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Kinetics of enhanced substrate consumption and endo-β-xylanase production by a mutant derivative of *Humicola lanuginosa* in solid-state fermentation

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Summary

Industrial byproducts namely canola meal, rice bran, sunflower meal, and wheat straw were used as substrates for endo-xylanase production by *Humicola lanuginose* mutant TH1 through solid substrate fermentation. The enzyme was secreted extracellularly by both wild and mutant cultures. Rice bran supported the maximum production of endo-xylanase followed by wheat straw, canola meal and sunflower meal. The highest activity was achieved after 72 h of culture and the highest yields from the above substrates were 842, 840, 610 and 608 IU per g substrate consumed respectively. The highest productivity (281 IU flask^{-1} h^{-1} corresponding to 5620 l^{-1} h^{-1}) of endo-xylanase by the mutant of *H. lanuginosa* was 1.6-fold more than that produced by the parental organism in solid-state fermentation of rice bran at 45 °C. Maximum specific activity (180 IU mg^{-1} protein) and substrate consumption rates were significantly more than those reported by previous researchers on *Humicola* sp. The mutant possessed markedly low accompanying cellulase activity. Thermodynamic studies revealed that the mutant required significantly lower activation energy for enzyme production and higher for thermal inactivation which signified that the endogenous metabolic machinery of mutant cells exerted more protection against thermal inactivation during product formation than that needed by its parental cultures.

Nomenclature

Q_x	Rate of cell mass formation (g cells l^{-1} h^{-1})
Q_s	Rate of substrate consumption (g substrate l^{-1} h^{-1})
μ	Specific growth rate (h^{-1})
Q_P	Rate of enzyme formation (IU flask^{-1} h^{-1} or IU l^{-1} h^{-1})
$Y_{P/S}$	Endo−xylanase yield (IU g^{-1} substrate utilized)
$Y_{P/X}$	Specific yield of enzyme production (IU g^{-1} cells)
q_P	Specific rate of enzyme production (IU g^{-1}cells h^{-1})
ΔH^x	Activation enthalpy for product formation (kJ mol^{-1})
ΔH^*_D	Activation enthalpy for product deactivation (kJ mol^{-1})
ΔS^*	Activation entropy for product formation (kJ mol^{-1} K^{-1})
ΔS^*_D	Activation entropy for product inactivation (kJ mol^{-1} K^{-1})

Introduction

Xylan, the most abundant of the hemicelluloses in plant cell walls, has a linear backbone structure consisting of β-1,4-linked xylosyl residues that, depending on its origin, may contain branches of L-arabinofuranosyl, acetyl, glucuronosyl and 4-*O*-methylglucuronosyl residues (Pandey 2002). Endo-β-xylanase (1,4-β-xylan xylanohydrolase; EC 3.2.1.8) and β-xylosidase (1,4-β-xylan xylohydrolase; EC 3.2.1.37) are the main hemicellulases that catalyse hydrolysis of xylans from different sources (Gilbert *et al*. 1993). Most of the endo-xylanases hydrolyse glycosidic linkages along the xylan backbone in a random cleavage mechanism, while β-xylosidases release D-xylose from short-chain xylo-oligomers. Biosynthesis of endo-xylanase is markedly dependent upon the type of inducer used. By conducting systematic screening studies, more efficient and cheaper supporters of enzyme production can be selected. It must be recognized that the major cost factor in producing commercial enzymes

is the carbon source (Pandey 2002). These costs are relatively low in developing countries. Thermophilic moulds produce endo-xylanase of high catalytic potential and stability (Anand & Vithayathil 1996).

Endo-xylanase can be produced in both solid and submerged fermentations but solid-state fermentation (SSF) offers advantages over liquid cultivation, in the form of more productivity, reduced energy requirements, lower capital investment, lower waste water output, high concentration of metabolites and low downstream processing costs (Nigam & Singh 1994). We therefore, studied xylanase production by the fungus in SSF.

Humicola spp. are thermophilic fungi that produce endo-xylanase with little accompanying cellulase activity and have been used for pulp improvement (Da Silva *et al.* 1994; Anand & Vithayathil 1996) and reduction of dependence on chlorine and chlorine dioxide for bleaching in the brightening process (Gilbert 1993). *H. lanuginosa* is a fast growing biocatalyst and produces hydrolases in the initial phase of growth (Adams 1994) but its potential for bulk production of xylanases has not been fully explored kinetically and is the subject of these studies.

2-Deoxy-D-glucose (DG), a toxic glucose analogue has frequently been employed to isolate glucose-deregulated mutants (Rajoka *et al.* 1998; Haq *et al.* 2001). Mutagenesis to confer deoxy-D-glucose resistance (DG^r) is a well recognized approach to control the formation of repressible proteins (CreA, CreB and CreC) (Lockington *et al.* 2002) and to enhance the expression of inducible enzymes as used for improved production of cellulases in different organisms (see Rajoka *et al.* 1998). Increases in xylanase expression and redirection of transport systems in this organism will enhance substrate utilization and product formation. In this work we report the isolation of a DG-resistant mutant of this organism in which 1.6-fold higher levels of endo-xylanase were achieved and to establish the optimal culture conditions. This mutant, deregulated for endo-xylanase has been stable for the last 3 years and fermented carbon sources faster than the wild-type in both liquid and solid-state fermentation. The kinetics of solid-state fermentation were examined to demonstrate the superiority of the mutant organism with respect to substrate consumption and product formation parameters. Thermodynamic studies were followed in order to understand the overall behaviour of mutant cultures with respect to demand of enthalpy and entropy of activation for product formation under a wide range of temperatures at which this organism can grow.

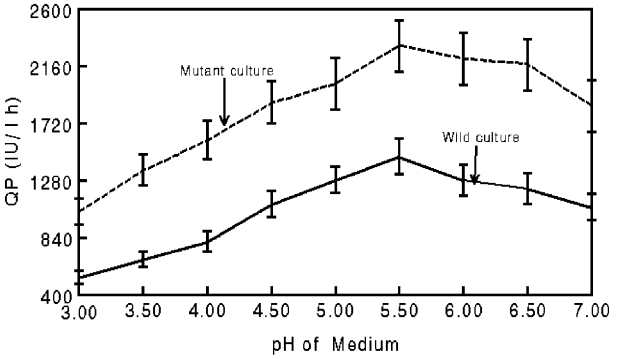

Figure 1. Effect of initial medium pH on endo–xylanase volumetric productivity (Q_P) of wild and mutated cultures grown on wheat straw medium at 45 °C.

Materials and methods

Micro-organism

The culture of *Humicola lanuginosa* (from our culture collection) was maintained on potato dextrose agar (PDA) slants and plates at 4 °C and subcultured every month. The fungus was resubcultured onto PDA plates and cultured at ambient temperature for 15 days before use as a source of inoculum.

Inoculum preparation

Ten ml of saline was added to 5- to 7 day-old slants. Spore suspension was prepared by scratching and shaking the tubes. One ml of spore suspension (containing 10^7 spores ml^{-1}, determined with haemocytometer) was aseptically transferred to each flask containing the fermentation medium and acid-washed glass beads to provide uniform suspension.

Mutagenesis using u.v. irradiation

Mycelial suspension (2.5 g dry wt l^{-1}) of *H. lanuginosa* in sterile phosphate buffer (pH 6) was treated by u.v. irradiation. An exposure dose of 1.2×10^6 J m^{-3} s^{-1} for 60 min resulted in cell viability loss of greater than 99%. The treated mycelia were allowed to express in the presence of 0.6% 2-deoxy-D-glucose (at which the wild mycelia did not grow) liquid medium on a rotary shaker at 45 °C. Appropriately diluted resistant phenotypes were plated onto xylan agar plates (1% xylan in basal medium + 0.1% Rose Bengal + 0.1% Triton-X-100 + 0.6% 2-DG) and incubated at 45 °C. The colonies with larger clear zones than wild-type cells grown in the absence of 2-deoxy-D-glucose (see Chadha *et al.* 1999) were subcultured and tested for xylanase and cellulase production in the optimized medium by solid-state fermentation.

Enzyme production by solid-state fermentation

The use of commercial xylan as a substrate is uneconomical for large scale production of xylanases, therefore, several abundantly available agro-industrial residues were included in these studies, such as wheat straw, sunflower meal, canola meal or rice bran. They were first dried (0.5% moisture) and converted to powder with a grinder and used at 5 g $flask^{-1}$. Wheat

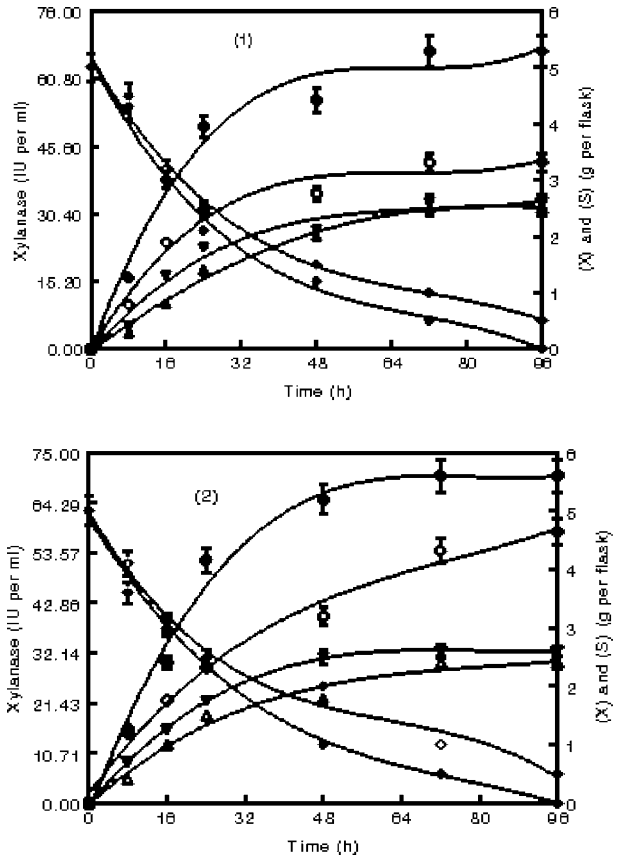

Figure 2. Kinetics of endo-xylanase production in solid state fermentation of two representative substrates, (1) rice bran, and (2) wheat straw. The initial pH of the medium was 5.5, inoculum size 4 ml per g substrate, and temperature 45 °C. Open o = xylanase, Δ = cell mass, and ◇ = substrate present in the fermentation medium of the parental cultures while closed symbols are for mutant cultures. Error bars show standard deviation among three replicates.

straw was also given alkali-pre-treatment (with 0.2% NaOH) as described earlier (Rajoka *et al.* 1997).

The optimized fermentation medium (added to solid substrate) contained (g l^{-1}) K_2HPO_4 1.0, $(NH_4)_2SO_4$ 0.5, KCl 0.5, $MgSO_4$ 0.2, $CaCl_2$ 0.1, yeast extract 0.5 and urea 10.0 (pH 5.5). Solid-state fermentation after Duenas *et al.* (1995) was employed. Five grams of dry substrate along with 7.5 ml of optimized fermentation medium were added to 250 ml conical flasks. They were plugged with cotton wool before autoclaving (121 °C, 1.05 kg cm^{-2} for 15 min). The flasks were allowed to come to ambient temperature and then inoculated with 4 ml of inoculum per g solid substrate. Final moisture content was 43%; at this moisture level, there was no free water in the flasks. The flasks were incubated at 45 °C for 72 h (previously found optimum). The flasks were run in parallel in triplicate. In time course studies, at predetermined time intervals (Figure 2), individual flasks in triplicate were withdrawn. For enzyme recovery, the fermented mass was suspended in 50 ml of de-ionized chilled water or buffer containing 1% (v/v) Tween 80. The suspension was homogenized and centrifuged (10 min, 4000 × *g*) to remove solid substrate. The residue (containing solid substrate) was shaken with chilled water containing 1% (v/v) Tween 80 for 30 min at 4 °C and clear supernatant was obtained by centrifugation (15,000 × *g*, 15 min). To recover the adsorbed portion of enzymes, solid mass was again suspended in 50 ml of deionized chilled water/buffer containing 1% (v/v) Tween 80, vigorously shaken on a rotary shaker and recentrifuged (10 min, 4000 × *g*) to remove solid substrate. Further fractions were collected until the enzyme activity was not observed in the supernatant. All washings were pooled for determining enzyme activities and compensated for the adsorbed portion of endo-xylanase and cellulases. The washed substrate was oven-dried to constant weight for further processing. The cell pellet was washed twice with saline, suspended in 10 ml distilled water and dried at 95 °C to a constant mass.

Determination of cell mass

Fungal growth was followed by measuring the N content of the centrifuged and washed solids of the fermented materials by a modified Kjeldahl's procedure (Duenas *et al.* 1995). The N values were multiplied by 6.25 to obtain protein content, and by 2.2 (dry biomass of culture on glucose contained 45% protein) to obtain the estimated biomass content.

Effect of nitrogen sources on xylanase production

Extensive work was carried out for screening and optimizing other cultural conditions. Different nitrogen sources, added to growth medium as above, were used to see their effect on enzyme production. These included $NaNO_3$, $(NH_4)NO_3$, $(NH_4)_2SO_4$, urea, sodium glutamate, corn steep liquor, and $(NH_4)_2HPO_4$. These all were used at a concentration to contain 0.12% nitrogen in the optimized medium added to solid substrate. Yeast extract (0.2%) and $NaNO_3$ (0.05%) served as the control. In all other media a basal level of yeast extract at 0.005% was used to provide micronutrients and growth factors. Urea (at 10%, w/v) supported maximum of 48 and 76 IU per ml endo-xylanase in both parental and mutated cultures and was used in all studies.

Effect of pH on xylanase production

The effect of initial pH of the medium was observed in the range 3.5–9.0; the pH was adjusted with 1 M NaOH or 1 M HCl.

Effect of temperature on xylanase production

The organisms were grown statically in optimized medium at different temperatures (30–60) °C in time course studies and data collected as described earlier.

Enzyme assays

The reaction mixture containing 0.5 ml of suitably diluted enzyme solution, 1 ml citrate phosphate buffer (pH 5.0), and 0.5 ml of xylan (oat spelt) was incubated

at 50 °C for 5 min. Xylose released by xylanase was assayed using DNS reagent as described previously (Rajoka *et al.* 1997). The enzyme activity was expressed in international units and was defined as the amount of enzyme required to release 1 μmol reducing sugar (xylose) per minute. Filter paper activity (FP-ase), the measure of whole cellulase (Duenas *et al.* 1995), β-glucosidase, and endo-glucanase activities were assayed as described previously (Rajoka *et al.* 1998). The total extracellular protein, indicative of the secretion ability of the organism, were assayed by Bradford method. Each variable was tested in triplicate and each assay was done in duplicate.

Determination of kinetic parameters

Enzyme activities (products per g substrate) were determined as mentioned earlier in this section. The 'growth yield coefficient' ($Y_{X/S}$) was calculated as the dry cell mass per mass of solid substrate utilized from the test substrates added to each flask. The volumetric rate of solid substrate utilization (Q_S) or enzyme productivity (Q_P) was determined from the maximum slope in plot of substrate and enzyme produced against time of fermentation. Cell mass productivity (g dry cells per flask per h) was maximum slope of a plot g cells vs. time of fermentation as described previously (Haq *et al.* 2002). Specific productivity (q_P) was a multiple of μ and $Y_{P/X}$ ($Y_{P/X}$ = product yield coefficient, IU per g dry cell mass) while $Y_{P/S}$ was IU per mass of substrate utilized from the test substrates.

Thermodynamic parameters

The empirical approach of the Arrhenius equations (Aiba *et al.* 1973) was used to describe the relationship of temperature-dependent reversible and irreversible inactivation of endo-xylanase production for temperature range 30–60 °C. For this purpose, specific rate of product formation (q_P, IU per g cells per h) was used to calculate these variables using equation (2):

$$q_P = T \cdot k_B/h \; e^{\Delta S^*/R} e^{-\Delta H^*/RT} \quad (1)$$

$$\ln(q_P/T) = \ln(k_B/h) + \Delta S^*/R - \Delta H^*/RT \quad (2)$$

Plot of $\ln(q_P/T)$ against $1/T$ gave a straight line whose slope was $-\Delta H^*/R$ and intercept was $\Delta S^*/R + \ln(k_B/h)$, where h (Planck's constant) $= 6.63 \times 10^{-34}$ J s and k_B (Boltzmann constant), $[R/N] = 1.38 \times 10^{-23}$ J K^{-1} where N (Avogadro's number) $= 6.02 \times 10^{23}$ mol^{-1}.

Statistical analysis

Treatment effects were compared by the protected least significant difference methods with ANOVA-II using MstatC software.

Results and discussion

Studies on production and secretion of endo-xylanase are important to develop enzyme systems which could be directly used for converting industrial wastes into enzymes for industrial purposes using wild or mutated cultures. Mutation alters the culture's behaviour and its potential to form metabolites. The 2-deoxy-D-glucose resistant mutants of *H. lanuginosa* were obtained by u.v. mutagenesis as described by Chadha *et al.* (1999). Well-developed zones of clearance on xylan plates appeared around three colonies and the mutant LH TH1 was selected for detailed enzyme production in solid-state fermentation.

Initially extensive screening and optimization studies were performed to select the best cultural condition for maximum production of endo-xylanase. Among carbon sources (Tables 1 and 2), both rice bran (281 IU / l^{-1} h^{-1}) and wheat straw (275 IU l^{-1} h^{-1}) supported

Table 1. Comparative substrate consumption kinetic parameters of *Humicola lanuginosa* (P) and its derepressed mutant TH1 (M) following growth on different substrates in solid state fermentation.

Carbon source/Strain	Q_X (g flask^{-1} h^{-1})	$Y_{X/S}$ (g g^{-1})	Q_S (g flask^{1} h^{-1})	Q_P (mg flask^{-1} h^{-1})
Canola meal				
P	0.027^{d}	0.50^{b}	0.026^{e}	1.0^{d}
M	0.032^{c}	0.51^{ab}	0.038^{b}	1.2^{c}
Rice bran				
P	0.037^{b}	0.51^{ab}	0.038^{b}	1.2^{c}
M	0.043^{a}	0.52^{a}	0.042^{a}	1.6^{a}
Sunflower meal				
P	0.022^{e}	0.50^{b}	0.031^{d}	1.0^{d}
M	0.032^{c}	0.50^{b}	0.034^{c}	1.2^{c}
Wheat straw				
P	0.032^{c}	0.51^{ab}	0.039^{b}	1.5^{b}
M	0.042^{a}	0.52^{a}	0.043^{a}	1.6^{a}

Each value is a mean of three replicates. Standard deviation among replicates were 7.5–10% of mean values and have been ignored. Values followed by different letters differ significantly at $P \leq 0.05$ using MstatC software. To calculate the values of volumetric productivities of cell mass and protein production and substrate utilization, multiply the values given in columns 1, 3 and 4 by 20.

Table 2. Endo-xylanase formation kinetic parameters of *H. lanuginosa* (P) and its derepressed mutant (M) following growth on different substrates in solid state fermentation at 45 °C.

Carbon source/Strain	Q_P (IU flask^{-1} h^{-1})	$Y_{P/S}$ (IU g^{-1} S)	$Y_{P/X}$ (IU g^{-1} cells)	Specific activity (IU mg^{-1} protein)
Canola meal				
P	95^{f}	400^{f}	909^{e}	95^{e}
M	180^{c}	588^{d}	1177^{c}	180^{c}
Rice bran				
P	181^{c}	641^{c}	1180^{c}	151^{c}
M	281^{a}	842^{a}	1615^{a}	171^{a}
Sunflower meal				
P	79^{g}	484^{l}	968^{d}	78^{f}
M	125^{l}	608^{c}	1216^{b}	117^{d}
Wheat straw				
P	177^{d}	639^{b}	1178^{c}	145^{c}
M	275^{b}	840^{a}	1612^{a}	172^{b}

Each value is a mean of three replicates. Values followed by different letters differ significantly at $P \leq 0.05$ using MstatC software. To calculate volumetric productivity per litre multiply the values in IU per flask per h by 20.

maximum endo-xylanase volumetric productivities. The latter was selected because it was the most inexpensive substrate and supported 98% of activity provoked by rice bran.

Effect of nitrogen sources on enzyme production

To see the improved production of xylanase by different nitrogen sources, easily metabolizable nitrogen sources like ammonium sulphate and ammonium nitrate were comparatively evaluated with reference to sodium glutamate, sodium nitrate, urea, and corn steep liquor when they were added to wheat straw at equimolar concentration (at 1.2 g nitrogen per l of medium). They supported endo-xylanase productivity of 195, 125, 210, 123, 275 and 234 IU l^{-1} h^{-1}, respectively. It was observed that urea, released NH_4^+ slowly and was the best nitrogen source. This was attributed to high urease activity of the organism and terminal pH values which were 4.5, 4.3, 5.3, 4.2, 5.5 and 6.5, respectively. Due to proper pH and high urease activity, sufficient nitrogen was available for utilization and supported production of more enzyme than that by other nitrogen sources. Urea (at 10%, w/v) supported maximum of 48 and 76 IU per ml endo-xylanase in parental and mutated culture respectively and was used in all studies. It was also shown that the 2-deoxy-glucose resistant mutant produced xylanase to a greater extent than the wild-type organism.

Effect of pH on xylanase production

The pH of the medium regulates product formation. pH conditions were optimized for maximum production of xylanase by *H. lanuginosa* and its mutant. Maximum activity was observed at pH 5.5 (Figure 1) and was maintained in all further studies. Further studies revealed that enzyme extraction was maximal when chilled water was replaced with phosphate buffer (pH 5.5). This was due to the fact that phosphate buffer extracted the enzyme from all fermented substrates by increasing the permeability of cell membrane by diffusion or osmosis.

Further studies were undertaken to produce enzyme in solid-state fermentation in time course under optimized conditions and representative batch culture kinetics of enzyme activity, cell mass and insoluble substrate present in culture broth of rice bran (1), and wheat straw (2) media under the conditions of solid-state culture are shown in Figure 2. The organism was capable of rapid fermentation at temperatures up to 45 °C with significantly ($P \leq 0.05$) higher cell mass productivity (Q_X), rate of substrate consumption (Q_S), protein productivity and cell yield (Table 1) better than those of its wild strain and other strains of *H. lanuginosa* grown at 45 °C (Da Silva *et al.* 1994; Anand & Vithayathil 1996) and *Aspergillus niger* (a fast growing mould) at 30 °C (Hanif *et al.* 2004) and better parameters of product formation (Table 2) than those by the wild-type cultures. The maximum enzyme activities were reached within 48–72 h, while other thermophilic fungi produced maximum activity after 120–140 h of fermentation (Chadha *et al.* 1999; dos Santos 2003). Enhanced substrate consumption, and cell mass formation rates by *H. lanuginosa* suggested its potential in commercial xylanase production process from agro-industrial residues available in Pakistan.

Maximum values of $Y_{P/X}$ (IU g^{-1} cells), or $Y_{P/S}$ (IU g^{-1} substrate consumed) of xylanase and specific enzyme activity by *H. lanuginosa* (Table 2) were several-fold improved over those from *Thielavia terrestris* and *Themoascus crustaceus* (Gilbert *et al.* 1993), and *Thermoascus auranticus* (500 IU g^{-1} bagasse) (dos Santos *et al.* 2003), *Humicola* spp. (Da Silva *et al.* 1994) and favourably comparable with that on *Thermomyces lanuginosus* (Cesar & Mrsa 1996). The *H. lanuginosa* mutant supported maximum volumetric endo-xylanase productivity of 5620 IU l^{-1} h^{-1} on rice bran which was 1.6-fold more than that by the wild strain (Table 2) and significantly more than those of other fungal strains (Kalogeris *et al.* 2003; Kang *et al.* 2004) in SSF process.

Table 3. Comparative volumetric productivity values of *H. lanuginosa* (P) and its mutant TH1 (M) for FP-ase CMCase and β-glucosidase following growth on different substrates in solid state fermentation at 45 °C in optimized culture medium.

Carbon source/ Strain	Q_p (IU l^{-1} h^{-1})		
	FP-ase	CMCase	β-Glucosidase
Canola meal			
P	1.00[g]	0.05	1.30[e]
M	0.80[h]	0.06	0.90[c]
Rice bran			
P	1.51[c]	0.00	1.90[c]
M	1.40[e]	0.00	3.50[a]
Sunflower meal			
P	1.42[d]	0.00	1.80[d]
M	1.16[f]	0.00	2.40[b]
Wheat straw			
P	1.76[a]	0.00	1.00[f]
M	1.60[b]	0.00	1.90[c]

Each value is a mean of three replicates. Values followed by different letters differ significantly at $P \leq 0.05$ using MstatC software.

Increased production of endo-xylanase by DG^r mutant was in line with increased cellulase production in *Neurospora crassa* DG^r mutants or invertase, maltase and phosphatase in *Saccharomyces cerevisiae* DG^r mutants (Rincon *et al.* 2001). Alteration in cell wall synthesis, protein synthesis or cell membrane permeability is a common mechanism of resistance to analogues (Rincon *et al.* 2001). Such permeability changes, may sometimes lead to increased production, presumably through increased rate of product export from the cell. It is conceivable that like the parental strain, the mutant derivative can effectively utilize all substrates, continue to grow, and secrete xylanase in the medium.

The productivity of FP-ase, CMC-ase and β-glucosidase by both wild and mutant cultures were very low on all substrates (Table 3). Endo-xylanase preparations free of cellulase activity are of particular interest for the pre-treatment of paper pulps to decrease the xylan content and, therefore, reduce the dependence on chlorine used for bleaching in the brightening process. This could have economic and environmental benefits to the paper and pulp industry. We intend to mutate this strain in order to further enhance the xylanase titres from cheaper feed-stocks.

Effect of temperature on xylanase production

The fermentation medium along with inoculum was incubated at 30, 35, 40, 45, 50, 55 and 60 °C (Figure 3a). The mutant supported maximum volumetric productivity values of 220, 254, 275, 260, 224 and 190 IU l^{-1} h^{-1}, respectively. Maximum enzyme production was supported at 45 °C. The temperature optimum for production of different xylanases from *H. lanuginosa* and its mutant was mainly between 45 and 50 °C (Da Silva *et al.* 1994). At lower and higher temperatures, the organism supported lower activity. At lower temperature, the transport of nutrients into the cells is hindered while at high temperature, maintenance energy requirement increases and less product formation occurs (Aiba *et al.* 1973).

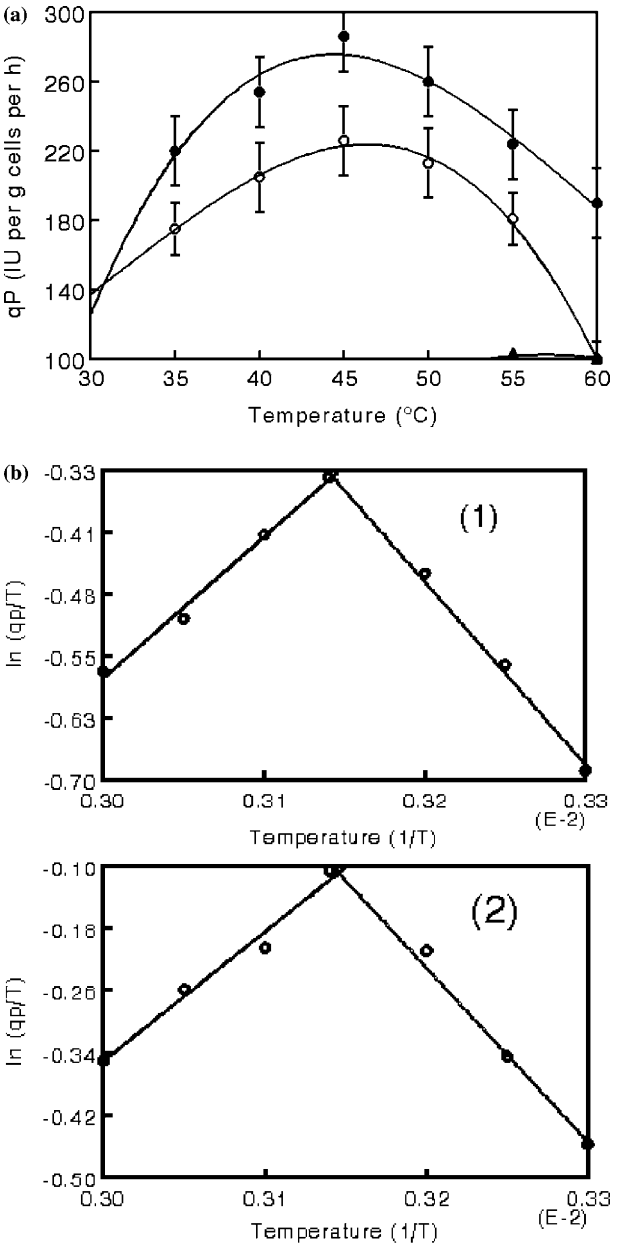

Figure 3. (a) Effect of fermentation temperature on endo-xylanase specific productivity (q_p) of wild and mutated cultures on wheat straw medium pH (5.5). (b) Arrhenius plot on enthalpy and entropy of endo-xylanase formation and its deactivation using specific productivity values of wild-type (1) and its mutant derivative (2) at different temperatures. For details, see Materials and methods.

Thermodynamic parameters of endo-xylanase production

In order to understand the mechanism of endogenous metabolism during xylanase formation, thermal energy demand for enzyme formation and its consequent inactivation were determined using the Arrhenius relationship as described previously for different enzyme production system (Aiba *et al.* 1973). Activation enthalpy and entropy of β-endo-xylanase production and inactivation were calculated from Figure 3b. The values

Table 4. Thermodynamic parameters* estimated by the Arrhenius approach for batch formation and deactivation of β-endo-xylanases from both parental and mutant cultures of *Humicola lanuginosa*.

	Enzyme formation	Thermal inactivation
Activation enthalpy (kJ mol^{-1}) for		
Parental	18.7 ± 1.3[a]	19.6 ± 1.2[b]
Mutant	13.7 ± 1.2 [a]	24.6 ± 1.5[b]
Activation entropy (kJ mol^{-1} K^{-1})		
Parental	(−) 0. 177 ± 0.014[a]	(−) 0. 242 ± 0.020[b]
Mutant	(−) 0.182 ± 0.012[a]	(−) 0.256 ± 0.021[b]

Each value is a mean of three independent studies. Values followed by different letters in each row are significantly different from each other at $P \leq 0.05$.
*Thermodynamic parameters were determined as described in Materials and methods.

(Table 4) indicated that the activation enthalpy of β-endo-xylanase formation by the mutant (ΔH^* = 13.7 ± 1.2 kJ mol^{-1}) is significantly lower than that for its wild organism, phytase production (70–80 kJ mol^{-1}) by *Aspergillus carbonarius* (Al-Asheh & Duvniak 1994) as well as other microbial processes, such as cell growth (34–74 kJ mol^{-1}) (Converti & Dominguez 2001). Activation enthalpy (ΔH_D^*) of thermal inactivation (24.6 ± 1.5 kJ mol^{-1}) (Table 4) is higher than that for its own production and that for enzyme from the wild organism but is comparable with that for β-galactosidase from a *Kluyveromyces marxianus* mutant (Rajoka *et al.* 2004). It is significantly ($P \leq 0.05$) lower than the values on glucose isomerase system (160–235 kJ mol^{-1}) (Converti & Del Borghi 1997). The activation entropy of β-endo-xylanase formation (−0.182 kJ mol^{-1} K^{-1}) is very low and even lower than that for its parental organism. The activation entropy value of thermal inactivation (−0.256 kJ mol^{-1} K^{-1}) is lower than that required by the enzyme from wild organism. On the other hand, its value is also very low (and has a negative sign) which signifies that this inactivation phenomenon implies a little disorder during its inactivation during growth on the substrate up to 60 °C and suggests a sort of higher protection exerted by the cell system of mutant cells compared with wild-type cells (possibly due to chaperone activity) against thermal inactivation.

These studies have provided insight into improvement in enzyme production by mutation leading to 2-deoxyglucose resistance. The mutation altered the values of both entropy and enthalpy of activation for product formation as reported earlier for β-galactosidase produced by a thermotolerant yeast *Kluyveromyces marxianus* (Rajoka *et al.* 2004).

Conclusion

The mutant of *H. lanuginosa* has the obvious advantage of enhanced production of xylanase and may serve as a starting strain for further genetic improvement. It is concluded that this organism may be exploited for bulk production of xylanase using inexpensive rice bran or wheat straw abundantly available in the country.

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References

Adams, P.R. 1994 Extracellular amylase activites of *Rhizomucor pusillus* and *Humicola lamuginosa* at initial stages of growth. *Mycopathologia* **128**, 139–141.

Aiba, S., Humphrey, A.E. & Millis, N.F. 1973 *Biochemical Engineering*. 2nd ed, pp. 92–127 New York: Academic Press. ISBN 0120450526.

Al-Asheh, S. & Duvniak, Z. 1994 Characteristics of phytase produced by *Aspergillus carbonarius* NRC 401121 in canola meal. *Acta Biotechnologica* **14**, 223–237.

Anand, L. & Vithayathil, P.J. 1996 Xylan-degrading enzymes from thermophilic fungus *Humicola lanuginosa*, action pattern of xylanase and β-glucosidase on xylans, xylooligomers and arabinooligomers. *Journal of Fermentation and Bioeng*ineering **81**, 511–517.

Cesar, T. & Mrsa, V. 1996 Purification and properties of the xylanase produced by *Thermomyces lanuginosus*. *Enzyme and Microbial Technology* **19**, 289–296.

Chadha, B.S., Jaswinder, K., Rubinder, K., Saini, H.S. & Singh, S. 1999 Xylanase production by *Thermomyces lanuginosus* wild and mutant strains. *World Journal of Microbiology and Biotechnology* **15**, 195–198.

Converti, A. & Del Borghi, M. 1997 Simultaneous effects of immobilization and substrate protection on the thermodynamics of glucose isomerase activity and inactivation. *Enzyme and Microbial Technology* **21**, 511–517.

Converti, A. & Dominguez, J.M. 2001 Influence of temperature and pH on xylitol production from xylose by *Debarryomyces hansenii*. *Biotechnology and Bioengineering* **75**, 39–45.

Da Silva, R., Yin, D.K. & Park, K.K. 1994 Application of thermostable xylanases from *Humicola sp*. for pulp improvement. *Journal of Fermentation and Bioeng*ineering **77**, 109–111.

dos Santos, E., Piovan, T. Roberto, I.C. & Milagres, A.M. 2003 Kinetics of the solid state fermentation of sugarcane bagasse by *Thermoascus auranticus* for the production of xylanase. *Biotechnology Letters* **25**, 13–16.

Duenas, R., Tengerdy, R.P. & Gutierrez-corea, M. 1995 Cellulase production by mixed fungi in solid-substrate fermentation of bagasse. *World Journal of Microbiology and Biotechnology* **11**, 333–337.

Gilbert, M.M., Yaguchi, D.C., Watson, K.K.Y., Wong, C., Breuil, & Saddler, J.N. 1993 A comparison of two xylanases from the thermophilic fungi *Thielavia terrestris* and *Thermoascus crustaceus*. *Applied Microbiology and Biotechnology* **40**, 508–514.

Hanif, A., Yasmin, A. & Rajoka, M.I. 2004 Production, repression and derepression of β-cellobiosidase synthesis in *Aspergillus niger*. *Bioresource Technology* **94**, 311–319.

Haq, I., Khurshid, S., Ali, S., Ashraf, H., Qadeer, M.A. & Rajoka, M.I. 2001 Mutation of *Aspergillus niger* for hyperproduction of citric acid following fermentation of blackstrap molasses. *World Journal of Microbiology and Biotechnology* **17**, 35–37.

Haq, I.U., Idress, S., & Rajoka, M.I. 2002 Production of lipases by *Rhizopus oligosporos* by solid-state fermentation *Process Biochemistry* **37**, 637–641.

Kalogeris, E., Iniotaki, F., Topakas, E., Christakopoulos, P., Kekos, D. & Macris, B.J. 2003 Performance of an intermittent agitation rotating drum type bioreactor for solid state fermentation of wheat straw. *Bioresource Technology* **86**, 207–213.

Kang, S.W., Park, Y.S., Lee, J.S., Hong, H.I., & Kim, S.W. 2004 Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass. *Bioresource Technology* **91**, 153–156.

Lockington, R.A., Rodbourn, L., Barnett, S., Carter, C.J. & Kelly, J.M. 2002 Regulation by carbon and nitrogen sources of a family of cellulases in *Aspergillus nidulans*. *Fungal Genetics and Biology* **37**, 190–196.

Miller, G.L. 1959 Use of dinitrosalicylic acid (DNS) for determination of reducing sugars. *Analytical Chemistry* **31**, 426–428.

Nigam, P. & Singh, D. 1994 Solid state (substrate) fermentation systems and their applications in biotechnology. *Journal of Basic Microbiology* **34**, 405–423.

Pandey, P. 2002 Production of cellulase free thermostable xylanase by an isolated of *Aspergillus niger PPI*, utilizing various lignocellulosic wastes. *World Journal of Microbiology and Biotechnology* **18**, 281–283.

Rajoka, M.I., Bashir, A. & Malik, K.A. 1997 Mutagenesis of *Cellulomonas biazotea* for enhanced production of xylanases. *Bioresource Technology* **62**, 99–108.

Rajoka, M.I., Bashir, A., Hussain, M.R.A. & Malik, K.A. 1998 Mutagenesis of *Cellulomonas biazotea* for improved production of cellulases. *Folia Microbiologica* **43**, 15–22.

Rajoka, M.I., Latif, F., Khan, S. & Shahid, R. 2004 Kinetics of improved productivity of β-galactosidase by a cycloheximide-resistant mutant of *Kluyveromyces marxianus*. *Biotechnology Letters* **26**, 741–746.

Rincon, A.M., Codon, A.C., Castrejon, F. & Benitez, T. 2001 Improved properties of baker's yeast mutant resistant to 2-deoxy-D-glucose. *Applied and Environmental Microbiology* **67**, 4279–4285.