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Full Length Research Paper

Kinetics and thermodynamics of high level β-glucosidase production by mutant derivative of *Aspergillus niger* under submerged growth conditions

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Gamma rays mediated mutagenesis and subsequent selection on 2-deoxy-D-glucose developed a highly catabolite de-repressed and stable β-glucosidase (BGL) hyper-producer mutant of local *Aspergillus niger* NIBGE, which has high potential for industrial applications. The M6 mutant showed highest constitutive production of BGL (14,900 IU/L) on glucose as compared to control (5,900 IU/L). Wheat bran was the best carbon source and BGL production by parent and mutant strain was 16,200 and 29,500 IU/L, respectively. Effect of inoculum density, pH, temperature and wheat bran concentration on the BGL production was determined in terms of kinetic growth parameters, that is, specific growth rate (μ), biomass doubling time (t_d), product yield coefficient with respect to cell mass ($Y_{p/x}$) and specific rate of product formation (q_p). Optimal conditions for BGL production by parent and mutant strain were same: inoculum level= 0.184 mg cells/mL (10% v/v); optimum temperature= 30°C and pH= 5.0; wheat bran= 3% (w/v). The apparent subunit molecular mass of BGL from both strains was also same (130 kDa). Determination of thermodynamic parameters led to consider that the hyper-production of BGL by the mutant was due to decrease in: activation energy ($E_{a(p)}$), change in enthalpy ($\Delta H^*_{(p)}$) and Gibbs free energy ($\Delta G^*_{(p)}$) for product (BGL) formation.

Key words: β-glucosidase, *Aspergillus niger*, activation energy, enthalpy, Gibbs free energy.

INTRODUCTION

Cellulose (the most abundant and renewable biomass on earth) is a linear polymer of D-glucose units linked by 1,4-β-D-glucosidic bonds. In nature, the hydrolysis of cellulose is carried out by the synergistic action of cellulases, which include: endoglucanase, exoglucanase and β-glucosidase (BGL) (Javed et al., 2009). Out of these, β-glucosidase (EC 3.2.1.21) hydrolyzes cellobiose to glucose and is generally responsible for the regulation of the whole cellulolytic process, being the rate limiting factor during enzymatic cellulose hydrolysis, as both endoglucanase and exoglucanase activities are often inhibited by cellobiose. Thus the function of BGL is not only to produce glucose from cellobiose but it also reduces the cellobiose inhibition, resulting in efficient functioning of endoglucanase and exoglucanase (Castro et al., 2010).

BGL from *Aspergillus niger*; a filamentous, non-pathogenic fungus (Javed et al., 2009) that belongs to

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Abbreviations: BGL, β-glucosidase; **2DOG,** 2-deoxy-D-glucose; **DNA,** deoxyribonucleic acid; **NIBGE,** national institute for biotechnology and genetic engineering; **CMC,** CM-cellulose sodium salt; **SDS-PAGE,** Sodium dodecyl sulphate polyacrylamide gel electrophoresis; **SmF,** submerged fermentation.

"Generally Recognized As Safe" group, are generally supplemented to *Trichoderma reesei* cellulase preparations (Kaur et al., 2007) and play an important role in cellulose saccharification on an industrial scale. This is a ubiquitous enzyme which occurs in all living kingdoms (ranging from bacteria to plants and mammals) for performing different biological functions. In micro-organisms, this enzyme is usually found as part of the cellulose-degrading enzyme complex while in plants, it is involved in number of functions including release of cyanides from cyano-glucoside precursors (acting as part of a defense system); lignification; cell wall catabolism; phytohormone conjugate activation; symbiosis etc. In animals (mammals) it plays important role in the breakdown of glycolipids and exogenous dietary glucosides (Nakkharat and Haltrich, 2006; Cairns and Esen, 2010).

In recent years, interest in BGL has again gained momentum, owing to its potential use in various biotechnological processes including degradation of biomass, fuel ethanol production from cellulosic agricultural residues (Jager et al., 2001), release of aromatic residues in the flavor industry, extraction of fruit juices and oil from seeds, production of fermented foods (such as Kao-liang chiu liquor, miso, soy sauce, vinegar), gelatinization of seaweeds and extraction of agar, synthesis of β-glucosides, and improving the digestibility of feed in the animal feed industry (Bhat and Bhat, 1997; Cairns and Esen, 2010).

In order to make the production of bio-ethanol an economically feasible process, the costs of the enzymes to be used for hydrolysis of the raw material need to be reduced and an increase in specific activity or production efficiency of cellulases is required (Camassola and Dillon, 2010). One obstacle associated with the higher production of cellulases is catabolite repression of cellulase genes. BGL are also important in the regulation of cellulase genes since they are the key enzyme in the synthesis of sophorose, an efficient inducer of the cellulolytic system (Amouri and Gargouri, 2006). One approach to alleviate this hindrance is the mutagenesis followed by selection on 2-deoxy-D-glucose (2DOG) to isolate BGL hyperproducer and catabolite repression resistant-mutants.

This sort of mutation also confers enhanced secretion of proteins into the culture medium (Bokhari et al., 2008). The reason is that the 2DOG is a toxic glucose analog, which, when incorporated into a suitable medium containing cellulose, causes catabolite repression of non-mutant strains, allowing only mutated strains resistant to this form of suppression to form colonies (Dillon et al., 2006).

So the need to still isolate and identify the new strains or to modify the already isolated strains which may become either hyper producer and/or sufficiently robust to withstand conditions of intended application is highly significant (Nadeem et al., 2009). In this context the present study was carried out to mutate the *A. niger* through exposure to highly ionizable gamma rays for higher BGL production and to determine the effect of these rays on the kinetics and thermodynamics of cell mass formation and β-glucosidase production. The gamma rays were used for their good cell penetration, precise doses, mutations within the genes through deoxyribonucleic acid (DNA) repair mechanism (Lamb, 2000) and most importantly result in highly persistent mutations (Parekh et al., 2000; Masurekar, 2005).

MATERIALS AND METHODS

Microorganism and chemicals

A pure local strain of *A. niger* NIBGE, available at National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan was maintained on potato dextrose agar plates and slants as described earlier (Nadeem et al., 2009). All chemicals were purchased from MP Biomedicals (USA) or as otherwise stated.

Medium, culture conditions and inoculum preparation

For inoculum preparation, a loopful of spores from a 5 to 6-day old *A. niger* slant was transferred into a 250 mL Erlenmeyer flask containing 50 mL of sterile Vogel's medium (Vogel, 1956) and 8-10 acid washed glass beads. The flask was incubated at 30±1°C on an orbital shaker at 120 rev/min for 36 h to get a homogenous cell suspension (Nadeem et al., 2009).

Mutagenesis, enrichment and selection

A. niger conidial suspension containing 3.6×10^6 CFU/mL was dispensed equally in 30 mL McCartney vials and exposed to different doses (0.4-1.4 kGray) of γ-rays (Awan et al., 2011) in a gamma cell radiation chamber (Mark-IV, Source ^{60}Co). A standard curve was prepared and mycelia exposed to 0.9 kGray dose (giving more than 3 log kill) were inoculated in broth containing 1% (w/v) 2DOG (in which the parent strain mycelia did not grow) and 1% (w/v) cellobiose, before plating on 2DOG-esculin-ferric ammonium citrate agar medium (Bokhari et al., 2008) from which colony showing biggest blackening zone in short time was selected.

β-glucosidase assay

β-glucosidase activity in 50 mM Na-acetate buffer (pH 5.0) at 40°C was determined using cellobiose as substrate. An appropriate amount of the enzyme was added in 1% (w/v) cellobiose solution dissolved in 50 mM Na-acetate buffer (pH 5.0) and incubated at 40°C for 30 min. The quenching was carried out by thermal denaturation of the enzyme by placing the tubes in boiling water for 10 min and cool immediately in ice cold water. The amount of released glucose was determined by incubating the appropriate amount of quenched reaction mixture (100 µL) with 1 mL of glucose measuring reagent (Fluitest® GLU, Biocon, Germany) at 37°C for 15 min, based on the D-glucose oxidase-peroxidase method (Jager et al., 2001). Absorbance (OD) was measured at 500 nm with spectrophotometer (Spectro 23RS).

One unit (IU) was defined as "the enzyme amount that releases 1 µmol of glucose/min from cellobiose under defined conditions of pH and temperature".

Protein and cell mass estimation

Kinetics of β-glucosidase (BGL) and cell mass production of the total protein was estimated as described by Bradford using bovine serum albumin as standard (Javed et al., 2009).

The biomass content was estimated on the basis of absorptiometry (cells turbidity) by measurement of the light transmitted at 610 nm (Pirt, 1975). The standard curve for biomass estimation was prepared as described earlier (Nadeem et al., 2009) and optical density was converted to dry weight of fungal cells (Powell et al., 2009).

In case of insoluble substrates (wheat bran), the enzyme production and cell mass were estimated as described earlier (Rajoka and Yasmeen, 2005).

SDS-DR-PAGE for subunit molecular mass determination and LC MS/MS analysis

The subunit molecular mass of BGL was determined by 10% sodium dodecyl sulphate denaturing-renaturing PAGE (SDS-DR-PAGE) as described earlier (Nadeem et al., 2009). However the protocol was modified for BGL activity staining. Briefly, the sample was prepared by incubating crude BGL at 45°C for 60 min with SDS sample loading buffer in the presence of β-mercaptoethanol (2% v/v) and was loaded on the gel in duplicate. PAGE was run at constant voltage (70 V) and the temperature was controlled (10°C) by using a refrigerated circulating water bath. Protein markers of Invitrogen (# 10747-012) with molecular mass ranging from 10 to 220 kDa were run as standards.

Standard curve, which was drawn between R_f values of standard protein bands versus their log molecular weight was used to determine the exact molecular mass. Half of the gel containing protein markers and the sample was stained with 0.1% (w/v) Coomassie brilliant blue R-250 for protein staining, while the other half of the gel was re-natured to remove the SDS by treating with 20% (v/v) isopropanol in 50 mM sodium acetate buffer, pH 5.0 (two washes of 30 min each). The gel was equilibrated with 50 mM sodium acetate buffer pH 5.0 to remove isopropanol (two washes of 30 min each). BGL activity staining was done as follows: The re-natured gel was overlaid onto an equally sized 0.8% (w/v) agarose gel prepared in 50 mM sodium acetate buffer pH 5.0 containing 0.4% (w/v) Esculin (6, 7-Dihydrocoumarin 6-glucoside) and 0.04% (w/v) Ferric ammonium citrate as a substrate (Kwon et al., 1994). The assembled gels were wrapped with cling film to avoid drying and incubated at 45°C for 30-45 min. After incubation, a dark brown band of BGL activity appeared, which was compared with the corresponding protein band for the determination of subunit molecular mass.

The desired coomassie brilliant blue–stained protein bands from the gel were also cut and processed for in gel digestion of proteins as described (Drbal et al., 2001). The resulting peptide mixture was analyzed on mass spectrometer (Finnigan™ LTQ™, Thermo Fisher Scientific).

Kinetics of β-glucosidase (BGL) and cell mass production

Both fungal strains were grown in submerged cultivation and conditions were optimized for the maximum production of BGL. The effects of carbon source, temperature, initial pH of growth medium, inoculum concentration and substrate concentration on the kinetics of BGL production were determined. The conditions were optimized by adopting search techniques of varying one parameter at a time as described earlier (Bhatti et al., 2007; Sharma et al., 2008; Awan et al., 2011). Once a parameter was defined, the same was followed for subsequent study as follows.

Effect of carbon source

Commercial grade glucose, cellobiose, CM-cellulose sodium salt (CMC) and wheat bran (2% w/v) were used as carbon source in Vogel's medium and the strains were grown in submerged conditions at 30°C, pH 5.0. Aliquots were aseptically withdrawn at regular time intervals in order to monitor the time for maximum BGL, cell mass and protein production. The inoculum strength for both strains was kept same by using their respective biomass standard curves in all studies. The best carbon source (substrate) achieved by this step was fixed for subsequent experiments (Ellaiah et al., 2002; Awan et al., 2011).

Effect of temperature

Fungi were cultivated on 2% (w/v) wheat bran at temperatures (24-36±1°C) with an increment of 3°C (Awan et al., 2011). The initial pH of growth medium was 5.0. Time course aliquots were withdrawn aseptically and analyzed for total proteins, enzyme activity and cell mass. The temperature that gave maximum BGL production was fixed for subsequent experimentation.

The effect of temperature on the rate of cell mass formation and enzyme production was also expressed in terms of temperature quotient (Q_{10}), which is the factor by which the rate changes for a rise in temperature by 10°C (Papagianni, 2004). Q_{10} was calculated by using the following equation.

$$\text{Temperature quotient } (Q_{10}) = (R_2/R_1)^{10/(T_2-T_1)} \quad (1)$$

Where, T_1 and T_2 are lower and higher temperatures (°C or K) and R_1 and R_2 are rate measurements at T_1 and T_2, respectively.

Effect of initial pH

For optimizing the initial pH of the medium for maximum BGL production, Vogel's media with initial pH from 3 to 7 were used (Awan et al., 2011) while keeping all other parameters at their optimum levels. The achieved optimum initial pH of the growth medium was fixed for subsequent experiments.

Effect of inoculum level

Various levels of inoculum (0.092 to 0.46 mg wet cells/mL of medium) were used, keeping all other conditions at their optimum levels (Ellaiah et al., 2002; Awan et al., 2011). The optimum level of inoculum achieved, was fixed for subsequent experiments.

Effect of substrate concentration

The effect of wheat bran (1, 2, 3, 4 and 5% w/v) concentration on BGL production was studied in submerged growth conditions. The cultivation of fungi was carried out in 250 mL Erlenmeyer flask at already optimized conditions. Time course aliquots were withdrawn and analyzed for total proteins, BGL activity and cell mass.

Determination of growth kinetic parameters

Growth kinetics of *A. niger* (P) and its 2DOG resistant mutant (M6) derivative was determined (Aiba et al., 1973; Pirt, 1975) as follows: Specific growth rate (μ), that is, rate of growth per unit amount of cell mass was calculated from slope of plot: ln x against time.

Biomass (cell mass) doubling time (t_d) = $\ln 2/\mu$ (2)

Product yield coefficient with respect to cell mass ($Y_{p/x}$) = dp/dx, (3)

Where, $dp = p_t - p_o$ and $dx = x_t - x_o$. The p_t and x_t are amount of BGL and biomass after specific time, while p_o and x_o are amount of BGL and biomass at zero time, respectively.

Specific rate of product formation (q_p) = $Y_{p/x} \times \mu$ (4)

Maximum specific rate of cell mass formation (μ_m) was determined from a double reciprocal plot: $1/\mu$ Vs $1/s$, which was equal to intercept on ordinate ($1/\mu_m$), whereas, substrate saturation constant (K_s) was equal to intercept on abscissa ($-1/K_s$). Similarly maximum rate of BGL production (q_{pmax}) was determined from plot: $1/q_p$ Vs $1/s$.

$E_{a(x)}$ and $E_{a(p)}$ for cell mass and product (BGL) formation were determined from Arrhenius plots of ln μ against 1/T and ln q_p against 1/T, respectively.

Thermodynamics of cell mass and BGL production

The thermodynamic parameters for cell mass formation and enzyme production by *A. niger* parent and mutant were calculated by using rearranged Eyring's absolute rate equation derived from the transition state theory (Eyring and Stearn, 1939).

$$k = (k_b.T/h).e^{(-H^*/RT)}.e^{(S^*/R)} \quad (5)$$

Where, k_b is Boltzmann's constant (1.38×10^{-23} J/K), T is absolute temperature (K), h is Planck's constant (6.626×10^{-34} Js), N is Avogadro's number (6.02×10^{23} /mol), R is the ideal gas constant (8.314 J/K/mol), H^* (kJ/mol) and S^* (J/mol/K) are change in enthalpy and entropy for product formation, respectively.

$$H^* = E_a - RT \quad (6)$$

$$G^* \text{ (Change in Gibbs free energy)} = -RT \ln (k.h/k_b.T) \quad (7)$$

$$S^* = (\Delta H^* - G^*)/T \quad (8)$$

The μ_m and q_{pmax} are equivalent to k (Pirt, 1975). H^*, G^* and S^* were calculated by applying Eqs., 6-8 with the modification that in Eq. 7 k was replaced with μ_m for cell mass formation and with q_{pmax} for BGL production. Moreover, in Eq. 6 E_a was replaced by $E_{a(x)}$ and $E_{a(p)}$ for the cell mass and product (BGL) formation, respectively.

RESULTS AND DISCUSSION

Mutation and selection Kinetics of β-glucosidase (BGL) and cell mass production

Microbes produce secondary metabolites as a matter of survival but in industry a high titer is usually a desired goal. To accomplish this goal, mutagenesis followed by selection on 2DOG has been widely used to isolate repression resistant mutants (Bokhari et al., 2008; Demain and Adrio, 2008).

The principle of using such toxic metabolite analogs is that the resistant mutants often possess enzymes that are insensitive to feedback inhibition, or enzyme forming systems resistant to feedback repression (Parekh et al., 2000; Demain and Adrio, 2008). Alteration in cell wall synthesis, protein synthesis or cell membrane permeability is also a common mechanism of resistance to analogues resulting in increased product accumulation in the medium presumably through increased rate of product export from the cell. The mutations in regions linked to the control of genes *cre* A, *ace*I and *ace*2 (transcriptional repressor of genes involved in metabolic processes other than glucose) may be a way to gain variant fungi with enhanced cellulase secretion (Bokhari et al., 2008). With these intentions, the *A. niger* conidial suspension was exposed to different challenging doses (0.4-1.4 kGray) and the survival curve was plotted. As a 3 log kill rate is suggested by many workers for the selection of improved mutants for industrially important enzymes (Rajoka and Yasmeen, 2005; Demain and Adrio, 2008), the mutants from a γ-ray dose of 0.9 kGray, giving more than 3 log kill (99.9%), were screened for the hyper-producer mutant as described in materials and methods. One of the derivative colonies (M6), producing larger black zone (24 mm) as compared to the parent (15 mm) was selected for further studies while other mutants (moderate to low producers of BGL) were discarded. Despite the cytotoxic effects of 2DOG on germinating conidia (Dillon et al., 2006), the selected mutant derivative (M6) showed growth even on solid Vogel's medium containing 2% (w/v) 2DOG as compared to parent (which only grew up to 0.8% w/v 2DOG). This result may be due to one of three types of genetic variations: an altered transport system resulting in reduced permeability for 2DOG, a low catabolite repression by 2DOG or an increase in 2DOG metabolizing efficiency within the mutant M6.

Molecular mass of β-glucosidase and LC MS/MS analysis

The apparent sub unit molecular mass of BGL from the parent and the mutated strains, determined on 10% SDS-DR-PAGE (Figure 1), was the same (130 kDa). The bands were also analyzed on mass spectrometer after tryptic digestion and the resulting spectrum also confirmed its identity as β-glucosidase enzyme. A range of molecular masses of BGL have been reported in various organisms like: 92 kDa in *Melanocarpus* sp., 50 kDa in *T. thermophilus* (Nakkharat and Haltrich, 2006), 43 kDa in *Candida peltata*, 120-160 kDa in *A. niger* (Dan et al., 2000) etc. The SDS-DR-PAGE is advantageous to use because it can be applied to unpurified proteins, being helpful in purification steps as early determination of molecular mass of desired protein will aid in the subsequent purification steps and can also be handy in small scale purification through elution of the desired protein from gel band through conventional methods such as diffusion and electrophoretic elution (Kwon et al.,

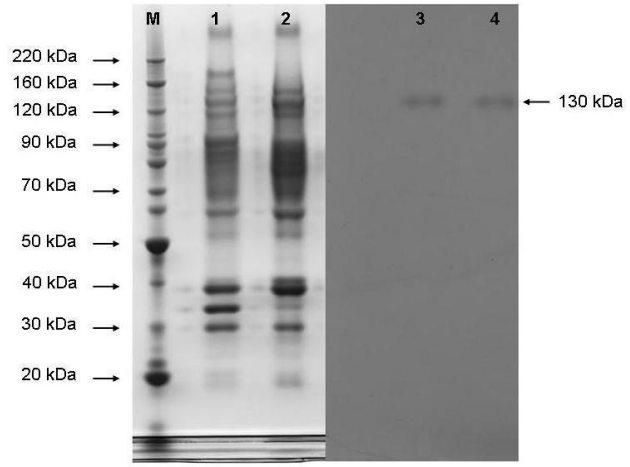

Figure 1. 10% SDS-DR-PAGE for sub unit molecular mass determination of BGL: Lane-M) Protein marker (Invitrogen, # 10747-012), Lane-1) Crude parent BGL, Lane-2) Crude mutant BGL, Lane-3) Zymograph of parent BGL, Lane-4) Zymograph of mutant BGL

Table 1. Effect of different carbon sources on the kinetic parameters for cell mass formation and β-glucosidase production by *A. niger* NIBGE (P) and mutant (M6) grown under submerged condition at 30°C, pH 5.

Carbon source	Strain	Enzyme titer (IU/L)	Total proteins (g/L)	μ (h^{-1})	t_d (h)	$Y_{p/x}$(IU/g)	q_p (IU/g cells/h)
Glucose	P	5900± 511	0.35±0.02	0.0860	8.06	304	26.13
	M	14900±1043	0.53±0.03	0.0925	7.49	564	52.13
Cellobiose	P	3840±192	0.37±0.04	0.0289	23.98	977	28.24
	M	7400±260	0.53±0.03	0.0389	17.82	1845	71.79
CMC	P	8923±625	0.53±0.03	0.0289	23.98	836	24.17
	M	15800±1106	0.5±0.03	0.0415	16.70	1357	56.33
Wheat Bran	P	15050±753	0.71±0.04	0.0839	8.26	1744	146.31
	M	27540±1377	0.62±0.03	0.0891	7.78	2854	254.28

Each value is a mean of three replicates ± standard deviation. μ = specific growth rate, t_d = biomass doubling time, $Y_{p/x}$ = product yield coefficient with respect to cell mass, q_p = specific rate of product formation.

1994).

Kinetics of β-glucosidase production and cell mass formation

Microbial production of enzymes through submerged cultivation (SmF) is easier to handle and the growth parameters can be closely monitored as compare to solid state fermentation (Daroit et al., 2007; Javed et al., 2009). Since any biotechnological process is likely to be based on crude enzymes preparations, it is necessary to improve their activities in the culture supernatants by selecting the best carbon source and optimizing the growth conditions (Gao et al., 2008).

Effect of carbon source

The cost of substrate has a significant role in the economics of enzyme production. The utilization of agro-industrial wastes as potential substrates for the production of BGL and other enzymes, including cellulases, hemicellulases and pectinases, has attracted much attention because it reduces the cost of enzyme production and also reduces environmental pollution (Daroit et al., 2007). Wheat bran is a cheap, abundant and easily available lignocellulosic biomass. It is a com-plex mixture rich in proteins, carbohydrates, minerals, fat and vitamin B (Leite et al., 2008). Wheat bran was used along with other carbon sources for BGL production. Glucose generally represses the production of BGL and also acts

Table 2. Effect of different temperatures on the kinetic parameters for cell mass formation and β-glucosidase production by *A. niger* NIBGE (P) and mutant (M6) grown under submerged condition on wheat bran, pH 5.

Temperature (°C)	Strain	Enzyme titer (IU/L)	Total proteins (g/L)	μ (h^{-1})	t_d (h)	$Y_{p/x}$ (IU/g)	q_p (IU/g cells/h)
24	P	3670±175	0.63±0.03	0.0665	10.42	677	45.06
	M	7230±362	0.55±0.02	0.0836	8.29	1149	96.09
27	P	5390±232	0.68±0.03	0.0712	9.74	993	70.67
	M	8950±314	0.58±0.02	0.0875	7.92	1416	123.91
30	P	15050±753	0.71±0.04	0.0839	8.26	1744	146.31
	M	27540±1377	0.62±0.03	0.0891	7.78	2854	254.28
33	P	13800±293	0.72±0.03	0.0912	7.60	1597	145.67
	M	26200±704	0.63±0.02	0.0920	7.54	2695	247.93
36	P	5500±275	0.68±0.03	0.0985	7.04	805	79.32
	M	8910±446	0.54±0.03	0.0958	7.23	1043	100.00

Each value is a mean of three replicates ± standard deviation. μ = specific growth rate, t_d = biomass doubling time, $Y_{p/x}$ = product yield coefficient with respect to cell mass, q_p = specific rate of product formation.

as its strong inhibitor. Therefore, from industrial view point, the microbes having low end product inhibition or showing high constitutive production of the enzyme are significantly important. The γ-rays mediated mutagenesis of *A. niger* strain resulted in hyper-production of BGL and the mutant *A. niger* (M6) showed quite high constitutive production of the enzyme (14,900 IU/L) as compared to control (5,900 IU/L) when grown on glucose as a carbon source (Table 1). This observed effect was due to catabolite repression resistance, conferred to some extent by mutation, and significantly enhanced secretion of the enzyme in the medium. The highest specific growth rate, μ (0.0925 h^{-1}) of mutant was also observed when glucose was used as a carbon source, however on wheat bran the specific growth rate was also comparable (Table 1). Torres et al. (1998) has reported 0.134 h^{-1} as specific growth rate of *A. niger* 10 on 3% (w/v) glucose at 35°C in 40 h of growth. Since the enzyme production ability of *A. niger* for BGL reaches its peak level during the stationary phase of growth (Sohail et al., 2009), therefore comparison of growth parameters for optimum BGL production up till stationary phase of growth is more practical.

The other kinetic growth parameters i.e. biomass doubling time (t_d), product yield coefficient with respect to cell mass ($Y_{p/x}$) and specific rate of product formation (q_p) for the BGL production also confirmed that, among the inductive substrates, wheat bran was the best (Table 1). Bokhari et al. (2008) has reported a constitutive production of 390 and 3900 IU/L of BGL from parent and mutated strains of *Humicola lanuginosa*. Although, in the current study the constitutive increase in enzyme production of mutant M6 as compared to the parent strain is not as high as reported by Bokhari et al. (2008), the parent strain itself is an efficient producer of BGL (5,900 IU/L), so an increase of approximately 2.5 fold in this case is quite significant.

Effect of temperature

Incubation temperature is a very critical parameter and a slight change in it sometimes affects the enzyme production considerably. The growth of *A. niger* parent and its mutant derivative (M6) on wheat bran was strongly affected by temperature. The strains showed a reasonable amount of BGL production at a temperature range of 27-33°C. At higher temperatures (above 36°C) the enzyme secretion was very low, while the optimum temperature for biomass and BGL biosynthesis for both strains was 30°C. With the increase in incubation temperature up to 33°C, an increasing trend in specific growth rate and specific rate of product formation was observed. The enzyme production and the growth kinetic parameters of *A. niger* mutant (M6) were also higher than those of its parent (Table 2), which again confirmed that the mutant was hyper-producer of BGL. Sohail et al. (2009) has reported 25°C as an optimum temperature for highest levels of BGL production from *Aspergillus niger* MS82 with a slight reduction in production rate at 30 and 35°C, whereas some other researchers have reported 30-35°C as an optimum temperature range for growth of *A. niger* strains (Torres et al., 1998; Ellaiah et al., 2002; Rajoka and Yasmeen, 2005; Sharma et al., 2008; Awan et al., 2011). The deleterious effects of high temperatures are considered to be due to enzyme and ribosomal denaturation and problems associated with the membrane fluidity (Bokhari et al., 2008), while low temperature may not permit the flow of nutrients across the cell membrane resulting in high demand for maintenance energy. When the organism is allowed to grow at a temperature higher than that of its optimum one, unfolding of the metabolic network enzymes is resulted in two steps, as shown below:

$$N \leftrightarrow U^* \rightarrow I$$

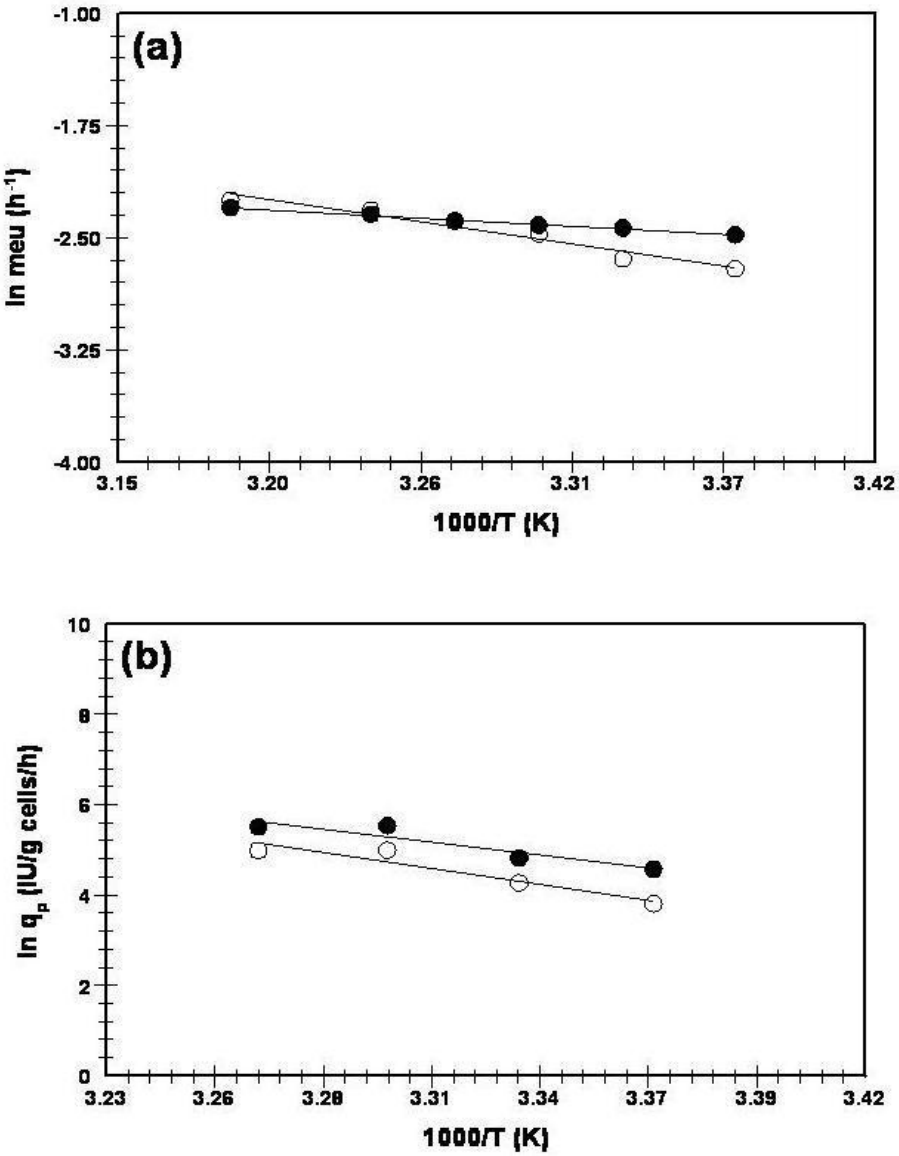

Figure 2. Arrhenius plots for the determination of activation energy for (a) cell mass formation ($E_{a(x)}$) and (b) BGL production ($E_{a(p)}$) by parent (○) and mutant (●) *A. niger.*

Where N are the native enzymes, U* are unfolded enzymes that can be reversely refolded on cooling and I are the inactivated enzymes that cannot be recovered on cooling due to prolonged exposure to heat. This thermal denaturation is accompanied by the disruption of non-covalent linkages including hydrophobic interactions with the increase in enthalpy of activation, while the opening of the enzyme structures is accompanied by an increase in disorder, randomness or entropy.

Arrhenius plots were applied to determine the $E_{a(x)}$ and $E_{a(p)}$ (Figure 2a and b). The activation energy $E_{a(x)}$ for cell mass formation of parent was 22.86 kJ/mol, whereas that of the mutant strain was significantly reduced to 8.48 kJ/mol. Hence, the mutant strain required low energy for cell mass formation, therefore, proved to be a faster growing strain. Similarly, the activation energy for product formation ($E_{a(p)}$) of mutant was lower (89.29 kJ/mol) than the parental strain (107.08 kJ/mol). As enzymes speed up the reactions by lowering down the energy of activation, the same principle can be applied on the microbial cells for enzyme production (Monod, 1942). The low $E_{a(p)}$ of mutant *A. niger* confirmed that it is better producer of BGL as compared to the parent strain. The low energy of activation (E_a) requirements for growth and product formation in case of mutant is also considered to be indices of thermostable enzymes (Adams and Kelly, 1998). Both parent and the mutant *A. niger* required much lower activation energies for cell growth and BGL production than reported for cell growth (78.59 and 46.56 kJ/mol) and BGL production (171.94 and 143.59 kJ/mol)

Table 3. Kinetics and thermodynamics of cell mass formation and β-glucosidase production by *A. niger* NIBGE (P) and mutant (M6) grown on wheat bran under submerged condition at 30°C, pH 5.

	Parameter	Parent *A. niger*	Mutant (M6) *A. niger*
Cell mass formation	μ_m (h^{-1})	0.1027	0.1085
	$K_{s(x)}$ (% w/v)	0.4386	0.4651
	$E_{a(x)}$ (kJ/mol)	22.86	8.48
	$H^*_{(x)}$ (kJ/mol)	20.34	5.96
	$G^*_{(x)}$ (kJ/mol)	100.61	100.47
	$S^*_{(x)}$ (J/mol/K)	-264.90	-311.92
	$Q_{10(x)}$	1.47	1.11
BGL production	q_{pmax} (IU/g cells/h)	357.14	625.00
	$K_{s(p)}$ (% w/v)	3.57	3.57
	$E_{a(p)}$ (kJ/mol)	107.08	89.29
	$H^*_{(p)}$ (kJ/mol)	104.57	86.77
	$G^*_{(p)}$ (kJ/mol)	80.07	78.66
	$S^*_{(p)}$ (J/mol/K)	80.86	26.77
	$Q_{10(p)}$	7.15	5.08

Where: activation energy for cell mass formation ($E_{a(x)}$) and BGL production ($E_{a(p)}$) for parent (P) and mutant (M6) were calculated from Figure 2a and 2b, respectively. The maximum specific rate of cell mass formation (μ_m) and substrate saturation constant ($K_{s(x)}$) for maximum cell mass formation were determined from Figure 3a, whereas, maximum specific rate of product formation (q_{pmax}) and substrate saturation constant ($K_{s(p)}$) for maximum product (BGL) production were calculated from Figure 3b. Q_{10} and thermodynamic parameters for cell mass formation ($\Delta H^*_{(x)}$, $G^*_{(x)}$ and $S^*_{(x)}$) and product (BGL) production ($\Delta H^*_{(p)}$, $G^*_{(p)}$ and $S^*_{(p)}$) were calculated as described in materials and methods.

of *Humicola lanuginose* wild and mutant cells, respectively (Bokhari et al., 2008) as well as other mesophilic organisms (138.9-177 kJ/mol). The reported values in the current study were even lower than (152 and 91 kJ/mol for parent and mutant cells, respectively) of *A. niger* NIAB 280 for α–galactosidase production (Rajoka et al., 2009). The temperature quotient (Q_{10} factor) for cell mass formation and BGL production by both strains was also calculated and presented in Table 3. Q_{10} values of 2-30 for filamentous fungi are common in literature (Papagianni, 2004).

Effect of initial pH

The effect of initial culture medium pH on the kinetic parameters of BGL production was investigated using wheat bran (2% w/v) as carbon source at 30°C. In the absence of pH control, the optimal kinetic parameters of both strains were achieved in the initial pH range of 4-6, while pH 5 was the best for BGL production (q_p= 146 and 254 IU/g/h for parent and mutant, respectively). However, the mutant performed better than parent strain at all studied pHs (Supplementary material, Table S1). Optimum pH is very important for the growth and metabolic activities of the microbes because metabolic activities are very sensitive to the change in pH. The optimum pH for BGL production by the parent and mutant *A. niger* was different from those reported for *Humicola lanuginose* (Bokhari et al., 2008), *Monascus purpureus* (Daroit et al., 2007), *Aspergillus terreus* M11 (Gao et al., 2008) and *A. niger* MS82 (Sohail et al., 2009).

Effect of inoculum level

Varying inoculum levels were tested to study their effect on BGL production by *A. niger* strains. An inoculum level of 0.184 mg wet cells/mL of medium (10% v/v) was optimum for BGL production by the both *A. niger* strains. A lower level of BGL production was observed when higher or lower inoculum level than optimal was used (Supplementary material, Table S2). Ellaiah et al. (2002) has also reported the inoculum level of 10% v/w for optimum production of glucoamylase from *Aspergillus* sp. A3, while Bhatti et al. (2007) has reported 15% as an optimum inoculum level for optimum glucoamylase production from *Fusarium solani*. Awan et al. (2011) has reported the 2.5×10^6 /mL conidial suspension (3 mL) as inoculum level for optimum α- and β–galactosidases production on wheat bran-corn steep liquor medium by *A. niger* NIAB 280. The provision of optimum inoculum size is essential because low inoculum density may give insufficient biomass causing reduced product formation and require more time for fermenting the substrate. Conversely, higher inoculum may deplete substrate for the nutrients necessary for product formation because of excessive biomass synthesis (Sharma et al., 2008).

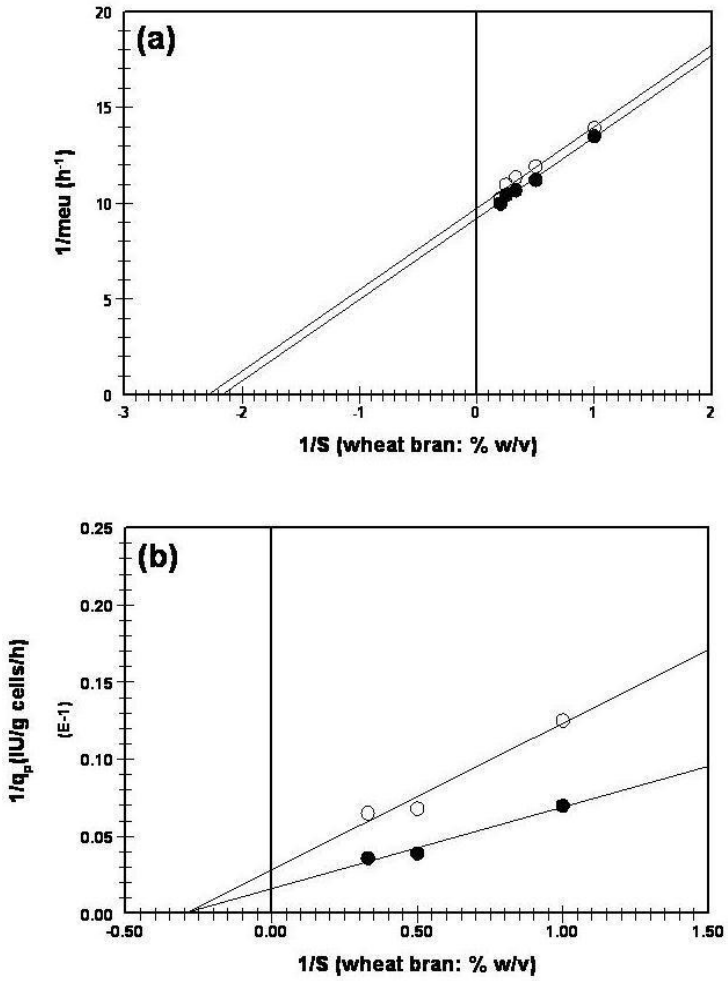

Figure 3. Double reciprocal plots for the determination of maximum specific rate of (a) cell mass formation (μ_m) and (b) BGL production (q_{pmax}) by parent (○) and mutant (●) *A. niger.*

Kinetics and thermodynamics of BGL production on wheat bran

The effect of various concentrations of wheat bran on BGL production was also studied. With the increase in substrate concentrations up to 3% w/v, all the kinetic parameters showed an increasing trend for BGL production from both strains, however, after that, the BGL production was lowered. The mutant performed better on all concentrations than did the parent (Supplementary material, Table S3). The declining trend at higher concentrations may be due to the increased viscosity of the medium and reduced aeration.

The μ_m and q_{pmax} were determined by applying double reciprocal plots (Figure 3a and b). The maximum specific rate of cell mass formation (μ_m) of both strains was almost same, whereas the substrate saturation constant ($K_{S(x)}$) to achieve μ_m of parent was slightly lower than the mutant. On the other hand, the maximum specific rate of product formation (q_{pmax}) of the mutant was about two fold higher than the parent *A. niger* while the substrate saturation constant ($K_{S(p)}$) was the same for both strains (Table 3).

Effect of random mutagenesis on the thermodynamics of cell mass and product formation was also determined and is presented in Table 3. The spontaneous occurrence of a reaction or process can be best determined from its change in free energy (ΔG^*). Lower the G^* value, the more likely is the reaction to take place. The $G^*_{(x)}$ for cell mass formation of the mutant was slightly lower than that of parent strain, which means the rate of cell mass formation of mutant was higher. This result was also supported by the enthalpy change $H^*_{(x)}$. Very low $H^*_{(x)}$ of the mutant *A. niger* confirmed that the mutant strain was more active in biomass formation.

Thermodynamics of BGL production by mutant *A. niger* ($\Delta G^*_{(p)}$ and $H^*_{(p)}$) were also very low as compared to the parental strain. The lower change in enthalpy and Gibbs

free energy values again confirmed that the mutant required small amount of energy for the BGL production hence, confirmed that it was hyper producer strain. The $S^*_{(x)}$ and $S^*_{(p)}$ for cell mass and BGL production were also determined. The thermodynamic growth parameters in submerged fermentation of the mutant were even lower than reported for thermotolerant *Kluveromyces marxianus*, *Humicola lanuginosa* (Bokhari et al., 2008) and *A. niger* NIAB 280 (Rajoka et al., 2009). Awan et al. (2009) has also reported change in enthalpy (21.6 and 19.1 kJ/mol) and entropy (-134.3 and -138.4 J/mol/K) of parent and mutant *A. niger*, respectively for α–galactosidase production in solid state fermentation using wheat bran supplemented with glucose as carbon source and corn steep liquor as a nitrogen source. Low magnitudes of activation energy, enthalpy, Gibbs free energy and entropy for product formation are considered to be indices for thermostability of metabolic pathway (Rajoka et al., 2009). Therefore, we suggest that the mutation had significant effect in thermostabilization of the metabolic network of mutant (M6) during BGL production, which might be due to either formation of chaperons or hyper-glycosylation of enzymes of the production metabolic network as reported earlier (Aiba et al., 1973).

Conclusions

Gamma rays mediated mutagenesis developed a highly catabolite de-repressed and stable BGL hyper-producer strain of *A. niger*, which have high potential for industrial applications. The possibility of using locally available substrates for enzyme production is highly promising and economical. This BGL hyper-production by the mutant was due to the decrease in: activation energy ($E_{a(p)}$), change in enthalpy ($\Delta H^*_{(p)}$) and Gibbs free energy ($\Delta G^*_{(p)}$) for product (BGL) formation. Our future plans are to identify mutations within the BGL gene and to determine the effect of these mutations on BGL stability–function relationship.

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