

Hyper-production of a thermotolerant β -xylosidase by a deoxy-D-glucose and cycloheximide resistant mutant derivative of *Kluyveromyces marxianus* PPY 125

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Production of β -xylosidase by a cycloheximide and 2-deoxy-D-glucose-resistant mutant of *Kluyveromyces marxianus* PPY125 was studied when cultured on growth media containing galactose, glucose, xylose, cellobiose, sucrose and lactose as carbon sources. Xylose, cellobiose, lactose and sucrose were the key substrates. Both *K. marxianus* PPY125 and its mutant (M 125) supported maximum β -xylosidase specific product yield ($Y_{P/X}$) following growth on xylose. Basal level of activity was observed in non-induced cultures grown on glucose. The mutant produced 1.5 to 2-fold more β -xylosidase than that produced by the wild cells. Synthesis of β -xylosidase was regulated by an induction mechanism in both wild and mutant cells. Addition of glucose did not inhibit the synthesis of β -xylosidase in both parental and mutant cultures in the presence of corn steep liquor. Partially purified enzyme showed good stability when incubated at 60°C and was quite stable at pH 5.0-7.0. Thermodynamic studies revealed that the enzyme derived by the mutant M125 was more thermostable as evidenced by higher midpoint inactivation temperature, lower activation energy demand for β -xyloside hydrolysis, as well as lower enthalpy and entropy demand for reversible denaturation of enzyme.

β -xylosidase (EC 3.2.1.37) is one of the component enzymes of the hemicellulase complex and is widely distributed in nature. It catalyzes the hydrolysis of alkyl- and aryl- glycosides as well as xylobiose and xylo-oligosaccharides to xylose. In industry, it is employed for hydrolysis of bitter compounds from grape fruit during juice extraction and liberation of aroma from grapes during wine making (Manzanares et al. 1999). β -xylosidase, in synergistic action with endo- β -xylanases, (EC 3.2.1.8) and debranching enzymes namely α -glucuronidases, esterases and glycosidases have potential application in production of biofuels and in the processing of food. Cellulase-free xylanases have an important role in reducing consumption of chlorine and chlorine dioxide in paper and pulp industry (Tsuji et al. 2001). Yeasts capable of producing glycosidases may be more suitable for the last application as they will require cloning of endo-xylanase gene only for this implication.

Kluyveromyces marxianus has been employed for production of biomass, enzymes and ethanol (Belem and Lee, 1998; Furlan et al. 2000). Recently production of β -xylosidase has been described in *K. marxianus* and other yeasts (Manzanares et al. 1999) but no detailed study is available on its production by wild or mutant cultures of *K. marxianus*. Xylose, xylobiose, synthetic β -xylosides and

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xylo-oligosaccharides are inducers of this enzyme in yeasts (Belem and Lee, 1998), but, like other enzymes, other carbon sources may serve as its inducers (Rajoka et al. 1997). The production of enzymes is influenced by induction and catabolite repression (de Groot et al. 2003). Carbon catabolite repression alters transcription and is regulated by CreA protein (catabolite repressible entities), a transcriptional repressor of glucose-repressible genes (de Vries et al. 1999). Increases in β -xylosidase production and redirection of transport system may enhance substrate utilization and product formation. 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). In this work we report, the isolation of a derepressed mutant of *K. marxianus* which was also cycloheximide (protein synthesis inhibitor) (Gerlinger et al. 1997) and to establish the optimal culture conditions. This mutant, deregulated for β -xylosidase synthesis is also believed to have better transport system (Rajoka et al. 1998). This work reports regulation and hyper-production of β -xylosidase following growth of *K. marxianus* PPY125 and its mutant M125 on different carbon sources under optimized fermentation conditions (initial pH 5.5, inoculum size 10%, and yeast extract 0.3%) with reference to its wild organism. Kinetic properties of partially purified enzyme were also studied to elucidate the thermo-tolerant/thermo-stable nature of this enzyme compared with those of thermostable or thermo stabilized enzymes reported in literature.

MATERIALS AND METHODS

Organism and growth conditions

Kluyveromyces marxianus PPY125, collected from Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan was maintained on malt extract plates and was grown in glucose yeast salts medium containing (g/l) KH_2PO_4 2.0, $(\text{NH}_4)_2\text{SO}_4$ 5.0, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.0, glucose 10, and yeast extract 3.0 at 35°C with shaking at 150 rpm. The cultures were centrifuged (10,000 g, 10 min), and used at 10% (v/v) containing 1 mg dry cells/ml.

Selection of mutant

The exposure of cell suspension (2×10^9 cells/ml) to γ -irradiation (24 hrs) of 800 Gy caused approximately a 3 logarithmic reduction in viable cells. The resistant cells were expressed in yeast medium containing 160 μg cycloheximide/ml. +0.6% (w/v) of 2-deoxy-D-glucose (a glucose analogue). After 48 hrs incubation, serial dilution of expressed cells was plated onto ortho-nitrophenyl β -D-xylopyranoside +cycloheximide +2-deoxy-D-glucose agar plates and putative mutant was selected after incubation at 50°C as described previously (Rajoka et al. 1997). The best mutant was designated M125 and tested for β -xylosidase

production using different carbon and nitrogen sources.

Table 1. Comparative kinetic parameters of *Kluyveromyces marxianus* ppY125 and its mutant derivative (M 125) for β -xylosidase formation following growth on different substrates (2%, w/v) in yeast fermentation medium (pH 5.5) at 35°C up to 48 hrs in time course studies.

C-source	$Y_{P/S}$ (IU/g)	Specific activity (IU/mg)	Q_p^{**} (IU/l h)	$Y_{P/X}$ (IU/.g cells)
Galactose				
P	41.7h	1.8g	11g	88j
M	84.0g	3.7f	23f	166i
Glucose				
P	1.1j	0.2i	1i	2.0 l
M	4.1i	0.6h	4h	11k
Xylose				
P	192.0d	15.8c	98b	333e
M	391.2a	24.4a	154a	653a
Cellobiose				
P	191.0d	15.0d	96b	315f
M	389.1a	23.2b	153a	425b
Lactose				
P	122.2f	7.5e	41e	244h
M	246.1c	14.8d	83d	365c
Sucrose				
P	135.1e	7.7e	43e	267g
M	273.1b	15.0d	87c	361c

Each value is a mean of three replicates. Values followed by different letters differ significantly from each other at $p \leq 0.05$ using MstatC software ** Q_p = volumetric productivity (IU/l.h), $Y_{P/X}$ = Specific product yield (IU/g cells), $Y_{P/S}$ = Product yield (IU/g substrate consumed).

Enzyme production

Growth kinetics of the organisms (in 1-l Erlenmeyer flasks in triplicate) was examined in the optimized medium (pH 5.5) containing glucose, cellobiose, lactose, sucrose and xylose (20 g/l each) as sole carbon sources and corn steep liquor as nitrogen source in place of ammonium sulphate unless mentioned otherwise (Rajoka et al. 2004). Inoculum was prepared in glucose medium at 35°C on a rotary shaker (150 rpm). The culture was centrifuged (10000 g, 30 min), and used at 10% (v/v) in saline containing 1 mg dry cells/ml. All experiments were carried out at 35°C (unless otherwise mentioned) in triplicate in time course studies on a gyratory shaking-incubator up to 48-72 hrs. The cells were recovered as above, probe sonicated, fractionated as described earlier (Rajoka et al. 1997) and cell extract was assayed for intracellular enzyme activities while cell mass was determined gravimetrically.

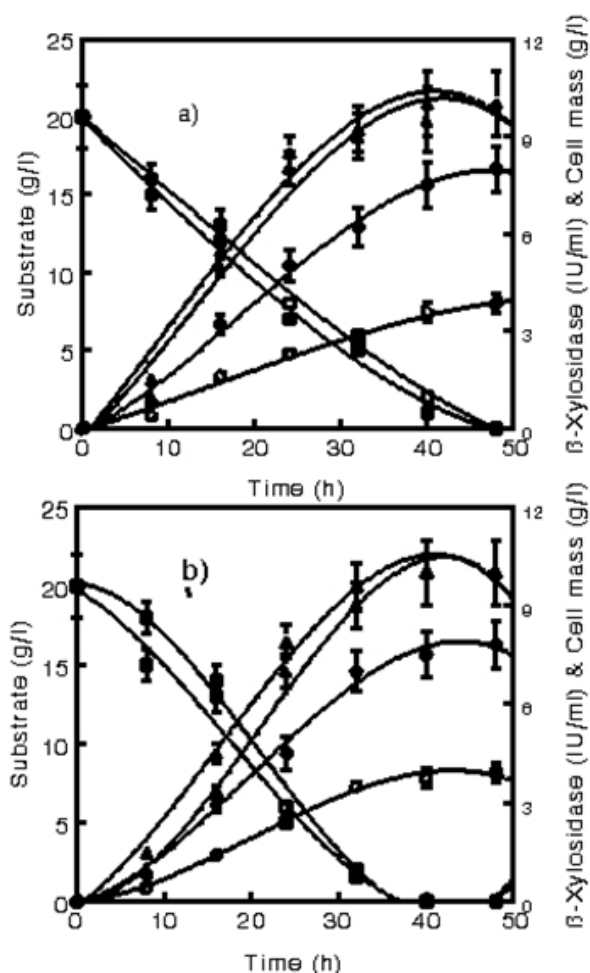


Figure 1. Kinetics of β -xylosidase production by *K. marxianus* PPY125 and its mutant M125 in shake flask fermentation of two representative substrates namely: (a) xylose, and (b) cellobiose

Each 2% using yeast basal salt medium. The initial pH of the medium was 5.5, inoculum size 10%, and temperature 35°C. O = β -xylosidase activity IU per ml (open, parent; closed, mutant); Δ = cell mass (g per l; open, parent; closed, mutant); \square = substrate (g per l) ; open, parent; closed, mutant). Error bars represent standard deviation among three replicates.

Effect of varying temperature on β -xylosidase production

To calculate kinetic and thermodynamic values, the data of batch fermentation were gained by performing experiments (three runs) on xylose-corn steep liquor media on an orbital shaker (150 rpm) at a temperature range 22-45°C (25, 27, 30, 32, 35, 37, 40, 43, 45°C).

Enzyme assays

β -Xylosidase was assayed using 1 mM *p*-nitrophenyl- β -D-

xylopyranoside as substrate in 50 mM sodium acetate buffer, pH 5.0 (unless otherwise stated). One millilitre of the properly diluted enzyme sample was incubated with 1 ml of substrate solution at 40°C for 10 min. The reaction was stopped by adding 2 ml of 1M sodium carbonate. The liberated *p*-nitro phenol was measured at 400 nm with a spectrophotometer. One IU of β -xylosidase was defined as the amount of enzyme that released 1 μ mol *para*-nitrophenol per min under the assay conditions.

Protein determination

The proteins were determined by Bradford method (1976) using bovine serum albumin as the standard.

Saccharides determination

Total saccharides were determined after Miller (1959). Different saccharides were analyzed by HPLC (Perkin Elmer, USA) with Aminex column HPX-87H (300 x 78 mm, Bio-Rad, Richmond, California) maintained at 45°C in a column oven. Sulphuric acid (0.002 N) served as a mobile phase at 0.6 ml/min. The samples were detected using refractive index detector and quantified using Turbochrom4 software of Perkin Elmer, USA.

Table 2. Kinetic and thermodynamic parameters for irreversible thermal inactivation of β -xylosidase from *K. marxianus*.

T	K_d $10^{-3} h^{-1}$	$t_{1/2}$ (h)	ΔH^* kJ/mol	ΔG^* kJ/mol	ΔS^* J.mol/K
323 P M	3.0g 2.31h	231b 300a	54.3a 48.3b	117.59b 117.61b	-195.9a -214.6h
328 P M	4.2e 3.5f	165d 200c	54.3a 48.3b	117.84b 118.34a	-193.7c -213.5g
333 P M	11.0c 9.2d	63f 75e	54.2a 48.2b	117.0c 117.5c	-188.6d -208.1f
338 P M	69.3a 36.5b	10h 19g	54.2a 48.2b	113.64e 115.44d	-175.9e -198.9b

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

K_d = First order rate constant for inactivation;

ΔH^* (kJ/M)= E_a (57 kJ/M)-RT where E_a is activation energy;

ΔG^* (kJ/M)= $-RT \ln \{(k_d \cdot h)/k_B \cdot T\}$;

ΔS^* is entropy of irreversible inactivation and was calculated from $\Delta S = (\Delta H^* - \Delta G^*) / T$.

Determination of kinetic parameters

All kinetic parameters were determined as described

previously (Lawford and Rousseau, 1993; Converti and Dominguez, 2001) in shake-flasks. Thermodynamics of enzyme production was performed as described previously (Converti and Dominguez, 2001). Kinetics of enzyme was performed as described previously (Siddiqui et al. 1997).

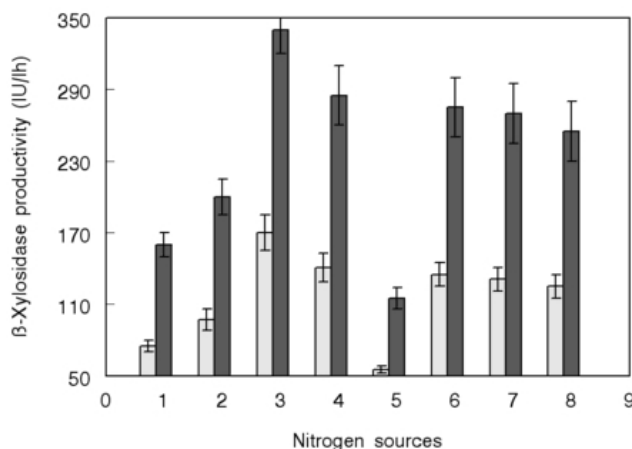


Figure 2. Effect of different nitrogen sources on β -xylosidase production by *K. marxianus* PPY125 (light squares) and its mutant M125 (dark squares). Organisms were grown on xylose (20 g/l, w/v) containing different nitrogen sources (1 = ammonium nitrate; 2 = ammonium sulphate; 3 = corn steep liquor; 4 = soybean meal; 5 = sodium glutamate; 6 = sodium nitrate; 7 = urea; and 8 = peptone) at a rate of 1.1 g/l equivalent to that contained in ammonium sulphate in triplicate. Flasks were harvested and processed as described in Materials and Methods. Each value is a mean of three independent experiments. Error bars represent standard deviation among three replicates.

pH and temperature tolerance

After 40 hrs of xylose fermentation, cells were harvested and β -xylosidase was extracted after probe-sonication according to Rajoka et al. (1997). The enzyme was partially purified by precipitation with 25% ammonium sulphate at 4°C, and centrifuged. The precipitate was discarded and more ammonium sulphate was added in supernatant to make up its concentration up to 50% and left overnight at 4°C. This time, precipitate was retained, dialyzed, and assayed after 30 min incubation in different buffers (containing phenyl methyl sulphonyl fluoride as protease inhibitor) as described earlier (Siddiqui et al. 1997).

The above enzyme containing 0.1 mM PMSF was incubated at different temperatures for 30 min (Siddiqui et al. 1997) and remaining β -xylosidase activity was determined. β -xylosidase was re-dissolved in 50 mM MES monohydrate buffer (pH 5.5) and assayed for thermostability (Siddiqui et al. 1997). Activation energy (E_a) was determined by using the data for optimum temperature. Arrhenius relationship was used to calculate the activation energy required by the enzyme to hydrolyze *p*-nitrophenyl- β -xylopyranoside as described earlier (Siddiqui et al. 1997).

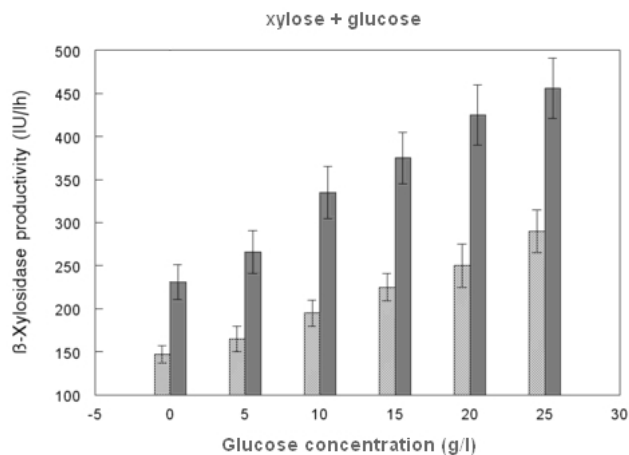


Figure 3. Effect of addition of glucose (5, 10, 15, 20 and 25 g/l) in the xylose-corn steep liquor medium on volumetric productivity of β -xylosidase produced by the parental (light squares) and mutant (dark squares) cultures. Each value is a mean of three independent experiments. Error bars represent standard deviation among three replicates

Thermodynamics of enzyme properties

Thermal inactivation of the enzyme was determined by incubating the enzyme solutions in above buffer at a particular temperature. Aliquots were withdrawn at different times, cooled on ice for 3 hrs and assayed for enzyme activity at 40°C as described above. This procedure was repeated at 5 different temperatures ranging from 45-70°C. The data were fitted to first order plots and analyzed. The first order rate constants (k_d) were determined by linear regression of $\ln(V)$ vs. time of incubation (t). The thermodynamic data were calculated by rearranging the Eyring absolute rate equation to study the overall thermodynamic C: parameters in the range 45-70°

$$K_d = T \cdot k_B / h \cdot e^{\Delta S^* / R} \cdot e^{-\Delta H^* / R \cdot T} \quad [1]$$

$$\ln(k_d / T) = \ln(k_B / h) + \Delta S^* / R - \Delta H^* / R \cdot 1/T \quad [2]$$

where k_d , T , k_B , h , ΔS^* , ΔH^* and R are specific reaction velocity, absolute temperature, Boltzmann constant, Planck's constant, entropy of activation, enthalpy of activation and gas constant respectively.

Statistical analysis

Treatment effects were compared by the protected least significant difference method and have been presented as two-factor factorial design in the form of probability (p) using MstatC software

RESULTS AND DISCUSSION

Kinetics of enzyme production following growth on different carbon sources

Initial studies were performed to optimize substrate and

substrate concentration. Among different substrates employed, 2% (w/v) xylose supported the maximum enzyme activity, followed by cellobiose. Representative kinetics of enzyme production following growth on xylose (a) and cellobiose (b) respectively is presented in Figure 1. These curves indicated that bulk production of β -xylosidase was predominantly growth-associated. Application of Luedeking and Piret model (Aiba et al. 1973), however, indicated that the enzyme production was both growth- as well as non-growth-associated (results not presented) and confirmed the work of Inchaurredo et al. (1994). The efficacy of inducers can be determined by both their actual concentration in side the cell and their binding affinity to regulatory macromolecules (Perez-Gonzalez, 1998; La Grange et al. 2001). This was determined by using inducers (xylose and disaccharides) and non-inducer (glucose). Specific enzyme yield ($Y_{P/X}$), in the case of wild culture, was 333 IU per g cells on xylose, and only 2 IU per g cells (as basal enzyme activity) on glucose (non- inducer). The induction ratio, defined as the ratio of activity on inducer to basal activity on non-inducer, was 165.5 and 140.0 on xylose and disaccharides (Table 1) in wild cells respectively and that mutant was significantly (up to 2.0-fold) improved for product formation parameters. The results of Table 1 suggest that biosynthesis of β -xylosidase was regulated by an induction mechanism that enhanced the specific enzyme yield ($Y_{P/X}$) of β -xylosidase up to 166.5-fold in induced over non-induced cultures in wild cells and up to 59-fold in mutant cells. There were no significant differences in the values of specific growth rate, specific substrate uptake rate and volumetric substrate uptake rates during growth on all carbon sources in the wild and mutant organisms (results not presented) still mutant 125 differed significantly ($p \leq 0.05$) over its wild cells for formation of β -xylosidase parameters. In the wild cells, the values of specific substrate uptake rate (q_s) from disaccharides (0.587 ± 0.162), xylose (0.73 g/g.h), and glucose (0.95 g/g.h) were significantly different from each other (results not presented) and product formation kinetic parameters (Table 1) were higher on xylose, followed by disaccharides, galactose and glucose. Thus product formation was inversely related to the values of q_s . These studies indicated that product formation was regulated by growth-dependent repression mechanism and confirmed the work of Li and Ljungdahl, 1994).

Best yield of β -xylosidase (Table 1) on xylose (in both organisms) is in good agreement with that reported by Perez-Gonzalez (1998). In other enzyme systems, disaccharides or high molecular weight substrates have been found to be the best inducers of β -xylosidase (Rajoka et al. 1997). Maximum values of product formation kinetic parameters in the mutant culture (Table 1) are higher than the calculated values on several *Saccharomyces cerevisiae* recombinants harboring heterologous genes of β -xylosidase from *Aspergillus niger*, different bacteria and *A. nidulans* (Perez-Gonzalez et al. 1998; La Grange et al. 2001), and *Cellulomonas* and other strains (Rajoka et al. 1997).

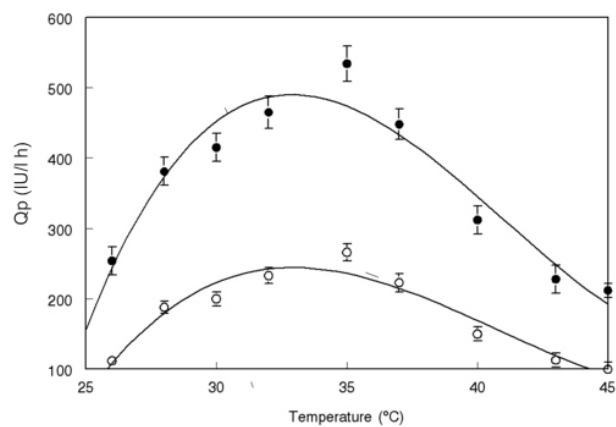


Figure 4. Effect of temperature on volumetric productivity (IU/h) of β -xylosidase during growth of *K. marxianus* PPY125 and its mutant (M125) cultures on xylose (2%) medium (pH 5.5). The organisms were grown as described in **Methods**. Each value is a mean of three independent experiments. Error bars represent standard deviation among three replicates.

Different nitrogen sources were employed to give equimolar amount of nitrogen maintained at 1.1 g/l, and xylose as carbon source (Figure 2). Corn steep liquor and soy bean were the best nitrogen sources followed by sodium nitrate, urea and peptone. (Rajoka et al. 1998) observed that an increase in NaNO_3 concentration greatly increased cellulase synthesis in *Cellulomonas biazotea*.

Catabolite repression plays an important role in the regulation and secretion of inducible enzymes. Such repression effect has been observed in several organisms (Li and Ljungdahl, 1994; Ponce-Noyola and de la Torre, 2001). Production of β -xylosidase by the organism on xylose-yeast medium using corn steep liquor as nitrogen source when glucose was added (5, 10 to 15 g/l final concentration respectively) at the time of inoculation (Figure 3), showed that there was significant enhancement in the enzyme synthesis rate in both wild and mutant cells and there was no inhibition of enzyme synthesis. Mixed inductive or repressive effect has been observed in other organisms (Li and Ljungdahl, 1994; Ponce-Noyola and de la Torre, 2001). When corn steep liquor was used as a nitrogen source, no inhibitory effect was observed when even glucose served as the carbon source. Corn steep liquor may not have supported the formation of Cre A protein as observed in its absence (Lockington et al. 2002).

Effect of initial temperature of fermentation on β -xylosidase production

Temperature is known to affect microbial growth, cell biomass and consequently it may also affect β -xylosidase production. Maximum specific productivity (q_p) of β -xylosidase occurred at fermentation temperature of 35°C (Figure 4). At higher or lower temperature, β -xylosidase

production by the cells was decreased. At lower temperature, the transport of substrate across the cells is suppressed and lower yield of products are attained (Aiba et al. 1973). At higher temperature, the maintenance energy requirement for cellular growth is high due to thermal inactivation of the enzymes of the metabolic pathway (Aiba et al. 1973) resulting in lower amount of product formation. The productivity decline observed at high temperature could be due to the reversible denaturation of enzyme formed on optimized-medium as described previously (Converti and Dominguez, 2001).

Enzyme properties

The enzyme showed maximum activity at 55°C. Thermal inactivation followed first order kinetics in the presence of PMSF. Half life of the enzyme was 231, 165 and 63 hrs at 50°C, 55°C and 60°C respectively (calculated as described by Rashid and Siddiqui, 1998), compared with half life of 4.85 h at 65°C exhibited by *E. coli* recombinant harboring heterologous β -xylosidase genes from thermophilic organisms (Tsuji et al. 2001) and confirmed the thermostability of mutant derived β -xylosidase.

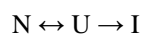
Thermostability

The protein mid-point inactivation temperature, activation energy, values of enthalpy and entropy of enzyme inactivation are considered potential determinants of enzyme thermostability. The protein mid-point inactivation temperature (T_m) was 65°C (results not shown). For wild-type nuclease at pH 7.0, the T_m of 53.3 was obtained (Shortle et al. 1988). Optimum activity was at 50°C for an incubation of 10 min while compensation temperature was 68°C (results not shown) which was higher than that for thermostable or thermo stabilized mutants (Chen et al. 1994; Rashid and Siddiqui, 1998; Declerck et al. 2003).

Activation energy for catalysis of pNBX β was 57 kJ/ mol (results not presented) which is significantly lower than required by native or chemically modified β -glucosidases (389 or 174 kJ/ mol) (Rashid and Siddiqui, 1998) and thermostable glucoamylase from a thermostabilized mutant derivative of *Aspergillus awamori* (Chen et al. 1994).

Thermodynamic studies of β -xylosidase

Thermal inactivation occurs in two steps as shown below:



where N is the native, U is the unfolded enzyme which could be reversibly refolded upon cooling and I is the inactivated enzyme formed after prolonged exposure to heat and therefore can not be recovered on cooling.

The thermal denaturation of enzymes is accompanied by the disruption of non-covalent linkages, including hydrophobic interactions, with concomitant increase in the enthalpy of activation (Daniel, 1996). The opening up of the enzyme structure is accompanied by an increase in the disorder, randomness or entropy of activation (Vieille and Zeikus, 1996). Thermal inactivation of the enzyme was determined by incubating the enzyme solutions in sodium acetate buffer (pH 5.5) at a particular temperature. Aliquots were withdrawn after every 15 min, cooled on ice and assayed for enzyme activity at 40°C as described in Materials and methods. The thermodynamic data were calculated as described previously (Rashid and Siddiqui,

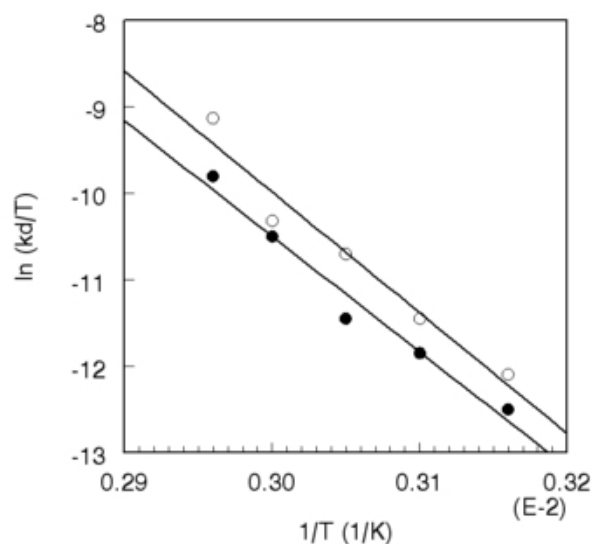


Figure 5. Arrhenius plots for calculating enthalpy and entropy for reversible inactivation of β -xylosidase. They were calculated by applying the equation 2 in Materials and Methods (Aiba et al. 1973).

The value of ΔH^\ddagger was calculated from slope of the straight line between $\ln(k_d/T)$ and $1/T$ while $\ln(k_b/h) + \Delta S^\ddagger/R = \text{Intercept}$ on ordinate. k_b , h , and R are $1.38 \times 10^{-23} \text{ JK}^{-1}$, $6.63 \times 10^{-34} \text{ J.S}$ and $8.314 \text{ JK}^{-1} \text{ mol}^{-1}$ respectively (Rashid and Siddiqui, 1998).

1998) in the range 45-70°C (Figure 5).

The values of thermodynamic parameters (calculated from Figure 5) were 73 kJ/ mol and 60 J/ mol K, for ΔH^\ddagger (enthalpy of deactivation) and ΔS^\ddagger (entropy of deactivation) respectively for the mutant-derived enzyme. These values were significantly lower than those for native enzyme (99 kJ/ mol and 75 J/ mol K respectively). The values of ΔS^\ddagger and ΔH^\ddagger of β -glucosidase from a thermophilic culture of *Aspergillus wentii* (Kvesitadze et al. 1990) were 125 kJ/mol and 65 J/mol K respectively. The test enzyme required significantly lower energy values for inactivation (a positive effect) as compared with that required by above organism, therefore, up to 70°C, the enzyme was substantially thermostable. These values are

also significantly lower than those reported on thermostable glucoamylase by a thermo stabilized mutant of *A. awamori* (Chen et al.1994). When enthalpy and entropy values for inactivation were calculated at each temperature, ΔS^* had negative values for both wild- and mutant-derived β -xylosidase (Table 2). This suggested that there was negligible disorder ness as was that of β -glucosidase from *A. wentii* or α -amylase from *Bacillus licheniformis* as revealed by its negative ΔS^* at 80°C (Violet and Meunier, 1989; Declerck et al. 2003) and other thermostable enzyme by other organisms (Chen et al. 2000; Chen and Stites, 2001; Tsujibo et al. 2001). Similarly enzyme was stable at pH 5.0-7.0 (results not presented) and corroborated the work of Tsujibo et al. 2001 who observed pH 6.0-7.0 optimum for *E. coli* recombinants.

CONCLUDING REMARKS

From the data on induction studies, it was concluded that the availability of an inducer in the fermentation medium is advantageous for β -xylosidase production. β -Xylosidase was also induced by cellobiose, sucrose, and lactose. Lactose in cheese whey or sucrose in molasses may be economically viable inducers for mass production of this enzyme. Further studies are needed to develop a continuous system for enzyme production which would fulfill process requirement. Thermodynamic studies provided sufficient insight into the thermostability of the enzyme and may be due to accompanying chaperone activity. Endo- β -xylanase and β -xylosidase act in synergism to breakdown xylan-lignin complex in paper and pulp industry (Tsujibo et al. 2001) *K. marxianus* produces β -xylosidase with out any accompanying cellulases (Belem and Lee, 1998) This organism produces β -galactosidase, β -glucosidase, and pectinase as well (Belem and Lee, 1998) and may be suitable for biobleaching of pulps if xylanase gene could be incorporated in *K. marxianus* as reported by other authors (La Grange et al. 2001).

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