biomass (upto 50 tons/ha/year) for biotechnical applications (Malik *et al*., 1986; Latif *et al*., 1994). Kallar grass can be grown on saline lands in other parts of the world as well because this can be cheaply produced from such soils without application of fertilizer. It is comparable or better than purified substrates, namely α-cellulose, filter paper, Sigmacell 100 and cotton wool, with respect to supporting productivities of FPase, endoglucanase and β-glucosidase (Table 2). These purified substrates are prohibitively expensive, whereas a renewable substrate obtained inexpensively ($5.20/dry ton biomass) from saline soil would be more suitable for large-scale production of cheaper enzymes. The cost of pretreatment is also low ($0.5/kg). Currently, we are characterizing the cellulase components participating in cellulose hydrolysis for exploiting their academic and commercial potential.

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