

Influence of various fermentation variables on exo-glucanase production in *Cellulomonas flavigena*

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The influence of carbon and nitrogen sources on the production of exo-glucanase was investigated. The enzyme production was variable according to the carbon or nitrogen source used. Levels of β -cellobiohydrolase (CBH) were minimal in the presence of even low concentrations of glucose. Enzyme production was stimulated by other carbohydrates and thus is subject to carbon source control by easily metabolizable sugars. In Dubos medium, on cellobiose, the cellobiohydrolase titres were 2-to 110-fold higher with cells growing on monomeric sugars and 2.7 times higher than cells growing on other disaccharides. α -Cellulose was the most effective inducer of β -cellobiohydrolase and filter paperase (FPase) activities, followed by kallar grass straw. Exogenously supplied glucose inhibited the synthesis of the enzyme in cultures of *Cellulomonas flavigena*. Nitrates were the best nitrogen sources and supported greater cell mass, cellobiohydrolase and FPase production. During growth on α -cellulose containing 8-fold sodium nitrate concentration, maximum volumetric productivities (Q_p) of β -cellobiohydrolase and FPase were 87.5 and 79.5 IU/L/h respectively and are significantly higher than the values reported for some other potent fungi and bacteria.

Cellulases and xylanases are implicated in baked foods, fruit processing, cloth cleaning, preparation of dehydrated vegetables and food products, preparation of essential oils, flavours, pulp and paper production, starch processing, preparation of botanical extracts, jams, baby foods, juices, degumming coffee extracts, improved oil recovery, waste treatment, textile refining and preparation of feed for farm animals (Kubicek et al. 1993; Hoshino et al. 1997; Lynd et al. 2002).

Cellulase production has attracted a world-wide attention due to the possibility of using this enzyme complex for conversion of abundantly available renewable lignocellulosic/lignocellulosic (LC) biomass for production

of carbohydrates for numerous industrial applications including bioethanol (Gadgil et al. 1995; Hayward et al. 2000). Economical production of cellulases is key for feasible bioethanol production from LC biomass using cellulase-based processes. Different fungi and bacteria have been used for production of cellulases and xylanases using different substrates (Bahkali, 1996; Magnelli and Forchiassin, 1999; Shin et al. 2000; Lynd et al. 2002). Previously we produced cellulases following growth of *C. flavigena* on different substrates produced on saline lands namely *Leptochloa fusca* (kallar grass) straw, *Panicum maximum*, *Sesbania aculeata* compared with bagasse straw, and wheat straw and found that kallar grass was superior to other substrates for supporting synthesis of cellulases (Rajoka and Malik, 1997). Cellulase system of *C. flavigena* which produced high activities of cellulases and xylanases during growth on kallar grass showed end-product inhibition-resistance and thermal stability at room temperature of 25°C (Rajoka, 1990). These enzyme characteristics prompted to further investigate the production potential of FPase, cellobiohydrolase or exoglucanase (EC 3.2.1.91) as it measures the complete cellulase complex (Duenas et al. 1995). Celluloses and the cellulosic components in LC substrates are essential for formation of mRNA to support maximum formation of cellulases at the transcription level (Gutierrez-Nova et al. 2003). Glucose, on the other hand, represses cellulase synthesis by a catabolite repression mechanism at the transnational level (Rajoka et al. 1998; Ponce-Noyola, 2001; Lockington et al. 2002). A study of β -cellobiohydrolase, measured on *r*-nitro-phenyl β -D-cellobiopyranoside and FPase, measured on filter paper (which correlated with substrate utilization parameters) by *C. flavigena* including its induction, repression, and production is presented.

MATERIALS AND METHODS

Micro-organism and growth medium

Cellulomonas flavigena NIAB 441 (Rajoka and Malik,

Table 1. Specific substrate consumption rate (q_s) specific growth rate (μ) volumetric intracellular protein production rate (Q_{IP}) volumetric intracellular production rate (Q_{EP}) and β -celllobiosidase (IU/mg protein) levels in *Cellulomonas flavigena* strain grown on different carbon sources in Dubos medium (pH 7.3) in shake-flaks batch cultures at 30°C. The values in columns have been calculated from means of three replicates; the standard deviations were 3.04% of these values and have not been presented. Values in parentheses indicate the specific activity of FPase using Whatman no. 1 filtre paper as substrate.

Carbon source	q_s (g/g/h)	μ (h ⁻¹)	Q_{EP} (mg/l/h)	Q_{IP} (mg/l/h)	β -Celllobiosidase (IU/mg protein)
Arabinose	0.15	0.1	20.0	21.0	0.05
Fructose	0.54	0.23	21.0	21.0	0.35
Glucose	0.52	0.25	19.0	18.7	0.00
Xylose	0.41	0.14	19.0	17.9	0.08
Lactose	0.42	0.12	15.9	16.0	0.45
Maltose	0.45	0.13	15.1	14.3	0.25
Sucrose	0.44	0.12	14.5	17.3	0.15
Cellobiose	0.52	0.23	18.1	14.3	0.55
Cellodextrin	0.24	0.13	15.1	17.6	0.85 (0.84)
α -Cellulose	0.23	0.12	14.3	15.4	1.33 (1.31)
CMC	0.21	0.10	11.5	11.3	0.65 (0.64)
Kallar grass	0.15	0.12	14.1	12.1	1.12 (1.06)

1997) was used in these studies. Stock cultures of the organism were stored in culture tubes using Dubos salt-cellulose agar plates after the instruction of the stock culture maintainers and grown in liquid cultures as described earlier (Rajoka and Malik, 1997). All chemicals were of analytical grade. Carboxymethylcellulose (Na-salt) of low viscosity and α -cellulose were from Sigma Chemical Co., Missouri, USA.

Evaluation of carbon sources for induction of exo-glucanase

A total of 12 different carbon sources in triplicate (Table 1) were chosen as test substrates on the basis of literature data and their availability. The organism was grown and enzyme preparations processed as described earlier (Rajoka and Malik, 1997).

Effect of nitrogen sources on cellulase production

Different nitrogen sources (Table 2) were tested to select the best nitrogen source keeping nitrogen concentration at 0.164 g/l. The cultures were grown in time course study as described earlier and processed for enzyme assays. Sodium nitrate proved to be the best nitrogen source and its concentration was optimized by changing its concentration in the medium while maintaining other optimal cultural conditions.

Growth studies

The ability of the organisms to utilize monosaccharides, disaccharides, kallar grass, with reference to cellobiose, CMC, or α -cellulose (Avicel) as sole carbon source 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 basal medium to give a saccharide level of 10 g/l. All monosaccharides and disaccharides were added to autoclaved medium after filter-sterilization. All media were adjusted to pH 7.3 with 1 mol NaOH or 1 mol HCl and were dispensed in 200 ml aliquots into 1-l Erlenmeyer flasks in triplicate.

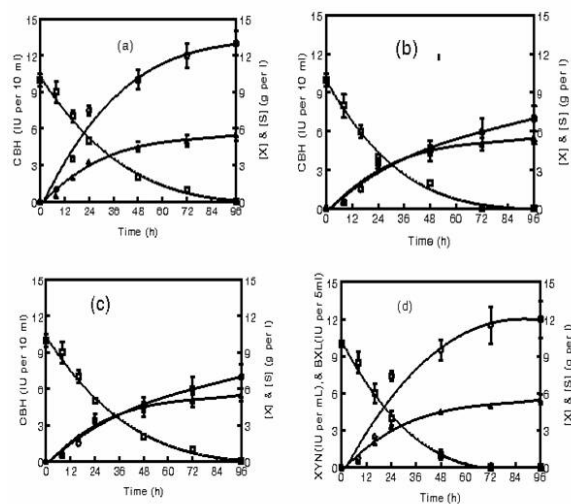


Figure 1. Kinetics of cellobiohydrolase (CBH) production in shake flask fermentation of four representative substrates. The initial pH of the medium was 7.3, inoculum size 10 %, on 1% (w/v) carbohydrates in substrates, and temperature 30°C. o = CBH, Δ = cell mass, and \square = substrate present in the fermentation medium. Error bars show standard deviation among three replicates.

- (a) Cellobiose;
- (b) CMC;
- (c) kallar grass;
- (d) Alpha-cellulose.

Table 2. Potential substrate consumption and FPase production parameters following growth of *C. flavigena* on α -cellulose-, CMC- or kallar grass-Dubos salts culture medium (pH 7.3-7.4) in shake-flask experiments conducted at 30°C. The organism was grown on carbon sources (containing 1% carbohydrates in basal Dubos medium. Kallar grass (*Leptochloa fusca*) is a salt tolerant grass grown on saline lands, (Rajoka and Malik, 1997). The values in columns have been calculated from means of three replicates; the standard deviations were 2.5-3% of these values and have not been presented. The values of CBH were not significantly different from those for FPase and have been omitted.

Parameters	Following growth on 1% carbohydrates in		
	α -cellulose	CMC	kallar grass
$Y_{X/S}$ (g cells/g)	0.550	0.450	0.540
Q_S (g S utilized/l/h)	0.145	0.135	0.121
Q_X (g cells/l/h)	0.240	0.200	0.234
Q_{RS} (mg/l/h)	14.000	34.567	20.25
Q_P (IU/l/h)	35.000	22.00	32.25
$Y_{P/S}$ (IU/g substrate consumed)	133.200	64.500	125.25
$Y_{P/X}$ (IU/g cells)	244.18	144.400	235.23
q_P (IU/g cells/h)	36.6	17.7	32.8

For determining enzyme synthesis, above medium was inoculated with 10% of inoculum containing 2.5 g dry cells/l and incubated at 30°C on a rotary shaker (150 rpm) after (Rajoka and Malik 1997). After 8, 16, 24, 48, 72, and 96 hrs of growth, flasks in triplicate were harvested. The amount of growth was measured as dry cell mass gravimetrically. The enzyme activity present in the cell-free supernatant or cell extract was assayed as the induction or repression indicator. When the organism was grown on insoluble substrates, the whole culture medium after fermentation was centrifuged (4000 g, 15 min) to remove particulate substrate. The residue was shaken twice with chilled water containing 1 % (v/v) Tween 80 for 30 min at 4°C and clear supernatant was obtained by centrifugation (15000 g, 30 min, 4°C). All washings were pooled for determining enzyme activities and correction was made for the adsorbed portion of FPase on the surface of the substrates. 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 a constant mass. Cell extract was obtained by ultra-sound disintegration and centrifugation (15000 g, 30 min) as described previously (Rajoka and Malik, 1997).

Enzyme assay

For cellulolytic enzyme assays, the appropriately diluted culture supernatant or cell extract (Rajoka and Malik, 1997) was used to determine FP cellulase (FPase) activity or cellobiohydrolase activity using filter paper no.1 or *para*-nitrophenyl- β -D-cellobioside (Sigma) respectively in 0.2 mol acetate buffer (pH 7) at 40°C after Nakamura and Kitamura (1988). In former tests, reducing sugars were estimated calorimetrically with 3,5-dinitrosalicylic acid method (Miller, 1959). One unit of enzyme activity is defined as the amount of enzyme, which releases one μ mol of glucose equivalents or *para*-nitrophenol per ml per min respectively under the assay conditions. It was found that FPase and cellobiohydrolase from both CMC and Avicel were almost equal. Therefore, only FPase activities following growth on insoluble substrates under various

conditions have been presented. While for soluble mono- and di-saccharides, *para*-nitrophenyl- β -D-cellobioside was used.

Saccharide determination

Saccharides were determined using 3,5-dinitrosalicylic acid after Miller (1959). Cellulose and hemicellulose were determined as described previously (Latif et al. 1994).

Protein determination

Cellobiosidase was mainly cell-bound while FPase was secreted in the medium. Therefore, the proteins in both extracellular and cellular fractions were determined according to Bradford method (1976) using bovine serum albumin as the standard. Protein content in the substrate and spent dry matter was determined by multiplying 6.25 with nitrogen content determined by Kjeldahl's method.

Determination of kinetic parameters

Allkinetic parameters for substrate consumption and product formation (Rajoka and Malik, 1997) were determined as described previously (Rajoka et al. 1998).

RESULTS AND DISCUSSION

Effect of carbon sources

Extensive screening of potential cellobiosidase, (CBH) or filter paperase (FPase) inducers showed that when *Cellulomonas flavigena* NIAB 441 was cultured on media containing monomeric saccharides, dimeric saccharides, carboxy methyl cellulose (CMC), α -cellulose or kallar grass (Table 1), it had a shorter lag period and more specific growth rate when grown on monosaccharides namely arabinose, xylose, glucose, galactose and fructose than those on disaccharides or polysaccharides, Figure 1, (Rajoka and Malik, 1997).

CBH was mainly present in the intracellular preparation. The organism synthesized CBH to a measurable level from

Table 3. Effect of addition of glucose to α -cellulose medium (containing 0.4% sodium nitrate in Dubos medium) on cellobiohydrolase formation parameters. Each value is a mean of three replicates. \pm stands for standard deviation among replicates. Means followed by different letters within each column differ significantly at $p \leq 0.05$ using one factor factorial design in MStatC software.

Glucose (g/l)	Q_p (IU/l.h)	Q_x (g cells/ l.h)	Q_s (g substrate/ l.h)
0.0	87.56.1 ^a	0.430.02 ^c	0.510.019 ^a
1	79.87.3 ^b	0.460.021 ^{bc}	0.470.023 ^a
2.0	65.2.56.5 ^c	0.490.023 ^{bc}	0.390.014 ^b
5.0	58.64.5 ^c	0.490.023 ^{bc}	0.35.0.017 ^d
10	52.64.7 ^e	0.570.033 ^a	0.32.0.011 ^c

disaccharides, otherwise only basal level of this enzyme was produced. Similarly FPase was produced in very small quantity below the detection limits of the assays or could not be quantified due to the presence of soluble sugars. Following growth on mono- and di-saccharides, cell extract containing cellobiohydrolase was collected and assayed for this activity. Carbon sources which supported rapid growth measured as q_s (specific substrate utilization rate), μ (h^{-1}) Q_{EP} (productivity of extracellular proteins) and Q_{IP} (productivity of intracellular proteins) were the worst repressors of enzyme synthesis. Among polymeric substrates, α -cellulose supported the maximum activity, followed by kallar grass. Overall cellulosic substrates induced high level of enzyme activities. Among monosaccharides, only fructose synthesized cellobiohydrolase and supported the work of Nochureet al. (1993). Among disaccharides, cellobiose was the best inducer, followed by maltose and lactose. There was greater enhancement in cellobiohydrolase productivity (2.4-fold enhancement) following growth on α -cellulose over that obtained from cellobiose (Table 1).

Additionally level of CBH varied over a 1.57- to 11-fold range with the non-inducing carbon sources namely,

xylose, arabinose, fructose and inducing carbon sources namely maltose, cellobiose, cellodextrin and cellulose (1.2- to 2.38- fold) and showed inverse relationship with values of q_s , μ , Q_{IP} and Q_{EP} .

FPase got immobilized on cellulosic substrates ($10 \pm 0.25\%$) and could be successfully eluted with Tween 80; all values presented in different figures and tables have been compensated to include substrate-bound FPase. Similarly some cells got adsorbed on the surface of insoluble substrates. These cells contained CBH activity and the values of CBH have also been compensated to contain cell-bound CBH activity in Table 1.

Effect of nitrogen sources on cellulase production

In *C. flavigena*, the effect of nitrogen sources was tested by replacing $NaNO_3$ in the medium with other compounds, maintaining equi-molar amount of nitrogen at 0.164 g/litre. The cultures were grown for 72 hrs, harvested and processed for enzyme assays (Table 3). Inorganic nitrogen sources including $NH_4 Cl$, $(NH_4)_2 SO_4$, $NH_4H_2PO_4$ and organic nitrogen sources namely corn steep liquor and urea were the poor nitrogen sources of FPase synthesis. $NaNO_3$, KNO_3 and NH_4NO_3 were the best sources since *C. flavigena* possessed strong nitrate reductase activity which was induced by NO_3 ions to an optimal level and repressed by free NH_4 ions in the growth medium. Spiridonov and Wilson (1998) found that NH_4 compounds are the most favourable nitrogen sources for protein and cellulase synthesis. Nakamura and Kitamura (1988) observed that polypeptone supported the maximum production of FPase and CBH by *C. uda* CB 4.

Effect of $NaNO_3$ on exoglucanase synthesis

The addition of $NaNO_3$ increased cell mass and FPase synthesis when added to the basal medium (Figure 2). FPase was secreted at elevated levels (up to 2.5-fold higher) compared with control when alpha-cellulose was used as a cellulosic substrate. However, concentrations higher than 0.45% lowered the FPase activity to some extent but it was still higher than that present in the control. The yield was two- fold that obtained in mutant derivative of *C. biazotea* (Rajoka et al. 1998). The Q_p levels of FPase on alpha-cellulose (87.5 IU/l/h) and kallar grass medium (79.5 IU/l/h) are greater than those reported on *C. biazotea* and its mutant derivative (18 IU/l/h and 21 IU/l/h; Rajoka et al.

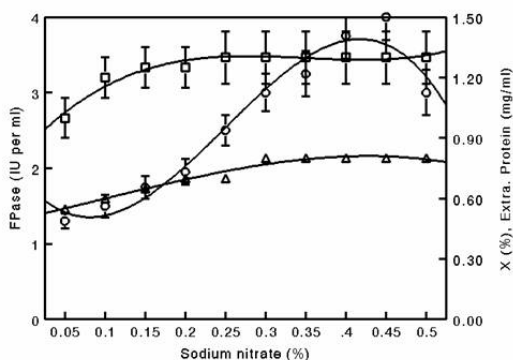


Figure 2. Effect of sodium nitrate concentration in Dubos salt medium on FPase (o), cell mass (Δ) and extracellular protein (l) production following growth on alpha-cellulose. The initial pH of the medium was 7.3, inoculum size 10%, on 1% (w/v) substrate, and temperature 30°C. Error bars show standard deviation among three replicates.

Table 4. Effect of nitrogen sources on production of FPase, cell mass and extracellular protein production relative to control which contained α -cellulose -Dubos salts +0.2% yeast extract +0.1% sodium nitrate. Control was composed of Dubos salts medium +0.2% yeast extract and supported 35.2 IU/ l/h, 21.02 mg/l/h and 0.255 g cells/l/h extracellular FPase, extracellular protein and cell mass productivity respectively. Standard deviation among 3 replicates was 2.0-3.5% of given values and have not been presented.

Nitrogen	Relative values (%) of control		
	FPase	Cell mass	Extracellular
Dubos medium			
Sodium nitrate in the control	100	100	100
Sodium nitrate (8-fold)	250	150	175
Potassium nitrate (2-fold)	125	125	135
Urea	85	78	80
Ammonium chloride	30	25	35
Ammonium sulphate	45	30	40
Diammonium hydrogen phosphate	70	65	75
Sodium glutamate	80	85	96
Ammonium nitrate	90	95	80
Corn steep liquor	45	60	70

1998) and other organisms (Spiridonov and Wilson, 1998; Kalogeris et al. 2003).

During growth of the organism on different cellulosic substrates, reducing sugars accumulated (Table 4) in the growth medium as unmetabolized principles. Those substrates which released more carbohydrates were stronger repressors. The values of fermentation parameters with respect to substrate utilization, namely maximum biomass and protein productivities, Q_s and $Y_{x/s}$ from α -cellulose, CMC and kallar grass (Table 2). Indicated that maximum growth in terms of dry cell biomass was significantly higher on α -cellulose while minimum values was obtained following growth on CMC. The $Y_{x/s}$ of 0.55 g cells/ g cellulose utilized was 98% of the maximum theoretical (0.565) of $Y_{x/s}$ from cellulose (Duenas et al. 1995). Over all, the values of all substrate consumption parameters were reasonably high. Like *C. biazotea* (Rajoka and Malik, 1997), *C. flavigena* was an active degrader of α -cellulose and gave high values of substrate consumption parameters comparable with their corresponding amounts of *C. biazotea* on cellobiose (Rajoka and Malik, 1997).

Volumetric productivity of CBH and growth parameters of the organism in Dubos- α -cellulose medium when glucose was added (1, 2.5, 5 and 10 g/litre final concentration) at the time of inoculation (Table 3), showed significant decrease in the enzyme synthesis. All treatments had statistically significant ($p < 0.05$) influence on CBH productivity, substrate consumption and cell# mass formation rates. It was found that glucose enhanced the cell mass productivity, but suppressed CBH productivity and substrate consumption rate. Mixed inductive or repressive effect has been observed in other organisms (Ponce-Noyola and De la Torre, 2001). Catabolite repression plays an important role in the regulation and secretion of inducible enzymes. Such repression effect has been also observed in other organisms (Spiridonov and Wilson, 1998; Gutierrez-Nova et al. 2003). The effect of different glucose concentrations on CBH activity was determined in order to differentiate it from the effect of glucose on the synthesis of

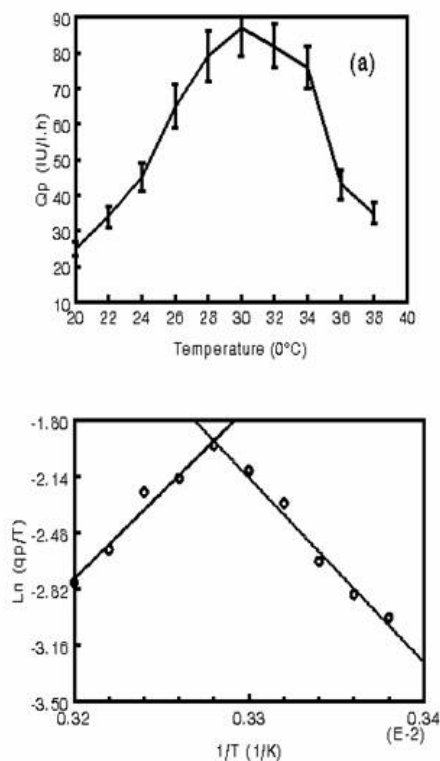


Figure 3.

(a) Effect of fermentation temperature (a) on volumetric rate of CBH production (Q_p) following fermentation of α -cellulose in Dubos medium. Error bars show standard deviation among three replicates.

(b) Arrhenius plot to calculate enthalpy (ΔH^\ddagger) and entropy (ΔS^\ddagger) of CBH formation and its deactivation applying relationship: $\ln(q_p/T) = \ln(k_B/h) + \Delta S^\ddagger/R - \Delta H^\ddagger/R \cdot 1/T$ (Table 5).

Table 5. Thermodynamic parameters estimated by Arrhenius approach for batch formation and deactivation of CBH by cultures of *Cellulomonas flavigena* Thermodynamic parameters were determined using following equation:

$\ln(q_p/T) = \ln(k_B/h) + \Delta S^\ddagger/R - \Delta H^\ddagger/R \cdot 1/T$; where q_p , T , k_B , h , ΔS^\ddagger , ΔH^\ddagger and R are specific productivity, absolute temperature, Boltzmann constant, Planck's constant, entropy of activation, enthalpy of activation and gas constant respectively. The values of k_B , h , and R are 1.38×10^{-23} J.K⁻¹, 6.63×10^{-34} J.s and 8.314 J/.K⁻¹.mol⁻¹ respectively.

ΔH^\ddagger was calculated as slope and $\ln(k_B/h) + \Delta S^\ddagger/R$ as intercept on Y-axis using **Figure 2b**.

	Enzyme formation	Thermal inactivation
Activation enthalpy (kJ/mol)	86.9 ± 3 ^b	92.6 ± 8 ^a
Activation entropy (J/mol/ K)	90.5 ± 5 ^a	(-) 498.5 ± 25 ^b

this enzyme; the same enzymatic activity values being obtained with these glucose concentrations studied, thus suggesting that the decrease in the content of enzyme, together with the increase in the initial concentration of glucose in the fermentation medium, was due to negative effect of sugar on the synthesis of this enzyme. Moreover, when the level of available sugar decreased as a result of culture growth, the synthesis of enzyme increased until 72 hrs of culturing. The effect of glucose was investigated with cells harvested during stationary phase in order to minimize the influence of growth. When glucose was added at the beginning of the studies, CBH production ceased even though the inducer (α-cellulose) was also present. However, when after 8 hrs of incubation, the cells were washed free of glucose and placed in glucose-free medium containing α-cellulose, the synthesis of CBH started again, thus proving the reversibility of the repression mechanism of CBH by glucose.

Effect of pH and temperature

Optimum pH for production of CBH is 7.3 (results not shown). Temperatures higher than 30°C suppressed production of exo-glucanase and supported the finding of Rajoka et al. (1998). Maximum specific productivity (q_p) of CBH occurred at fermentation temperature of 30°C ([Figure 3a](#)). At higher or lower temperature, CBH production by the cells was low. At lower temperature, the transport of substrate across the cells is suppressed and lower yield of products are attained. At higher temperature, the maintenance energy requirement for cellular growth is high due to thermal denaturation of enzymes of the metabolic pathway (Aiba et al. 1973) resulting in minimum amount of product formation.

Effect of temperature was shown by calculating activation enthalpy of CBH production graphically from [Figure 3b](#) by the application of Arrhenius approach (Aiba et al. 1973). The values of the activation enthalpy ([Table 5](#)) of CBH production ($\Delta H^\ddagger = 86.9$ k J/mol) are lower than that for glucose isomerase production reported by Converti and Del Borghi (1997). The phenomena responsible for thermal inactivation of enzyme is characterized by an activation enthalpy of $\Delta H_D^\ddagger = 92.6$ k J/mol and is comparable with that

for CBH production. This suggests that the productivities decline observed at high temperature could be due to the reversible denaturation of enzyme formed on Avicel medium. The activation entropy of CBH formation (0.095 k J/mol K) compares favourably with that of phytase formation (Converti and Dominguez, 2001). The activation entropy value of thermal inactivation (-0.498 kJ/mol. K) was also very low and had negative symbols which suggested that this inactivation phenomenon implied a little randomness/disorderness during the activated state formation and compared favourably with that of β-galactosidase formation (Rajoka et al. 2003). They further led to suggest that the phenomenon limiting CHB production could be enzymatic reaction/s under varying fermentation conditions as observed for other enzymes (Converti and Dominguez, 2001).

CONCLUDING REMARKS

These studies led us to conclude that the carbohydrate and nitrogen sources play a vital role in the production of CBH or FPase by *Cellulomonas*. α-Cellulose was the best carbon followed by kallar grass but the former is an expensive substrate, therefore, kallar grass could be used for mass production of cellulases. In these studies, efforts were made to increase the enzyme production by manipulating above aspects only. The scope to increase the production by the use of genetical, biochemical and microbial engineering techniques to make use of full potential of this organism are to be studied. Gamma ray mutation followed by chemical mutagenesis have given mutant derivatives for improved cellulolysis (Gadgil et al. 1995; Rajoka et al. 1998) and will be used in further studies. Thermodynamic studies suggested that cells needed lower thermal energy for product formation and that the cell system exerted defence against thermal inactivation.

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