KINETICS OF HIGH-LEVEL OF ß-GLUCOSIDASE PRODUCTION BY A 2-DEOXYGLUCOSE-RESISTANT MUTANT OF HUMICOLA LANUGINOSA IN SUBMERGED FERMENTATION

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Submitted: November 13, 2007; Returned to authors for corrections: February 05, 2008; Approved: November 10, 2008.

ABSTRACT

A 2-deoxyglucose-resistant mutant (M7) of Humicola lanuginosa was obtained by exposing conidia to γ-rays and permitting expression in broth containing 0.6% 2-deoxyglucose (DG) and cellobiose (1%) before plating on DG esculin-ferric ammonium citrate agar medium from which colonies showing faster and bigger blackening zones were selected. Kinetic parameters for enhanced ß-glucosidase (BGL) synthesis by M7 were achieved when corncobs acted as the carbon source. The combination between corncobs and corn steep liquor was the best to support higher values of all product formation kinetic parameters. Effect of temperature on the kinetic and thermodynamic attributes of BGL production equilibrium in the wild organism and M7 was studied using batch process at eight different temperatures in shake-flask studies. The best performance was found at 45°C and 20 g L⁻¹ corncobs in 64 h. Both growth and product formation (17.93 U mL⁻¹) were remarkably high at 45°C and both were coupled under optimum working conditions. Product yield of BGL from the mutant M7 (1556.5 U g⁻¹ dry corncobs) was significantly higher than the values reported on all fungal and bacterial systems. Mutation had thermo-stabilization influence on the organism and mutant required lower activation energy for growth and lower magnitudes of enthalpy and entropy for product formation than those demanded by the wild organism, other mesophilic and thermo-tolerant organisms. In the inactivation phase, the organisms needed lower values of activation energy, enthalpy and entropy for product formation equilibrium, confirming thermophilic nature of metabolic network possessed by the mutant organism.

Key words: ß-Glucosidase, enthalpy, entropy, hemicellulose, kinetics, regulation, submerged fermentation

INTRODUCTION

ß-Glucosidase (EC. 3.2.1.21) occurs in several organisms, performing several functions in nature. It catalyzes the hydrolytic cleavage of ß-glycosidic linkages in glycosides and cellobiose to glucose. Hydrolytic capacity is dependent on the source of enzyme, physiological function and the localization of enzyme. Fungi and bacteria producing high ß-glucosidase (BGL) activities have attracted considerable attraction because of potential applications to release flavour compounds such as terpenes from odorless non-volatile glycosidic precursors in fruit juices and wines (33), release phenolic compounds with antioxidant, neutraceutil and flavouring properties from their glycosilated forms in plants among other industrial applications (9,32).

Renewable natural resources such as cellulose and xylans (42) are abundant in many agricultural wastes. The enzymatic degradation of waste cellulose by fungal enzymes has been suggested as a feasible alternative for the conversion of lignocellulosics into fermentable sugars and fuel ethanol. This bioconversion is conducted through the combined action of cellulolytic enzymes. Since the activities of cellulase complex are relatively low, this vast resource is not properly utilized. Major constraint is low titre of BGL, which hydrolyzes cellobiose to D-glucose and relieves the end product inhibition of exo-glucanase (EC. 3.2.1.91), and endo-glucanase (EC. 3.2.1.4), aids in cellulase induction and cellulose hydrolysis. BGL from Aspergillus spp. are supplemented to commercial cellulase preparations from Trichoderma reesei, deficient in BGL. Aspergillus spp. are
mesophilic moulds and their BGL may be less thermo-stable than that derived from thermophilic moulds (1). Enzymes isolated from thermophilic microorganisms are not only thermo-stable, but also are often resistant to and active in the presence of organic solvents and detergents (3). The technological use of thermophiles still faces several challenges, since knowledge on the diversity, genetics, and physiology of such organisms is generally poor (36). Thermophiles or the genes derived from them are, however, still the preferred source for thermo-stable enzymes.

**Humicola lanuginosa**, a thermophilic mould, has developed a complex battery of glycosidases to deal with substrates possessing extensive micro-heterogeneities. Strains of *H. lanuginosa* have been applied for biobleaching of pulp (18,42) and may be good sources of thermostable ß-glucosidase for supplementation of cellulase preparation of *Trichoderma reesei*.

The production of enzymes is influenced by induction and catabolite repressions, which alter transcription with CreA protein, a transcriptional repressor of genes involved in metabolic processes other than glucose (10,19,20) and AceI and Ace2 proteins (6,7) in *T. reesei*. Thus mutations in regions linked to the control of genes creA, aceI and ace2 may be a way to gain variant fungi with enhanced cellulase secretion. Mutagenesis followed by selection on DG has been widely used to isolate repression-resistant variants (5). This sort of mutagenesis also confers enhanced secretion of proteins in the culture medium (4).

In Pakistan, large amounts of agro-industrial residues namely sugarcane bagasse, wheat straw, wheat bran, and corncobs are generated every year by diverse economic activities, supporting the biotechnological interest on their utilization as raw materials in biotechnological processes (37). They could be utilized for bulk production of industrial products to meet the increasing consciousness of energy conservation. Submerged fermentation is an attractive method since all process variables can be easily controlled in this fermentation procedure while in SSF, the lower mass transfer processes, related to gases and nutrients diffusion, and temperature take place (4).

This work examines the potential of a DG resistant mutant of *H. lanuginosa* for production of BGL and evaluates the key role of substrate, nitrogen source, pH of the medium and temperature in the life of a thermophilic mould during growth and to maximize the production of BGL as reported in other mutant organisms (12,15,21).

**MATERIALS AND METHODS**

**Chemicals**

All chemicals used in these studies were purchased from Sigma Chemical Company, Saint Louis, Missouri, USA.

**Micro-organism**

Strain of *H. lanuginosa* NIBGE F220 was maintained on potato-dextrose agar plates and slants (30). For inoculum preparation, Vogel’s medium (containing 0.5% trisodium citrate, 0.2% NH₄NO₃, 0.5% KH₂PO₄, 4% (NH₄)₂SO₄, 0.02% MgSO₄·7H₂O, 1% glucose, 0.1% yeast extract) 90 mL (pH 6.5) HCl-washed glass beads (20 in number), was made up in 500 mL flasks. The whole contents were sterilized at 121°C for 30 min. The flasks were inoculated with 10 mL of spore suspension (3 × 10⁸ CFU mL⁻¹) of *H. lanuginosa* strains and incubated overnight at 45°C on a gyratory shaker (150 rpm). For enzyme production one gram of solid substrates (Table 1) (100-500 µm particle size) were added to 45 mL Vogel’s medium (pH 6.5) in 250 mL Erlenmeyer’s flasks in triplicate and whole contents were sterilized. Non-induced (glucose-grown inoculum) and washed conidial suspensions (5 mL) at 3 g cells L⁻¹ were used to inoculate the enzyme production medium. The inoculated flasks were incubated at 45 ± 2.0°C for 72 h in an orbital shaker (150 rpm). After each 8 h, the contents were homogenized and then passed through Whatman 40 filter paper to remove substrate. The filtrate was centrifuged (12,000 x g for 10 min, 10°C) and substrate free enzyme extract was used for following the enzyme activities. Substrate was washed thoroughly and dried at 95°C to a constant weight. The moisture content was determined from the weight loss after drying the sample at 80°C for 2 days.

**Mutagenesis and selection**

*H. lanuginosa* conidial suspension maintained at 3 × 10⁸ CFU mL⁻¹ was dispensed equally in 30 mL McCartney vials and exposed to different doses (0.4-1.4 kGray) of γ-rays in gamma cell radiation chamber (Mark-IV). The survival curve was prepared and exposure dose of 1.2 kGray giving 3-log kill was selected for mutation of the organism. The mutants were selected by permitting expression in broth containing DG (0.6%) and cellobiose (1%) before plating on DG-esculin-ferric ammonium citrate agar medium (37) from which colony showing faster and bigger blackening zones was selected.

**Enzyme assays**

ß-Glucosidase activity was determined using *p*-nitrophenyl ß-D-glucopyranoside (pNPG) as substrate (37). The reaction mixture (600 µL) contained 200 µL of citrate buffer (50 mM, pH 4.5), 200 µL of pNPG (5 mM), and 200 µL culture supernatant. After incubation at 40°C for 10 min, the reaction was stopped by adding 3 mL of cold sodium carbonate (500 mM, pH 10) and volume was made up to 6 mL with distilled water. The activity of BGL was estimated spectrophotometrically by reading the absorbance of the liberated *p*-nitrophenol at 400 nm. One U of BGL has been defined as the amount of enzyme that releases 1-µmol para-nitrophenol equivalents per mL per min.

**Effect of varying pH and temperature on enzyme production**

The effect of initial pH of the fermentation medium on enzyme production parameters was studied in shake flasks...
by varying pH (5.0-7.5) while maintaining optimum temperature and other growth supporting conditions. For studying the effect of temperature, the experiments were repeated in shake flasks (as above) and incubated on an orbital shaker (120 rpm) at 30, 35, 40, 45, 50, 55, 60 and 65ºC for up to a period of 72 h.

### Protein and saccharide determination

The protein in the enzyme preparation was quantified by the Bradford method (11) using bovine serum albumin as the standard. Reducing sugars were estimated colourimetrically with 3,5-dinitrosalicylic acid after Miller (34) using xylose as a standard. Solid material in the fermentation broth was determined gravimetrically.

### Mycelial biomass

Mycelial biomass was determined indirectly from the measurement of protein content (22).

### Determination of kinetic parameters

All kinetic and thermodynamic parameters were determined as described previously (2).

### Statistical analysis

MStat C software was used to do the statistical analysis as described earlier (39).

### RESULTS AND DISCUSSION

Glucose in the growth medium of moulds generally represses enzyme production, and only after exhaustion of glucose, the fungi start the production of β-glucosidase (BGL). In agreement with these results, the wild culture of *H. lanuginosa* produced a constitutive basal level of BGL (0.39 U mL⁻¹) in the presence of glucose (Fig. 1), and the higher enzyme production started only after glucose consumption. But in the case of DG-resistant mutant (M7), BGL formation started from the initial stages of growth and that mutant organism supported 10-fold higher BGL production on glucose in basal Vogel's medium. The mutation conferred catabolite repression resistance to some extent on M7 and significantly enhanced transport of enzyme in the medium. Thus, the production of extra-cellular BGL by *H. lanuginosa* was inducible and controlled by catabolite repression in the wild organism, caused by mono-saccharides (Table 1). The product formation parameters like enzyme formation rate (QP), specific enzyme yield (YP/X), enzyme yield based on substrate utilized, and qP = specific enzyme formation rate.

### Table 1. Comparative β-glucosidase formation parameters of *H. lanuginosus* (P) and its DG mutant M7 (M) following growth on different substrates in Shake flask fermentation at 45°C in Vogel’s basal salt medium.

| Substrate     | Qp (U L⁻¹ h⁻¹) | Yp/X (U g⁻¹) | Yp/S (U g⁻¹) | qP (U g⁻¹ h⁻¹) | Enzyme titer (U mL⁻¹) |
|---------------|----------------|--------------|--------------|----------------|-----------------------|
| Arabinose     | 18.1±142.2     | 68.7±416.7   | 27.5±175.0   | 13.1±83.3      | 0.55±3.5            |
| Xylose        | 77.3±180.2     | 239.6±560.0  | 115.0±280.0  | 45.5±117.6     | 2.32±5.6            |
| Sorbose       | 9.0±78.9       | 51.2±487.8   | 20.5±200.0   | 9.7±10.2       | 0.41±4.0            |
| Mannose       | 14.4±60.5      | 48.9±316.3   | 23.0±155.0   | 10.8±72.8      | 0.46±3.1            |
| Fructose      | 37.7±126.4     | 180.8±479.2  | 85.0±230.0   | 36.2±100.6     | 1.72±4.6            |
| Galactose     | 16.0±127.4     | 42.7±408.2   | 20.5±200.0   | 9.4±93.9       | 0.41±4.0            |
| Glucose       | 16.2±111.3     | 39.0±367.9   | 19.5±195.0   | 8.6±88.3       | 0.39±3.9            |
| Lactose       | 43.2±196.6     | 325.0±829.3  | 130.0±340.0  | 58.5±165.9     | 2.60±6.8            |
| Cellobiose    | 43.7±204.9     | 317.1±825.6  | 130.0±355.0  | 57.1±198.1     | 2.61±7.1            |
| Maltose       | 98.8±190.6     | 262.5±817.1  | 105.0±335.0  | 47.2±155.3     | 2.12±6.7            |
| Sucrose       | 40.9±167.0     | 295.9±549.0  | 145.0±280.0  | 68.1±131.8     | 2.91±5.6            |
| Corn cobs     | 182.3±212.8    | 1716.9±2614.0| 844.0±1342.3| 274.7±470.5   | 9.25±14.9           |
| Wheat straw   | 130.0±158.3    | 1454.5±2434.8| 695.6±1154.6| 232.7±413.9   | 6.43±12.6           |
| Xylan         | 122.2±194.8    | 580.0±1019.2 | 290.0±530.0 | 110.2±203.8   | 5.88±10.6           |

LSD (P ≤ 0.05) 6.49 30.210 20.600 17.690 0.764

Each value is a mean of three replicates. Values followed by different letters differ significantly at \( p \leq 0.05. \)

QP = enzyme formation rate, Yp/X = specific enzyme yield, Yp/S = enzyme yield based on substrate utilized, and qP = specific enzyme formation rate.
Production of β-glucosidase by H. lanuginosa

medium containing different carbon sources in time course studies as described earlier (15) and all kinetic parameters for product formation were determined (Table 1). Interaction of all treatments and all kinetic parameters was highly significant. The specific growth rate (μ), cell mass formation rate (Q_X), substrate uptake rates (Q_S), cell yield coefficient (Y_X/S) and specific substrate uptake rates (q_S) are shown in Table 2. These parameters were considered the factors that might control enzyme synthesis by different substrates (29). There were significant variation in the specific growth rate (μ), cell mass formation rate (Q_X) and the substrate uptake rate (Q_S) during growth on all carbon sources and permitted significantly (p≤0.05) larger variations on synthesis of BGL. The values of q_S from glucose (0.45 g g⁻¹ h⁻¹), cellobiose (0.47 g g⁻¹ h⁻¹) and corn cobs (0.36 g g⁻¹ h⁻¹) were significantly (p≤0.05) different and exerted remarkable impact on the enzyme titre (U mL⁻¹), enzyme yields and productivities (Table 1).

Specific enzyme yield (Y_P/X) was 1716.9 U g cells⁻¹ on corn cobs and only 39.0 U g cells⁻¹ (basal enzyme activity) on glucose (non-inducer). The induction ratio, defined as the ratio of activity supported by inducers to basal activity, was 2.46, 7.70 and 32.06 on mono-saccharides, di-saccharides and polysaccharides respectively in the case of wild organism. Mutation narrowed down this induction ratio, which on mono-saccharides, disaccharides and complex substrates was 1.06, 7.70 and 32.06 in the case of wild organism.

Table 2. Comparative substrate consumption parameters of H. lanuginosus (P) and its DG mutant M7 (M) following growth on different substrates in shake flask fermentation at 45°C in Vogel's basal salt medium

| Substrate    | µ (h⁻¹) | Q_X (g L⁻¹ h⁻¹) | Q_S (g L⁻¹ h⁻¹) | Y_X/S (g g⁻¹) | q_S (g g⁻¹ h⁻¹) |
|--------------|---------|----------------|----------------|---------------|-----------------|
| Arabinose    | PM      | 0.19±0.20abcd  | 0.28±0.32abc   | 0.68±0.77cd   | 0.40±0.42de     | 0.47±0.48ef     |
| Xylose       | PM      | 0.19±0.21ab    | 0.23±0.35ab    | 0.55±0.80a    | 0.48±0.50cd     | 0.40±0.42d      |
| Sorbose      | PM      | 0.19±0.21ab    | 0.31±0.39ab    | 0.61±0.78a    | 0.40±0.41ab     | 0.48±0.51a      |
| Mannose      | PM      | 0.22±0.23abc   | 0.37±0.41abc   | 0.76±0.93b    | 0.47±0.49bc     | 0.47±0.47bc     |
| Fructose     | PM      | 0.20±0.21ab    | 0.26±0.35ab    | 0.63±0.85a    | 0.47±0.48ef     | 0.43±0.44cd     |
| Galactose    | PM      | 0.22±0.23abc   | 0.38±0.45bc    | 0.76±0.96c    | 0.48±0.49abc    | 0.46±0.47bc     |
| Glucose      | PM      | 0.22±0.24abc   | 0.37±0.47ab    | 0.72±0.97a    | 0.50±0.53b      | 0.44±0.45bc     |
| Lactose      | PM      | 0.18±0.20abcd  | 0.26±0.33ab    | 0.52±0.69b    | 0.40±0.41ab     | 0.45±0.48ab     |
| Cellobiose   | PM      | 0.18±0.20abcd  | 0.24±0.32ab    | 0.58±0.73a    | 0.41±0.43df     | 0.44±0.47bc     |
| Maltose      | PM      | 0.18±0.19df    | 0.26±0.32ab    | 0.68±0.72a    | 0.40±0.41ab     | 0.46±0.46cd     |
| Sucrose      | PM      | 0.23±0.24abc   | 0.38±0.47ab    | 0.76±0.97a    | 0.49±0.51bc     | 0.47±0.47bc     |
| Corn cobs    | PM      | 0.16±0.18ab    | 0.12±0.16a     | 0.31±0.40a    | 0.48±0.50cd     | 0.33±0.36       |
| Wheat straw  | PM      | 0.16±0.17bc    | 0.16±0.25ab    | 0.32±0.44a    | 0.47±0.47bc     | 0.34±0.36       |
| Xylan        | PM      | 0.19±0.20abcd  | 0.26±0.32ab    | 0.60±0.76b    | 0.50±0.52ab     | 0.38±0.38       |
| LSD (p≤0.05) |          | 0.01636        | 0.01636        | 0.01636       | 0.01636         | 0.01636         |

Each value is a mean of three replicates. Values followed by different letters differ significantly at p≤0.05.

µ = specific growth rate, Q_X = cell mass formation rate, Q_S = substrate uptake rate, Y_X/S = cell yield coefficient and q_S = specific substrate uptake rate.
2.05 and 5.50 respectively, in the case of mutant M7. Thus mutation decreased induction ratio by a factor 2.3, 3.8, 5.8-fold in the M7 and is a significantly high achievement. Corn cobs is an insoluble substrate and is too difficult to pump through pipelines for continuous fermentation. Cellobiose was the best soluble disaccharide but is extremely expensive. Among disaccharides, sucrose (the one of the best substrate among soluble substrates) in molasses may be economically viable inducer for mass production of this enzyme as already reported in *Kluyveromyces marxianus* (24). Further studies are needed to develop a continuous process for enzyme production that would fulfill this requirement of the process. Best yield of BGL (Table 1) on corncobs is in good agreement with that reported earlier (17). The results presented are of considerable significance for further development of a suitable large-scale production process for maximal production of BGL by *H. lanuginosa* strains.

**Time course of production of β-glucosidase and carbon source utilization**

Enzyme production and substrate utilization from one representative substrate namely, xylan in shaken cultures is presented in Fig. 2. This figure and others (not presented) revealed that optimal production of BGL was reached within 64 h while other thermophilic fungi produced maximum activity after 120-140 h of fermentation (8,18,21). These curves also indicated that production of enzyme was apparently growth-associated. Among potential inducers (Table 1) of BGL, lignocellulosic substrates, xylan, and xylose both induced the cells to produce BGL activity (because of lower substrate consumption parameters). It has been observed (26) that during growth on such polymeric carbon sources, high amount of mRNA for a particular enzyme is produced. This is attributed to reducing sugars accumulating slowly in the growth medium as unmetabolized principles to cause induction of xylanases as reported earlier (26).

Cost of substrate plays a major role in the economics of enzyme production (15). Hence approach to use cheaper lignocellulosic (corncobs) rather than opting for expensive cellobiose will decrease cost of BGL production. We may also opt for sucrose in molasses as a cheaper substrate for BGL production in continuous culture.

**Effect of nitrogen sources**

Among the various nitrogen sources (ammonium nitrate, ammonium sulfate, di-ammonium phosphate, urea, and corn steep liquor) added at equimolar concentration to medium containing corncobs (2% w/v), corn steep liquor (CSL) favoured maximum BGL production, followed by urea in the absence of pH control. In this work, inorganic nitorgen sources (ammonium compounds) did not support a high level of BGL production comparable to CSL (Table 3). On the other hand, some studies have found that inorganic nitrogen sources resulted in equal or higher BGL production than organic ones (9). Urea (0.25% w/v) favoured maximum pectinase production in *Streptomyces* sp. RCK-SC (27). The results (Table 3) indicate that the presence of CSL in the culture medium was essential to support enhanced global kinetic parameters. CSL, when used alone as carbon and nitrogen source, supported one tenth of the maximum activity supported by corncobs. In the presence of CSL, higher enzyme synthesis may have occurred due to the up-regulation of global nitrogen metabolism regulator, AreA (8,19,26) and supported the work of other authors (8,9,12,17). Corn steep liquor is an example of cheap nitrogen source successfully employed as complex nitrogen source in BGL production, contributing to further lower down the cost of production. The utilization of agro-industrial residues as potential substrates for the production of BGL and other cellulase, hemicellulase and pectinase components has attracted much attention (17, 36), since it can contribute to lower the costs of enzyme production and also reduce the environmental pollution caused by the accumulation of lignocellulosic wastes. It was demonstrated that the best substrate-nitrogen source combination for BGL production (15.2 U mL⁻¹) was the corncobs and CSL. The results showed that extra-cellular BGL activity continues to grow until the 3rd day of cultivation, a considerable fall in the productivity is observed at this point. In this sense,
the cultivation period of 2 days was chosen for extra-cellular BGL production by *H. lanuginosa* strain. Corncobs and CSL are waste products of starch industry, with more than 16 million tons produced annually. Thus, they can be employed in composting processes, as sources of antioxidant compounds, for enzyme, ethanol and citric acid production, among other applications.

Product yield (YP/S), specific product yield (YP/X) and product formation rates (QP) of BGL of *H. lanuginosa* (Table 3) are significantly higher than the values reported for *Aspergillus* spp., *Trichoderma reesei* RUT C30, other fungal cultures, different bacteria (37,45), *E. coli* and *Saccharomyces cerevisiae* recombinants harboring heterologous bgl gene (38), *Thielavia terrestris* and *Themoascus crustaceus* (41), *Thermoascus aurantiacus* (25), *Humicola* spp. (23), *Thermomyces lanuginosus* (31), other fungi and their DG resistant mutants (8,21,45) though substrates used in referred studies were different. This comparison shows the superiority of DG resistant mutant (M7) for production of BGL.

**Effect of medium pH**

The effect of initial culture medium pH on the global kinetic parameters of BGL production was investigated using corncobs (2%) as the carbon source and CSL as the nitrogen source. The results in Fig. 3 show that optimal kinetic parameters were achieved in the range 6.5-6.75. Therefore pH 6.5 was used in all further studies. The optimum pH of enzyme productivity was significantly different than that reported for most other BGL-producing microorganisms, namely, *Monascus purpureus* (17) and *Myceliophthora* sp. (8).

![Figure 3](image_url)

**Figure 3.** Effect of medium pH on product yield (YP/S, Δ), volumetric productivity (QP, □) and specific productivity (qP, ○) of β-glucosidase following growth of parental (open symbols) and mutant M7 (closed symbols) of *H. lanuginosa* on corncobs-CSL medium using optimized conditions. Error bars show standard deviation among three observations.

**Effect of temperature**

Temperature is one of the most important variables. Temperature effects on fermentation performance of some selected organisms for product formation were reported previously (35,43). Only few investigations were reported on the influence of temperature on the dynamic behaviour of *H. lanuginosus* during fermentation processes. Global fermentation parameters and thermodynamic parameters determination when product formation is occurring, may give information on the organism’s metabolic network. The influence of temperature on the formation rate of BGL by mutant (M7) and wild strain of *H. lanuginosus* were studied with regard to the kinetic parameters related to biomass and BGL production and substrate utilization. The mutant organism (M7) was capable of rapid fermentation at temperature up to 65°C with significantly higher specific growth
rate (Fig. 4a) which was better than those of other strains of H. lanuginosa grown at 45°C (3). The maximum specific growth rate, specific death rate of cells and the maximum product formation rate of both cells increased as the temperature increased up to 45-50°C. Expressed by the Arrhenius relationship, the temperature dependence of these parameters is shown in Fig. 4a, 4b. The estimated values of activation energies for cell growth (46.56 and 78.59 kJ mol⁻¹ for the mutant (M7) and wild cells respectively), BGL formation (143.59 and 171.94 kJ mol⁻¹) and for cell death (42.20 and 60.86 kJ mol⁻¹ respectively) and product inactivation (108.62 and 128.70 kJ mol⁻¹ respectively) contrary to the higher values reported in literature for mesophilic organisms (138.9-177 kJ mol⁻¹) (35). The values of activation energy during deactivation phase were lower than the values in the growth phase and confirm the findings of other authors for thermophilic organisms (2). Requirement of lower energy of activation (Eₐ) for growth and product formation is considered indices of thermostable enzymes (1). The values of enthalpy for product formation (Table 4) indicated that the activation enthalpy (140.92 kJ mol⁻¹) and activation entropy of formation of BGL (250.2 J mol⁻¹) for the mutant M7 is lower than that for the wild organism and those for mesophilic organism (14) and thermotolerant Kluyveromyces marxianus (40).

The deleterious effects of high temperature were considered to be due to the denaturation of ribosomes and enzymes and problems associated with the fluidity of membranes (43). Thermal inactivation of metabolic network, when the organism is permitted to grow at temperature higher than that for maximum growth and product formation, may result in unfolding the enzymes of the metabolic network and normally occurs in two steps as shown below:

\[ N \leftrightarrow U \rightarrow I \]

Where N are the native enzymes, U are the unfolded enzymes that could be reversibly refolded upon cooling and I are the inactivated enzymes formed after prolonged exposure to heat and therefore can not be recovered on cooling.

The thermal denaturation of enzymes of the metabolic network is accompanied by the disruption of non-covalent linkages, including hydrophobic interactions, with concomitant increase in the enthalpy of activation (16). The opening up of the enzyme structures is accompanied by an increase in the disorder, randomness or entropy of activation (46). The related values of thermodynamic parameters were calculated from Fig. 5. The

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**Figure 4.** Effect of fermentation temperature on specific growth rate (a) and specific product formation rate (IU g cells⁻¹ h⁻¹) (b) of β-glucosidase from corncobs-CSL medium, following growth of the parental (open symbols) and mutant M7 (closed symbols) of *H. lanuginosa.*

**Figure 5.** Determination of enthalpy and entropy for activation for product formation and inactivation equilibria of β-glucosidase in *H. lanuginosa* (open symbols) and its mutant M7 (closed symbols) following growth on corncobs-CSL medium (pH 6.5) under optimized conditions.
mutant M7 needed 111.38 kJ mol⁻¹ and -609.33 J mol⁻¹ K⁻¹ values for enthalpy of deactivation (ΔHₚ) and entropy of deactivation (ΔSₚ) respectively. The values of ΔHₚ and ΔSₚ of BGL from a thermophilic culture of Aspergillus wentii were 125 kJ mol⁻¹ and 65 J mol⁻¹ K⁻¹ (28). Thus difference of values for the BGL inactivation during growth for Aspergillus wentii BGL and H. lanuginosa mutant (M7) is markedly high, therefore, up to 65ºC, the process of BGL formation in mutant M7 was reasonably thermo-stable. These values are also significantly lower than those reported on thermo-stable glucoamylase from a mutant derivative of A. awamori (13) and those for the wild organism reported in this study (Table 4).

When enthalpy and entropy values for inactivation were calculated at each temperature, ΔS*, ΔH* and ΔG* for BGL formation by mutant M7 had again lower values (Table 5) than those for the wild organism. This suggested that there was negligible defolding of enzymes of the metabolic network in the inactivation phase equilibrium as was that of BGL from A. wentii (28). All these findings suggested that mutation had significant effect in thermo-stabilization of the metabolic network of the mutant M7 during BGL production. This may have occurred due either to formation of chaperones or hyper-glycosilation of enzymes of the production metabolic network as reported earlier (2).

Table 4. Thermodynamic parameters* estimated by Arrhenius approach for batch formation and inactivation of β-glucosidase formation metabolic network in cultures of H. lanuginosa (P) and its DG mutant M7 (M) calculated from a graph between ln (qP/T) and 1/T as described in materials and methods.

|                      | Enthalpy (kJ mol⁻¹) | Entropy (J mol⁻¹ K⁻¹) | Eₐ for growth(kJ mol⁻¹) |
|----------------------|---------------------|-----------------------|------------------------|
| β-glucosidase formation | PM 183.05±140.92     | 379.45±250.25         | 78.59±46.53            |
| Thermal inactivation | PM 131.47±111.38     | -542.90⁻609.33        | 60.86⁻42.20            |
| LSD (P ≤ 0.05)       | 11.870               | 18.830                | 3.766                  |

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 ≤ 0.05.
*Thermodynamic parameters were determined following growth on corn cobs and CSL medium in temperature range of 30-65ºC.

CONCLUSION

From the data on induction studies, it was concluded that the availability of an inducer at a low level over a longer period of fermentation time would be more advantageous for β-
glucosidase production. Further studies are needed to develop a continuous process for enzyme production that would fulfill this requirement of the process. A mutant strain of *H. lanuginosa*, producing β-xylosidase and other glycosidases (18) was also found to have improved BGL enzyme production. The possibility of using locally available substrates (Table 2) for enzyme production was promising in that induction on wheat straw and corncobs yielded BGL to a level greater than 2.95 and 3.17-fold of that induced by cellobiose. Corn steep liquor was essential for rapid uptake of substrates and microbial activity. The organism is prone to mutagenesis and will be more suitable for its application in futuristic bulk production of BGL for biofuels industry in Pakistan.

Thermodynamic studies provided sufficient insight into the cellular functions under varying conditions of fermentation temperature. They led to suggest that the phenomenon limiting BGL production metabolic network could be enzymatic reaction/s under all varying fermentation conditions as observed for xylitol production (14). It was also concluded that the cell system of the mutant M7 exerted more protection against thermal inactivation, probably by acquiring chaperones or hyper-glycosilation.

**ACKNOWLEDGEMENTS**

Pakistan Atomic Energy Commission, Islamabad and Pakistan Agricultural Research Council, Islamabad supported this work. Some chemicals were purchased from a USAID grant under PCST Proposal 6.163. This work formed a part of Ph.D. thesis work of Syed Ali Imran Bokhari who was financially supported by Higher Education Commission, Government of Pakistan through Indigenous 5000 PhD Fellowship Programme. Technical assistance of Mr. Maqsood Ahmed and Miss Fatima Jalal is appreciated.

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