African Journal of Microbiology Research Vol. 6(22), pp. 4639-4646, 14 June, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.754
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Gamma ray mediated mutagenesis of *Phialocephala humicola*: Effect on kinetics and thermodynamics of α-amylase production

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Accepted 18 November, 2011

The current report dealt with the development of the fungus *Phialocephala humicola* through gamma ray treatment for the hyper production of α-amylases, which have diverse industrial applications. Improvement of microbes for the over production of industrial products has been the hallmark of all commercial fermentation processes. Three mutants (M13, M16 and M24) were selected based on the hyper-production of α-amylases. The mutants had gamma ray exposure of 100 and 140 krad (killing rate ≥ 99%). The mutants showed ≥ 2-fold improvement in enzyme production. The effects of inoculum size, temperature, pH and starch concentration on the growth kinetic parameters [μ, t_d, $Y_{p/x}$, q_p, μ_m, $K_{s(x)}$, q_{pmax}, $K_{s(p)}$] and thermodynamic parameters [$\Delta G^*_{(x)}$, $\Delta H^*_{(x)}$, $\Delta S^*_{(x)}$ and $\Delta G^*_{(p)}$, $\Delta H^*_{(p)}$, $\Delta S^*_{(p)}$] were determined. Optimal conditions for the amylase production by the mutants were: inoculum 10% (v/v); pH range of 4-5 and temperature 37°C. The activation energy for cell mass [$Ea_{(x)}$] of parent, M13, M16 and M24 was 3.095, 15.73, 3.68 and 8.03 kJ mol^{-1}, respectively, whereas their $Ea_{(p)}$ for the enzyme production was 69.42, 22.94, 17.04 and 20.78 kJ mol^{-1}, respectively. The maximum specific rate of cell mass formation (μ_m) of all mutated strains were greater than that of the parent strain, while specific rate for the maximum product formation (q_{pmax}) of the mutants was about two fold higher than the parental strain.

Key words: Activation energy, cell mass estimation, entropy, gibbs free energy growth kinetics.

INTRODUCTION

Starch, the most important carbohydrate, is a major constituent of many processed and traditional foods (Rubio et al., 2009). It contributes 50-70% of the energy in the human diet, as a direct source of glucose, which is an essential substrate in brain and red blood cells for generating metabolic energy (Perry et al., 2007). Being an important industrial component, 60 million tones of starch are extracted worldwide annually from various cereals, tuber and root crops (Burrell 2003). At molecular level, two glucose polymers are the major constituents of Starch, that is, linear amylose (25–28%) and the highly branched amylopectin (72–75%). Amylose consists of linear chains of 500–6000 α-(1, 4)-linked D-glucopyranosyl units, with a fraction of the amylose molecules being slightly branched by α-(1, 6)-linkages (Hizukuri et al., 1981; Shibanuma et al., 1994).

The gelatinization and hydrolysis of starch granules require heating and a long reaction time. Therefore, as an alternative to such an expensive and energy-consuming process, using enzymes capable of digesting starch directly would be more successful due to specificity of reaction, stability of the generated products, lower energy requirements and elimination of the neutralization steps (Satyanarayana et al., 2005). The starch polymer requires a combination of different enzymes for its

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complete hydrolysis. These include alpha amylase, glucoamylase and isoamylase, which comprise 30% of the world's enzyme consumption (Sharama and Shukla, 2007).

Alpha amylase (1, 4-α-D-glucan-glucanhydrolase, EC.3.2.1.1) is most commonly used in starch industry for liquefaction of gelatinized starch (Mitssuiki et al., 2005; Sujka et al., 2006). The catalytic mechanism of the α-amylase family is α-retaining double displacement (Van der Maarel et al., 2002). Alpha amylases catalyze the hydrolysis of internal α-1, 4-O glycosidic bonds in polysaccharides and are universally distributed throughout the animals, plants and microbial kingdoms. However, enzymes from microbial sources have dominated their applications in industrial sectors. Filamentous fungi have mostly been used for the production of alpha amylase for centuries, having wide applications in beverages, food, paper, textile and detergent industries (Pandey et al., 2000).

Improvement of microbial strains for the over production of industrial products has been the hall mark of all the commercial fermentation processes. Modification and improvement of the strains through mutations are specifically achieved by subjecting genetic material to a variety of physical or chemical agents called mutagens (Parikh et al., 2000). These mutagens are known to act in different ways to cause DNA lesions. Physical mutagens include UV (Ultra Violet), gamma and X rays. Ultra violet rays have medium effect, which induces pyrimidine dimerization by frame shift transition from GC to AT base pair (Chopra 2005). Among these radiations, gamma rays are the most energetic and highly ionizing radiations, which cause mutations as single or double strand breakage of DNA by deletion or structural change, DNA-protein cross links, oxidized bases and basic sites (Cadet et al., 1999; UNSCEAR, 2000). Gamma rays have drawn extensive attention of biotechnologists and breeding experts due to their prominent role in strain improvement for higher yield of the products.

Mutagenesis followed by selection on 2-Deoxy-D-glucose (2DG) has been widely used to isolate repression-resistant mutants (Anwar et al., 1996). 2DG is a toxic glucose analogue, which has frequently been employed to isolate glucose-deregulated mutants. Mutagenesis to confer 2deoxy-D-Glucose resistance is a well recognized approach to control the formation of repressible proteins (Cre A, Cre B and Cre C) (Lockington, 2002) and to enhance the expression of inducible enzymes in different organisms (Rajoka et al., 2005).

The current report deals with the development of 2DG resistant mutants of *P. humicola*, which were able to produce higher levels of alpha amylase. The effects of temperature, pH, inoculum size and substrate, have been determined in terms of growth kinetic parameters. Moreover, here we report for the first time thermodynamics of alpha amylase production by *P. humicola*.

MATERIALS AND METHODS

All chemicals were of analytical grade and mostly were purchased from MP Biomedicals. The raw starch was obtained from Rafhan Maize Products (Pvt) Ltd, Faisalabad.

Microbial strain

The culture of *P. humicola,* was taken from First Fungus Culture Bank, Department of Mycology, Punjab University, Lahore. The culture was maintained on potato dextrose agar (PDA) slants and plates at 4°C (Rashid, 1998).

Inoculum preparation

For inoculum preparation, 100 ml of Vogel's medium (containing 0.5% trisodium citrate, 0.2% NH_4NO_3, 0.5% KH_2PO_4, 4% $(NH4)_2SO_4$, 0.02% $MgSO_4.7H_2O$, 1% glucose, 0.1% yeast extract) in 500 ml conical flasks with 6-10 chromic acid washed glass beads were used. The pH of the medium was adjusted to 5 using 1M HCl/1M NaOH and sterilized at 121°C (1.1 kg cm^{-2}) for 10 min. A platinum wire loop full of *P. humicola,* spores was transferred aseptically to each flask and the flasks were kept in an orbital shaker at 110 rpm at 37°C for 1-2 days. In order to transfer an equal amount of inoculum to the growth medium, the cell mass content was estimated by using the standard curve as described by Nadeem et al. (2009).

Mutagenesis through gamma ray treatment

Freshly grown *P. humicola,* cells were centrifuged at 13000 rpm (39,200×*g*) for 15 min and washed with saline solution. The colony forming unit (CFU ml^{-1}) of *P. humicola* cell suspension was maintained at 5×10^3 cells ml^{-1}. Aseptically, the suspension was dispensed equally in 30 ml McCartney vials and exposed to different challenging doses (20-140 krad) of gamma ray in a gamma cell radiation chamber (Mark-IV) as described previously by Hassan (2001). The survival curve was prepared and doses of gamma ray exposure 100 and 140 krad giving 1.69 and 2.39 log kill, respectively were taken for mutant selection.

Selection of DG resistant mutants based on thermo-tolerance

The irradiated and parent (untreated) fungal cells were grown on Vogel's medium containing 1.5% (W/V) 2-deoxy-D-glucose (2DG) at 37°C for 48 h to isolate the 2DG resistant mutants. Serial dilutions of the parent and mutant strains from each dose were streaked on PDA plates containing 1% (W/V) soluble starch, then the plates were incubated at various temperatures (37- 45°C) with an increment of 2°C, to find thermo-tolerant mutants. Viable cell counts from each dose were determined and colony forming unit (CFU) ml^{-1} was calculated.

Selection of mutants based on hyper alpha amylase production

Out of 200 mutant colonies representing various gamma ray doses, about twenty four best grown mutants were selected which were

incubated at 37°C. The mutants were grown on Vogel's medium containing 1% (W/V) starch under submerged conditions to screen for hyper producing strains of alpha amylase. The mutant derivatives were selected, characterized in solid and liquid culture as described previously by Haq et al. (2001).

Screening of mutants on the basis of clearance zone

The cells from selected doses were diluted up to 10^{-6} fold. The petri plates containing 1.5% (W/V) 2DG, 1% (W/V) soluble starch and 0.45% (W/V) ox gall in Vogel's medium, were aseptically inoculated by touching the tip of sterilized tooth pick containing the spores of parent and mutant strains in the center of the plates. The agar plates were kept upside down during inoculation. The plates were incubated in inverted position and picked with much care to avoid the falling of spores on the plates. The plates were stained with iodine solution and the diameter of the clearance zone was measured, which was formed due to the digestion of starch by the secreted alpha amylase. Three 2DG resistant mutants, based on the largest clearance zone (Rajoka et al., 2005), were selected and designated as M-13, M-16 and M-24. The mutants were stored at 4°C in a refrigerator.

Alpha amylase assay

The amylase activity was determined as described by Riaz et al. (2007) by using soluble starch as substrate with the difference that the released product was estimated by using Di-Nitro-Salicylic acid (DNS) method. The reaction mixture (2100 µL) contained 1750 µL of sodium phosphate buffer (50 mM, pH 7), 250 µL of soluble starch (1% *W/V*), and 100 µL enzyme extract. After incubation at 45ºC for 20 min, the reaction was stopped by adding 2 ml of DNS and volume was made up to 4.1 ml. The activity of alpha amylase was estimated spectrophotometrically by reading the absorbance at 550 nm. Appropriate blanks were used to correct the non-enzymatic release of sugars. One unit of alpha amylase was defined as the amount of enzyme that released reducing sugars equivalent to 1 µmole maltose per minute under the standard assay conditions.

$$U\ mL^{-1}\ min^{-1} = \frac{\Delta A_{550}\ of\ sample \times Maltose\ standard\ factor\ (7.24 \mu mol) \times Total\ Reaction\ mixture\ volume}{Enzyme\ (0.1\ mL) \times Incubation\ (20\ min) \times Reaction\ mix\ volume\ for\ color\ development}$$

Total protein content in the enzyme preparation was estimated by Bradford method (1976) using bovine serum albumin as the standard.

Effect of inoculum level

To determine the suitable level of inoculum for alpha amylase production, various levels of inoculum (5, 10 and 15% V/V) were used, keeping all other conditions at their optimum levels (Ellaiah et al., 2002). The optimum level of innoculum achieved was fixed for subsequent experiments.

Effect of initial pH

While optimizing the initial pH of the Vogel's medium, the pH of aqueous solution was varied from 3.0 to 7.0 with 1 M HCl or 1 M NaOH keeping all other conditions at their optimum levels. The achieved optimum initial pH of the growth medium was fixed for subsequent experimentations.

Effect of temperature

Fungi were cultivated on 2% (W/V) starch at temperatures (25, 30, 35, 37, 40°C). 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 for maximum alpha amylase production was fixed for subsequent experiment.

The effect of temperature on the rate of enzyme and cell mass formation was also expressed in terms of temperature quotient (Q_{10}), which is the factor by which the rate increases due to a rise in the temperature by 10°C. Q_{10} was calculated by using the following equation, given by Latham and Burgess (1977).

$$Q_{10} = \text{Inverse ln } \{E/R\ (1/T_1\text{-}1/T_2)\} \quad (1)$$

Where: E: E_a = Activation Energy (Joules); *R:* Gas constant = 8.314 J K^{-1} mol^{-1}; T= Absolute temperature.

Effect of substrate

The effect of soluble starch concentration (1-6% W/V) on α-amylase production was studied under submerged growth conditions. For this study pH and temp were kept constant i.e. 5 and 37±1°C, respectively. Time course aliquots were withdrawn aseptically after appropriate time intervals and analyzed for total proteins, biomass estimation and α-amylase activity.

Determination of growth kinetic parameters

Growth kinetics of *P. humicola* (P) and its DG resistant mutants (M13, M16 and M24) were determined as described by Pirt (1975) and Aiba et al. (1973). The kinetic parameters were determined as follows:

Specific growth rate (μ) i.e. rate of growth per unit amount of cell mass (x) was calculated from slope of plot ln x vs. time.

$$\text{Biomass (cell mass) doubling time} = t_d = \ln 2/\mu \quad (2)$$

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

Where, $dp = p_t\text{-}p_o$ and $dx = x_t\text{-}x_o$. The p_t and x_t are amount of alpha amylase and biomass after specific time, while p_o and x_o are amount of alpha amylase and biomass at zero time, respectively.

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

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

Similarly maximum rate of alpha amylase production (q_{pmax}) = (plot: $1/q_p$ vs. $1/s$)

$E_{a(x)}$ and $E_{a(p)}$ for cell mass and product (alpha amylase) formation were determined by applying Arrhenius plots (plot: ln μ vs. 1/T) and (plot: ln q_p vs. 1/T), respectively.

Thermodynamics of cell mass and alpha amylase production

The thermodynamic parameters for cell mass formation and the enzyme production by *P. humicola* parent and mutants were

calculated by using rearranged Eyring's absolute rate equation derived from the transition state theory.

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

Where:

k_b: Boltzmann's constant (R/N) = 1.38×10^{-23} JK^{-1}; T= Absolute temperature (K); *h:* Planck's constant = 6.626×10^{-34} Js; *N:*Avogadro's number = 6.02×10^{23} mol^{-1}; *R:* Gas constant = 8.314 J K^{-1} mol^{-1}; ΔH^*: Enthalpy of activation for product formation (kJ mol^{-1}); ΔS^*: Entropy of activation for product formation (J mol^{-1} K^{-1});

$$\Delta H^* = E_a - RT \quad 6$$

$$\Delta G^* \text{ (Free energy of activation)} = -RT \ln (k.\, h/k_b.T) \quad 7$$

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

The μ_m and q_{pmax} are equivalent to k (Monod, 1942). ΔH^*, ΔG^* and ΔS^* were calculated by applying Equations 6 to 8 with the modification that in Equation 7 k was replaced with μ_m for cell mass formation and q_{pmax} for α-amylase production. Moreover, in Equation 6 E_a was replaced by $E_{a(x)}$ and $E_{a(p)}$ for the cell mass and product (alpha amylase) production, respectively.

RESULTS AND DISCUSSION

Various chemical and physical factors have been known to effect the production of alpha amylase such as temp, pH, period of incubation, moisture, agitation and carbon sources etc with regards to solid state fermentation and submerged fermentation. Interactions of these parameters are reported to have significant influence on production of enzyme (Sivaramakrishnan et al., 2006).

Optimization of the growth parameters and manipulation of media are one of the most important techniques used for the over production of enzymes to meet industrial demands (Tanyildizi et al., 2005). Production of alpha amylase in fungi is known to depend on both morphological and metabolic state of the culture. Growth of mycelia is crucial for the production of an extra cellular enzyme like α-amylase (Carlsen et al., 1996).

Mutation and selection

Mutations alter the organism's behavior and its potential to form metabolites. Mutagenesis followed by selection on 2DG has been widely used to isolate repression resistant mutants (Bokhari et al., 2009). The principle of using such toxic metabolite analogs is that the resistant mutants often posses enzymes that are sensitive to feed back inhibition or feed back repression (Parekh et al., 2000). Therefore, mutation of wild type *P. humicola* was carried out at different doses (20-140 krad) in a gamma cell radiation chamber (Mark-IV). For screening of hyper producer strains of alpha amylase, four 2DG resistant best grown colonies from each dose of gamma ray were selected. Hence, a total of 24 mutants were selected, which were then grown on starch under submerged conditions to analyze their capability for enzyme production. Three DG resistant mutants (M13, M16 and M24) of *P. humicola* were selected and the α-amylase production of M16 (24,840 U L^{-1}) and M24 (24,960 U L^{-1}) was highest compared to the control and all other mutants. The selected mutants i.e. M13 and M16 had 100 krad, while M24 had 140 krad gamma rays exposure.

The mutants of gamma rays doses 100 and 140 krad gave ≥ 99% killing rate, which is extremely high and the mutations could be considered as stable. Survival curves are routinely used for the selection of microbial mutants (Rajoka and Yasmeen, 2005). The mutants M13, M16 and M24 were further tested by clearance zone assay and they again proved to be hyper producer strains of alpha amylase. On the whole, the selected mutants were 2 fold hyper producer strains of alpha amylase as compared to control. Furthermore, the mutants required shorter period of time span (72-80 h) for the optimal production of the enzyme compared to the control (96h). Similar findings were reported by Hiller and Waseem (1996) for *B. amyloliquefaciens*.

Effect of inoculum size

Optimum inoculum level is required to get the best product yield. Low inoculum density may give insufficient biomass resulting in reduced product formation. In contrast, higher inoculum level decreases the product formation by rapidly depleting nutrients due to excessive biomass synthesis (Sharma et al., 2008). Various inoculum levels (5, 10 and 15%) were tried to study their effect on α-amylase production. It was found that 10% (V/V) inoculum level was the optimum for α-amylase production.These findings are in agreement with other reports (Bhatti et al., 2007; Ellaiah et al., 2002).

Effect of pH

Among the physical parameters, the pH of the growth medium plays an important role by inducing morophological changes in the organism. It regulates production of enzymes and the growth rate of the microbes. The parent and mutant strains of *P. humicola* showed optimal production of alpha amylase at pH 5.0.

Effect of initial culture medium pH on the kinetic parameters of α-amylase production was investigated using soluble starch (2%) as carbon source at 37°C. The results indicated that optimal kinetic parameters of parent and mutant strains were achieved in the pH range of 4-5, while pH 5 was the best for alpha amylase production (q_p = 265, 419, 650 and 809 U g^{-1} h^{-1} for parent and mutants M13, M16 and M24, respectively). However, the mutants performed better than parent strain at all pHs as the doubling time for parent was greater compared to that of

mutant strains. 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 the yields of α-amylase by *Aspergillus* sp., *A. oryzae*, *A. ficcum* and *A. niger* in submerged fermentation was 5.0 (Carlsen et al., 1996; Djekrif-Dakhmouche et al., 2005). In contrast, the optimum pH for α-amylase production by the parent and mutant *P. humicola* was different from those reported for *Humicola lanuginosa* (8.1) (Bokhari et al., 2009), *Aspergillus terreus* M11 (2.0) (Gao et al., 2008) and *Aspergillus niger* (6.5) (Rajoka and Yasmeen, 2005).

Effect of temperature

Temperature affects the fermentation performance of microorganisms for product formation. The influence of temperature on amylase production is related to the growth of microorganism. Global fermentation and thermodynamic parameters determination of product formation may give information on the organism's metabolic network. To check the influence of temperature on the production rate of α-amylase, a study was designed on mutant and wild strains of *P. humicola* within temp range of (25, 30, 35, 37, 40°C), with regard to the kinetic parameters related to biomass and product formation. The results indicated that the mutant organisms were capable of rapid fermentation at temperatures up to 37°C with significantly higher specific growth rates compared to the parent strain. The enzyme activity of M24 was higher than those of its parent and all other mutant strains at all temperatures. The maximum specific growth rate and the maximum product formation rate of both parent and mutants increased as the temperature increased upto 37°C. The optimum growth temperature was in agreement with previous report on *A. fumigatus* strain (Cherry et al., 2004).

Arrhenius plots were applied to determine the $Ea_{(x)}$ and $Ea_{(p)}$ (Figure 1). The activation energy for cell mass formation of parent was 3.095 kJ mol^{-1} whereas for that of the mutant strains it was significantly increased to 15.73 kJ mol^{-1} in the case of M13 strain, while slightly increased for M16 and M24 (3.68 and 8.03 $kJmol^{-1}$), respectively. Hence the mutant strain required greater energy for cell mass formation. Similarly the activation energy, for product formation was 8.35 kJ mol^{-1} for parent strain, while it was 2.76, 2.05 and 2.5 kJ mol^{-1} for mutants M13, M16 and M24, respectively. As enzymes speed up the reactions by lowering 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 mutants of *P. humicola* confirmed that they are more efficient in alpha amylase production compared to the parent strain. The parent and mutant strains of *P. humicola* required much lower activation energies than those reported for *Humicola lanuginosa* and other mesophilic organisms. Requirement of lower $E_{a(p)}$ by mutants is considered indices of thermostable enzymes (Bokhari *et al*. 2009). The temperature quotient (Q_{10} factor) for cell mass formation and alpha amylase production for all strains was also calculated and presented.

Kinetics and thermodynamics of α-amylase production

Starch has been reported as the best substrate (among wheat bran, potato peel and corn cob) based on several aspects such as specific cell growth rate, enzyme activity level, specific enzyme activity level, specific enzyme formation rate, etc. Soluble starch has been found as the best substrate for the production of α-amylase by *B. stearothermophilus* (Srivasava and Baruah, 1986) and *A. oryzae* (Lachmund et al., 1993; Syu and Chen, 1997; Jensen et al., 1987). So starch was selected as substrate for alpha amylase production in submerged fermentation studies. The level of substrate is vital in submerged fermentation as it influence the porosity and aeration of the medium. Different concentrations (1-6%) of starch were used to study their effect on enzyme production. The results indicated that all the mutant strains showed higher yields of alpha amylase at all substrate concentrations compared to parent. Alteration in cell wall synthesis, protein synthesis or cell membrane permeability is a common mechanism of resistance to analogues (2-Deoxy D Glucose) (Rincon et al., 2001). Such permeability changes, may sometimes lead to increased production, presumably through increased rate of product export from the cell. It is conceivable that like the parental strain, the mutant derivatives can effectively utilize starch, continue to grow, and secrete α-amylase in the medium. These results are in good agreement with the work reported by Allen and Roche (1989). The declining trend at higher concentrations may be due to the increased density of the medium and reduced aeration. Similar effects of the substrate on enzyme production have already been reported (Bhatti et al., 2007; Ellaiah et al., 2002).

Double reciprocal graph also depicts the values of q_{pmax} and μ_m (Figure 2). The maximum specific rate of cell mass formation ($\mu_{m)}$ of all mutated strains was greater than that of the parent strain, whereas the substrate saturation constant ($K_{s(x)}$) to achieve μ_m of parent was lower than the mutants. Hence, the affinity of parent strain to starch for the maximum cell mass formation was higher as compared to the mutants. On the other hand, the maximum specific rate of product formation (q_{pmax}) of the mutants was about two fold higher than the parent *P. humicola*. The substrate saturation constant ($K_{s(p)}$) for the maximum alpha amylase production was lower for parent than the mutant strains. The $K_{s(p)}$ values explained that

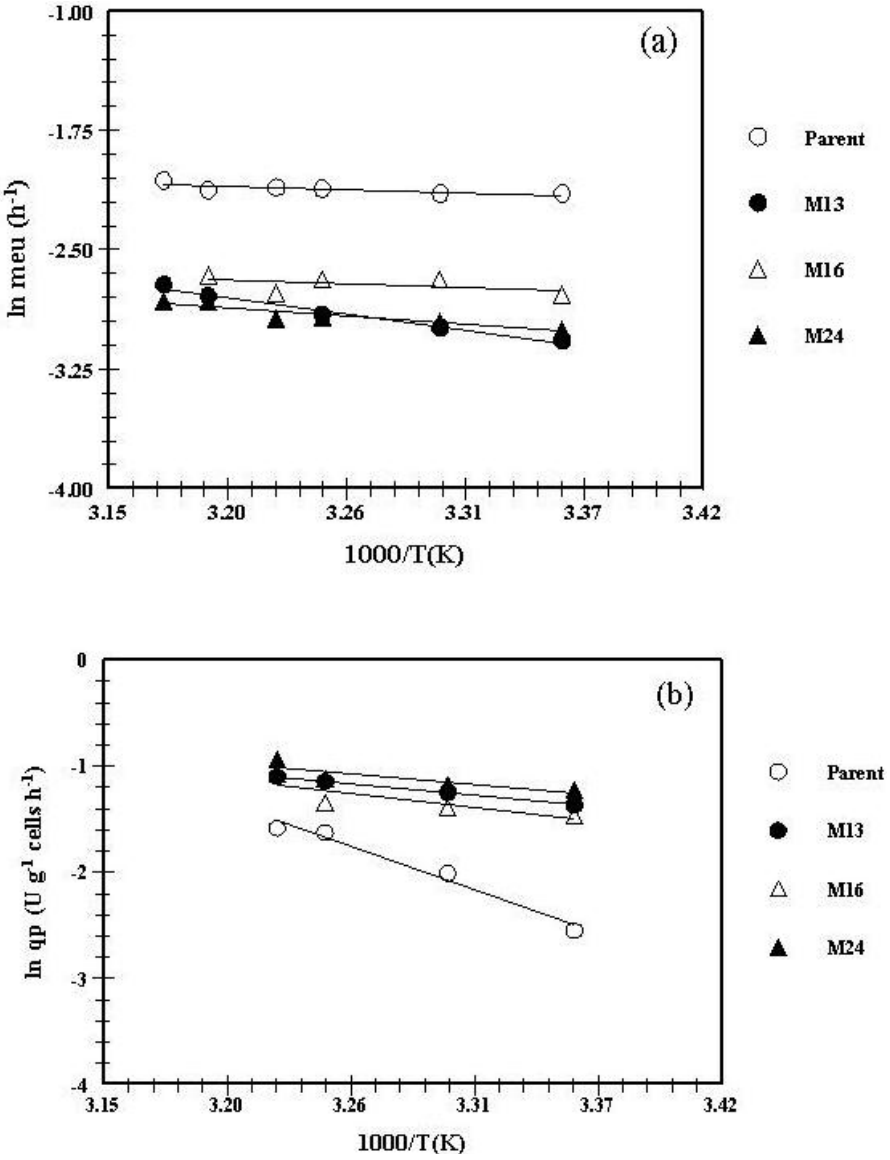

Figure 1. Arrhenius plots for the determination of activation energy: a) cell mass formation ($Ea_{(x)}$), b) alpha amylase production ($Ea_{(p)}$) by parent and mutant of *P. humicola.*

the affinity of starch to the parent was greater as compared to mutants of *P. humicola*. The spontaneous occurrence of a reaction or process can be best determined from its change in free energy (ΔG^*). Lower is the ΔG^* value, more likely is the reaction to take place. The $\Delta G^*_{(x)}$ for cell mass formation of the mutant was slightly lower than that of parent strain, which means the rate of cell mass formation of mutants was higher. This result was also supported by the enthalpy change $\Delta H^*_{(x)}$. Very low $\Delta H^*_{(p)}$ of the mutants *P. humicola* confirmed that the mutants were more active in product formation.

Thermodynamics of alpha amylase production by parent and mutant *P. humicola* is presented in. Both $\Delta G^*_{(p)}$ and $\Delta H^*_{(p)}$ of the mutants were low as compared to the parental strain. The lower enthalpy and Gibbs free energy values again confirmed that the mutants required small amount of energy for the alpha amylase production, hence, confirmed that these were hyper producer strains. The $\Delta S^*_{(x)}$ and $\Delta S^*_{(p)}$ for cell mass and α-amylase production were also determined. The thermodynamic growth parameters of the mutant were lower than those for mesophilic organism, thermotolerant *Kluveromyces marxianus* and *Humicola lanuginosa*. It is suggested that the mutation might have significant effect in thermostabilization of the metabolic network of the mutants (M13, M16 and M24) during α-amylase production, which might be due to either formation of chaperons or hyper-glycosylation of

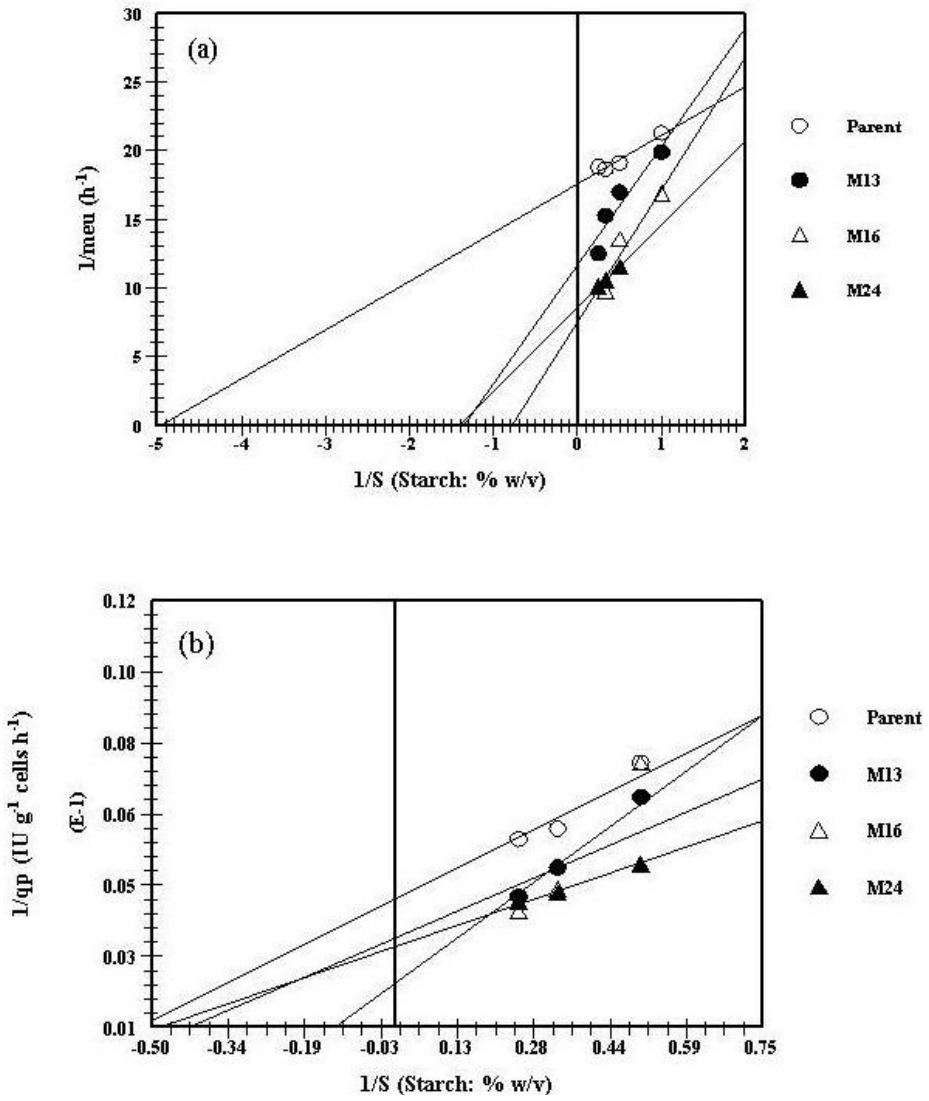

Figure 2. Double reciprocal plots for the determination of: a) maximum specific rate of cell mass (μ_m), and b) specific product (q_{pmax}) formation by parent and mutants of *P. humicola.*

enzymes of the production metabolic network (Bokhari et al., 2009). We could not find any report on the μ_m, q_{pmax}, K_s and Q_{10} from any other mutated alpha amylase producing microbes; therefore we report for the first time these parameters for mesophilic strain of *P. humicola* and its derivative mutants.

Conclusion

A number of microbial sources exist for the efficient production of α-amylase, but only few strains of fungi meet the criteria for commercial production. Gamma ray induced mutation of *P.humicola* resulted into generation of stable and viable mutants for hyper production of α-amylase (>2.0-fold), which were also resistant to catabolite repression**.** Therefore, these mutants have potential for industrial applications. The mechanism underlying this hyper secretion is of paramount significance and needs further study. Our future plans are to purify and characterize the alpha amylases from the parent and mutants of *P. humicola* to investigate the effect of gamma ray mediated mutagenesis on the stability-function relationship of alpha amylases.

ACKNOWLEDGEMENTS

This work was performed as a part of Ph.D. thesis work of Tayyaba Huma who was financially supported by Pakistan Atomic Energy Commission, Government of Pakistan. Technical assistance of Ghulam Ali Waseer is appreciated.

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