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ENHANCED PRODUCTION OF ALKALINE PROTEASE ENZYME BY MUTATED *ASPERGILLUS ORYZAE* DEVELOPED BY RANDOM MUTAGENESIS

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Abstract

Thermostability is an important attribute and a prerequisite for the enzymes used in industrial processes. *Aspergillus oyzae* has the potential to produce proteases for industrial applications. In this study, *A. oryzae* mutants were used to identify hyper-producers of thermostable proteases. Initially, 22 mutants were selected on the basis of their growth performance, cultured on 1% skimmed milk media plates and *Clearing Indexes* (*CI*). Five mutants with higher '*CI*' were selected for submerged fermentation employing 1% casein as substrate and following growth conditions were used: pH 6.5, temperature 30°C and shaking at 150 rpm for 84 hr. Irreversible thermostability was determined at 65°C using pseudo-first order plots. Mutant strain M-120(3) showed highest production and thermostability of protease compared to control and other mutants with half-life (t½) of 125 min. Effect of temperature and substrate concentration on growth kinetics of Mutant M-120(3) were determined showing optimum temperature of 32°C with a specific growth rate (μ) of 0.037h^{-1} and specific rate of product formation (q_p) of 0.31 (U $g^{-1}h^{-1}$). Kinetics and thermodynamic parameters confirmed that mutant strain M-120(3) required less activation energy (E_a) to produce protease enzyme (E_{ap} = 65.22 kJ mol^{-1}) and cell mass (E_{ax} = 29.074 kJ mol^{-1}) than control strain. It had low maximum specific rate of cell mass formation (μ_{max}) 0.043 kJ mol^{-1} and higher maximum specific rate of product formation (q_p) 1.07 kJ mol^{-1}. These parameters confirmed that Mutant M-120(3) had great potential for commercial scale production of thermostable proteases for industrial application.

Key words: Growth kinetics, Gibbs energy, Doubling time, Enthalpy, Activation energy.

Introduction

Proteases represent an important class of enzymes that hydrolyze protein's substrates into amino acids and peptides. Due to its significance in industrial applications, it commands a market share of about 60% of total enzyme sales and is expected to reach annual growth of nearly 6.1% in the period of 2019-2024 (Matkawala *et al*., 2021) Proteases are one of the large families of enzymes that are classified into different groups on the basis of pH (alkaline pH 8.0 to 13.0, neutral pH 6.0 to 8.0 and acidic pH 2.0 to 6.0), cleavage on the specific position (exopeptidase and endopeptidase) and presence of amino acids residues in the active site (asparagine proteases, aspartic proteases, cysteine protease, glutamic proteases, serine proteases, threonine proteases and mixed proteases) (Inácio *et al*., 2015; Souza *et al*., 2015) (Solanki *et al*., 2021). Each of the types has different applications in industries such as food, leather, waste processing, pharmaceutical, and laundry (Naeem *et al*., 2022). Among these proteases, the alkaline proteases alone have 40% of total global enzyme sales due to high stability and activity in harsh conditions (Rekik *et al*., 2019). They are active in neutral to alkaline pH and widely used in detergent, food, pharmaceutical, beef, silk, paper and pulp, cheese making, meat processing, silver recovery, photographic film, bioremediation and leather industries (Arya *et al*., 2021; Sharma *et al*., 2017).

Proteases can be produced by plants, animals and microbes but due to many technological, economical and ethical issues microbial sources are always considered the best choice for their production (Sharma *et al*., 2019; Negi *et al*., 2020; Salwan *et al*., 2020). Proteases produced from microbes are preferred for industrial usage due to their specificity, high production, stability under various conditions of pH and tolerant to heavy metals, surfactants and oxidizing agents and high productivity (Elumalai *et al*., 2020) (Adetunji & Olaniran, 2020). Protease enzymes are produced in a wide variety, characterized by compact structures. They are mostly generated as extracellular proteins, which facilitates their downstream processing (Palsaniya *et al*., 2012).

Among microbes, fungi are preferred organisms for protease production due to numerous economic and technical advantages (Snyman *et al*., 2019). Fungi are good producers of a wide range of proteases of alkaline, acidic and neutral nature and many from these groups have been commercialized (Inácio *et al*., 2015). Fungal strains can grow on numerous substrates under diverse conditions (Siqueira *et al*., 2020). Fungi have developed adaptive strategies for the exploitation and utilization of diverse resources available in the surrounding environment for survival. Secretion of hydrolytic enzymes is a part of such strategies, which plays the main role by making interaction with surrounding resources and causing damage to those organisms that pose threat to them (Snyman *et al*., 2019). *Aspergillus oryzae* is a well-known filamentous fungus widely used in Japanese traditional fermentation industries for the production of soya sauce and sake. Due to its great importance in Japanese cultural food *A. oryzae* has been designated as Japan's national microorganism in 2006 by Japan's brewing society (Kitamoto, 2015; Ichishima, 2016). It also has great potential for producing various secretory enzymes. In addition, recent progress in genetic engineering technology has opened new dimensions in the production of industrial enzymes. Lipase was first example of heterogeneous enzymes produced commercially in 1988 for

laundry detergent from *A. oryzae* (Machida *et al*., 2005, 2008). *A. oryzae* secretes a wide variety of proteases that play an important role in making fermented foods by releasing amino acids from digesting proteins. Previous studies suggest that it produces alkaline proteases in abundance which is highly associated with the utilization of raw materials and the quality of soy sauce (Gao *et al*., 2019).

The present study aimed to screen for hyperproducer mutants of thermostable proteases derived from a stock of Koji (*A. oryzae*) mutants developed through gamma ray mediated random mutagenesis. The present study is novel as it provides a comprehensive analysis of the growth kinetics and thermodynamics of protease production by mutants of *A. oryzae*. Pakistan dedicates a substantial proportion of its reserves towards the import of enzymes from international sources. According to World Trade Map (2022), Pakistan has spent 29.126 million USD on importing enzymes, and currently there is no domestic industrial unit producing the enzymes. Therefore, the current initiative will contribute to the advancement of protease production in local industries that will help Pakistan economically to save the foreign exchange worth of millions of US$. The findings may boost the local production of proteases in reasonble price, which will be applicable in various industrial processes like: food processing, leather, pharmaceutical, detergents, etc.

Material and Methods

Growth and revival of koji mutants: The Koji (*A. oryzae*) mutant strains (total = 22), irradiated with Caesium-137 (Cs-137), were obtained from Industrial Enzymes and Biofuel Group (IEB) of National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, which were developed and revived as described (Aleem *et al*., 2018). For revival, the mutants were spread on petri plates containing solid fungal growth media (SFGM) and incubated for 72 h at 30°C. The composition of media was: 10g glucose, 3g $NaNO_3$, 50ml Salt solution (% w/v: 2.6 KCl, 2.6 $MgSO_4.7H_2O$, 7.6 KH_2PO_4), 01ml Trace elemental solution (% w/v: 1.11 H_3BO_3, 0.11 $Mo_7O_{24}.4H_2O$, 5 EDTA, 0.16 $CoCl_2$. $6H_2O$, 0.5 $FeSO_4.7H_2O$, 2.2 $ZnSO_4.7H_2O$ and 0.5 $MnCl_2.4H_2O$), and 15 g agar and 750ml distilled water. Media pH was adjusted to 6.5 with 1M NaOH/HCl and later the media volume was adjusted to 1L distilled water. Finally, media was autoclaved for 15 min at 121°C and transferred aseptically in petri plates.

Screening of protease producing strains (Clearing Zone Index): The screening approach for the desired strains followed the fundamental methodology outlined in (Damare *et al*., 2006), with custom modifications made to align with the specific growth conditions of the mutant strains. Commercial skimmed milk powder of "Marvel Original company" was used for the screening of mutants. The composition of skimmed milk per 100g was: 0.6g Fat, 50.3g Carbohydrates, 1.3g Fibers, 35.9g Proteins, 1g Salt, 575µg Vitamin A and 1.5µg Vitamin D. The skimmed milk (1% w/v) medium was autoclaved separately at 121°C for 15 min. The SFGM was also autoclaved at 121°C for 15 min without glucose. After autoclaving SFGM and skimmed milk solution were mixed and poured into Petri plates aseptically. The Koji mutants' colonies were picked with the help of sterile toothpicks from the revived cultures in SFGM and transferred to the center of skimmed milk screening media petri plates with a single touch. The plates were incubated at 30°C and observed on regular basis for the appearance of the clearing zones around colonies. The *Clearing indexes* (*CI*) were measured from one edge of the zone to the other with a measuring scale. The *CI* was calculated by dividing the total halo zone diameter with the colony diameter (Aleem *et al*., 2018).

$$\text{Clearing Zone Index (C1)} = \frac{\text{Halo zone diameter + Colony diameter}}{\text{Colony diameter}} \quad (1)$$

Inoculum preparation: 100 ml of liquid fungal growth medium (LFGM) containing 0.3g $NaNO_3$, 2g Glucose, 5ml salt solution and 100 µl trace element solution was made in 250 ml Erlenmeyer flasks for every selected mutant strain. Eight distilled water washed glass beads (~8 mm in size) were added to each flask to break the fungal mycelia. The flasks were autoclaved at 121°C for 15 min. *A. oryzae* spores about 2 to 3 of platinum wire loop full were added in LFGM, aseptically. The flask was incubated in an orbital shaker at 30°C for 150 rpm. The growth was checked regularly until full growth, which was obtained at 36 h. After incubation, 1 ml of medium containing cells was poured in three microfuge tubes with micropipette using the cut tips i.e., to broaden their neck for easy collection of cells aseptically to estimate the wet cell mass. Tubes were centrifuged for 10 min at maximum speed and the supernatants were discarded. The total weight of fungal cells was calculated by subtracting the weights of empty microfuge tubes from tubes containing fungal cell mass. Then, equal weight of cell mass was transferred to protease production media for growth kinetics studies.

Protease production in submerged conditions: The protease production medium was prepared for each strain containing 1% casein Hammerstein grade, 0.3g $NaNO_3$, 5ml Salt solution 100ul trace element solution and the pH 6.5 was adjusted by adding 1M NaOH/HCl. Eight washed glass beads (~8 mm in size) were added to each flask to break the fungal mycelia. The media was autoclaved at 121°C for 15 min. Then equal fungal cell mass 0.4% w/v (wet packed cells) from the inoculum was transferred in production media and the flasks were incubated in orbital shaker at 30°C for 150 rpm for 84 hr at 150 rpm. The mutant strains M-60(5), M-100(12), M-120(2), M-120(3), M-120(5) and M-120(11) that gave higher clearing zone indexes along with control strain were used for the protease production in submerged conditions.

Sample collection for protease and cell mass estimation: Samples were collected at different intervals of time. The packed wet cell mass was estimated as described earlier. Supernatants were transferred to other labeled microfuge tubes for protease assays and tubes containing fungal cells were weighed.

Tyrosine standard curve for protease assay: Tyrosine standard curve was made with some modifications as described (Cupp-Enyard, 2008). Tyrosine stock of 0.01M was prepared in distilled water and the standard solution ranging from 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 75 and 100 µl were pipetted out into a series of test tubes. The volume was made-up to 2.6 ml by adding distilled water. The mixture was incubated for 60 min at 30°C. Then 2.5 ml solution containing 0.11 M trichloroacetic acid (TCA), 0.33 M sodium acetate and 0.22 M acetic acid was added for quenching of the reaction. Then the tubes containing reaction mixture were mixed and vortexed thoroughly, and placed at 4°C for better precipitation for 15 min. Afterwards, 1.2 ml was taken in microfuge tubes and centrifuged at maximum speed for 10 min, and then 1 ml of supernatant from each microfuge tube was transferred to new test tubes and 2.5 ml of 0.55 M Na_2CO_3 was added and mixed thoroughly. Then, 500 µl of three times diluted Folin-Ciocalteu's phenol reagent was added to all tubes for color development and absorbance were measured at 660 nm with spectrophotometer (Spectro 23RS).

Protease assay: Protease assay was performed according to the classical Anson method (Anson, 1938) which was slightly modified. For the assay, the reaction mixture was made containing 100 µl enzyme solution, 0.6% Casein (Hammerstein grade) already prepared in 0.05M phosphate buffer (pH 7.5). For each reaction mixture, a blank reaction mixture was prepared for control, which contained distilled water instead of enzyme and all other above reagents of the reaction mixture. The reaction mixtures were incubated at 30°C for 60 min. The reaction was quenched by adding 2.5 ml of solution containing (0.11M Trichloroacetic acid (TCA), 0.22M Acetic acid and 0.33M Sodium acetate) and allowed to stand at 4°C for 15 min. After the quenching step, 1.3 ml of quenched reaction mixtures were taken in microfuge tubes and centrifuged for 10 min at maximum speed. Afterwards, 1ml supernatant from every microfuge tube was taken and put into new test tubes and 2.5ml of 0.55 M Sodium carbonate (Na_2CO_3) were added and thoroughly mixed, followed by the addition of 500 µl of three-time diluted (Folin-Ciocalteu's phenol) reagent. With the addition of reagent blue color appeared and was measured on a spectrophotometer. One unit of protease activity was defined as the amount of enzyme producing a change of absorbance equivalent to 1 µmole of tyrosine per min under defined assay conditions.

$$\text{Units/ml/min} = \frac{\Delta A^\circ \text{ x Tyrosine S. factor (1.82) x Total RM (5.1 ml)}}{\text{Enzyme (0.1 ml) x RM for color development (1 ml) x Time (60 min)}} \quad (1)$$

Where,
ΔA° = Optical density (OD) at 660 nm = 'Experimental' OD – Blank 'OD'
RM = Reaction mixture

Irreversible thermostability: Irreversible thermostability of proteases produced by parent and mutated strains of *A. oryzae* was determined by taking 1.0 ml of protease enzyme in various labeled microfuge tubes and kept at 65°C in the water bath. After time intervals like: 5, 10, 20, 30, 40, 50, and 60 min, the representative microfuge tube was taken out from the water bath and placed in crushed ice for 30 min and assayed for protease activity. This experiment was performed for all the selected mutants. Finally, protease activity and denaturation rate constant (K_d) were determined by applying pseudo first-order plot.

Effect of temperature on protease production: Potent Koji mutant M-120(3), selected on the basis of hyper production, growth performance and irreversible thermostability was grown in the protease production media at temperatures *viz.* 26, 28, 30, 32, and 34°C. Other growth conditions were: pH 6.5 and casein Hammerstein grade (1% w/v). Samples were taken from the production media at regular time intervals: 12h, 24h, 36h, 48h, 60h, 72h and 84h, and cell mass estimation and protease assay were performed. The cell mass estimation, protease assay, inoculum and protease production media were prepared as described earlier.

The activation energies for protease and cell mass formation $Ea_{(p)}$ and $Ea_{(x)}$ were calculated from Arrhenius plots i.e., between $\ln q_p$ Vs 1/T and $\ln \mu$ Vs 1/T, respectively.

Effects of substrate on protease production: The mutant M-120(3) was grown in enzyme production media on different casein Hammerstein grade concentrations (0.5, 1.0, 1.5, 2.0, & 4.0% w/v) and the pH and temperature was 6.5 and 30°C. Samples were taken from the production media at various time intervals (12h, 24h, 36h, 48h, 60h, 72h and 84h) and cell mass estimation and protease assays were performed. The cell mass estimation, protease assay, inoculum and protease production media were prepared as described earlier.

Growth kinetic parameters: The growth kinetics parameters were determined according to (Muhammad *et al*., 2011) The specific growth rate (μ) was calculated from slope of the plot (lnx vs Time) and doubling time (t_d) of cell biomass was determined by using the formula ($t_d = \ln 2/\mu$).

The product yield coefficient ($Y_{p/x}$) with respect to cell mass was calculated from $Y_{p/x} = dp/dx$, where 'p' was the yield of product (protease units), while 'x' was the cell mass content at the time interval of maximum protease production. Moreover, $dp = p_t - p_0$ and $dx = x_t - x_0$. The p_t & x_t were the amount of protease and cell mass at maximum level of product formation, whereas, p_0 and x_0 were the amount of protease and cell mass at zero time, respectively. The specific rate of product formation (q_p) was determined using equation $q_p = Y_{p/x} \times \mu$.

The maximum specific rate of Koji cell mass production (μ_{max}), maximum specific rate of protease production (q_{pmax}) and the substrate saturation constants (K_s) were determined through direct fit method using Prism software.

Thermodynamics of cell mass and protease production: The thermodynamic parameters of cell mass formation and protease production were determined as described by (Riaz *et al*., 2014). The Eyring absolute rate equation, derived from transition state theory was used (Eyring & Stearn, 1939).

$$k = (k_B.T/h).e^{(-\Delta H^*/RT)}.e^{(\Delta S^*/R)} \quad (3)$$

where, k_B is the Boltzmann's constant (1.38×10^{-23} J K^{-1}), T is absolute temperature, h is Plank's constant (6.626×10^{-34} Js), N is Avogadro's number (6.02×10^{23} mol^{-1}), R is the ideal gas constant (8.314 J K^{-1} mol^{-1}), ΔH^* and ΔS^* (kJ mol^{-1}) are the change in enthalpy and entropy for product formation.

According to μ_{max} and q_{pmax} are equivalent to k, so to calculate change in Gibbs free energy (ΔG^*) for the cell mass formation & protease production the k was replaced with μ_{max} and q_{pmax} in the equation mentioned below:

$$\Delta G^* = -R\text{T} \ln (k.h/k_B.\text{T}) \quad (4)$$

Similarly, E_a was replaced by $E_{a(x)}$ and $E_{a(p)}$ in ($\Delta H^* = E_a - RT$) to calculate the energy of activation for cell mass and protease production. The change in enthalpy (ΔS^*) was calculated by the equation:

$$\Delta S^* = (\Delta H^* - \Delta G^*)/\text{T}) \quad (5)$$

Results and Discussion

A. oryzae is being used to produce many enzymes because it is non-pathogenic and considered generally as safe (Payne *et al*., 2006). Due to this quality, it has been the preferred organism in the traditional Japanese food industry for decades (Machida *et al*., 2005). Pakistan is agriculture-based country that produces about 50 to 60 million tons of agricultural waste annually. Wheat straw, wheat bran, rice straw, rice bran, rice husk, rice polishing, molasses, corn steep liquor, bagasse and banana waste are notable agricultural waste produced in Pakistan (Mukhtar & Haq, 2013). Such huge amount of agricultural waste caused various environmental problems like global warming is caused by burning of wastes (Levine, 1996). This waste can be utilized to produce valuable products such as biofuels, chemicals and enzymes. So, this industrial waste can be suitable substrate for the low-cost protease enzyme production (Ahmad *et al*., 2020). Pakistan also imports huge quantity of enzymes produced by microbes annually for textile industries (Chatha *et al*., 2017).

Many studies have been reported on the hyper-production of enzymes but the problem of thermostability still persists. Thermostable enzymes have high stability in the working environment of industries at high temperatures (Wu *et al*., 2021). It is the beauty of evolution that microbes modify their genomes through mutations to survive in unfavorable environments (Lenski, 2012). These mutations can be responsible for high production and thermostable enzymes (Sharma *et al*., 2019). To find such microbes in an environment is like finding a needle in a haystack. So there are other strategies for the creation of mutants in the laboratory that can provide thermostable enzymes such as high ionization radiations, which can cause mutations in the genome of organisms (Basu, 2018).

Koji mutants screening based on clearing zones: Screening of Koji mutants was performed to select the potent proteases hyper producer strains. To assimilate skimmed milk proteins during growth *A. oryzae* produces protease which hydrolyzes the surrounding proteins. Hence, clearing zones around the colonies presented the extent of protease production. The clearing zone index (*CI*) of mutant M-120(3) was largest (2.35) among the tested Koji mutants (Total = 22), which were grown on commercial grade dried skimmed milk (Table 1). Protease clearing zones of six potent Koji mutant strains have been shown (Fig. 1).

Table 1. clearance zones index of protease production by *Aspergillus oryzae* mutant strains grown on 1% (w/v) skimmed milk.

A. oryzae strain	Zone Dia. + Colony Dia. (cm)	Colony Diameter (cm)	Clearing Zone Index (CI)
Control	2.65 ± 0.15	2.25 ± 0.12	1.18
M-60(2)	2.1 ± 0.12	2.00 ± 0.11	1.05
M-60(5)	4.05 ± 0.15	2.5 ± 0.11	1.62
M-60(9)	1.4 ± 0.08	1.2 ± 0.06	1.17
M-60(10)	1.9 ± 0.11	1.5 ± 0.08	1.27
M-60(12)	1.8 ± 0.1	1.4 ± 0.08	1.29
M-80(3)	2.1± 0.13	1.8 ± 0.09	1.16
M-80(4)	2 ± 0.11	1.9 ± 0.1	1.05
M-80(5)	1.9 ± 0.15	1.7 ± 0.08	1.11
M-80(7)	2.4 ± 0.13	2.8 ± 0.15	0.86
M-80(8)	1.75 ± 0.1	1.51 ± 0.08	1.16
M-80(11)	3 ± 0.17	2.6 ± 0.14	1.15
M-80(12)	3.5 ± 0.23	3 ± 0.16	1.16
M-100(3)	2.85 ± 0.16	2.6 ± 0.14	1.1
M-100(6)	3.65 ± 0.2	3.2 ± 0.17	1.14
M-100(9)	3.3 ± 0.18	2.9 ± 0.16	1.14
M-100(10)	1.45 ± 0.08	1.41 ± 0.08	1.03
M-100(12)	4.4 ± 0.25	3.1 ± 0.21	1.41
M-120(2)	4.25 ± 0.09	2.4 ± 0.08	1.8
M-120(3)	4.35 ± 0.18	1.85 ± 0.11	2.35
M-120(5)	3.75 ± 0.16	2.4 ± 0.12	1.56
M-120(11)	4.7 ± 0.23	3.3 ± 0.2	1.42

Where, Clearance zone index (CI) = Halo zone diameter + colony diameter/colony diameter. Data presented is average values of n = 3 experiment and ± was Standard deviation (SD)

Protease production in liquid media: Mostly enzymes are produced through submerged fermentation, therefore production in liquid media offers the advantage of high recovery in downstream processes (Ali Al-Maqtari *et al*., 2019). To confirm hyper production of enzymes in liquid media, the potent strains having higher clearing indexes were used (Aleem *et al*., 2018). Therefore, *A. oryzae* mutant strains M-60(5), M-100(12), M-120(2), M-120(3), M-120(5) and M-120(11) were selected on the basis of clearing zone index and grown in liquid media for the production of proteases. Total protease activity of Koji mutants was calculated along with the control strain. Protease production in liquid media showed the same pattern of production as in the solid growth conditions. Mutant M-120(3) gave hyper protease production comparatively to other mutant strains, which was 1.65-fold higher. However, increase in protease productivity of other mutants like: M-60(5), M-100(12), M-120(2), M-120(5) and M-120(11) compared to control was 1.38, 0.09, 0.12, 1.28 and 1.15 folds, respectively (Fig. 2; Table 2).

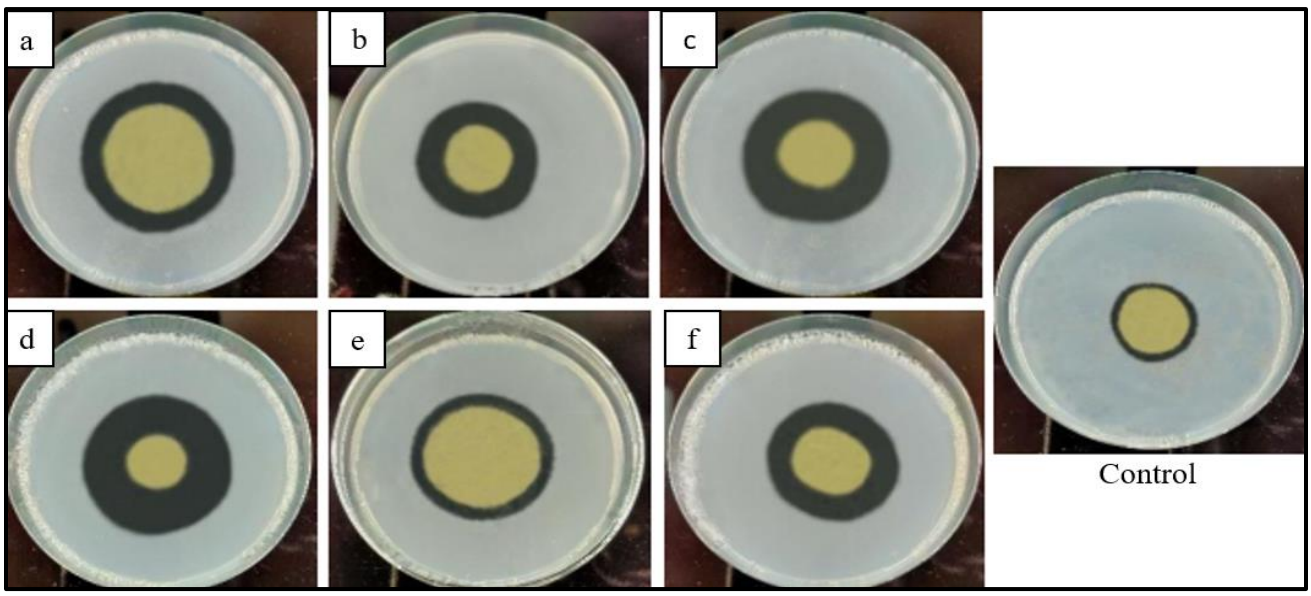

Fig. 1. Clearing zones of potent protease hyper producing mutant strains grown on skimmed milk at 30°C, pH 6.5: **a**) M-120(11), **b**) M-120(5), **c**) M-120(2), **d**) M-120(3), **e**) M-100(12), **f**) M-60(5).

Table 2. Comparison of *A. oryzae* control and mutant strains for protease production.

A. oryzae	Units ($U.ml^{-1}.min^{-1}$)	Folds increase
Control	1.30 ± 0.104	1.00
M-60(5)	1.80 ± 0.120	1.38
M-100(12)	0.12 ± 0.012	0.09
M-120(2)	0.16 ± 0.17	0.12
M-120(3)	2.15 ± 0.133	1.65
M-120(5)	1.67 ± 0.143	1.28
M-120(11)	1.50 ± 0.009	1.16

Where, Folds increase = Protease Units of Strain/ Protease Units of Control strain

Table 3. Irreversible Thermostability of proteases produced by *A. oryzae* mutants at 65 °C.

γ-ray Exposure	*K*d (min^{-1})	t½ (min)
Control	0.01023	68
M-60(5)	0.01808	38
M-100(12)	0.03428	20
M-120(2)	0.04789	14
M-120(3)	0.005549	125
M-120(5)	0.01183	59
M-120(11)	0.01155	60

K_d (first order rate constant of inactivation) determined from Fig. 3 and t½ (half-life) = $0.693/K_d$

Irreversible thermostability of koji proteases: Thermostability is an important property of enzymes for industrial applications. Thermostable enzymes work in harsh conditions and this property is very useful in industries to ensure reusability and cost-effectiveness (Xu *et al*., 2020). On the basis of clearing zone index and protease production in liquid conditions six potent mutants *viz*.: M-60(5), M-100(12), M-120(2), M-120(3), M-120(5) and M-120(11) were selected. The proteases produced by these mutants along with control were subjected to thermostability experimentation, pseudo-first order plots were drawn between *ln* (Protease activity) and time. The potent mutant M-120(3) having highest thermal stability i.e., half-life 't½' = 125 min at 65°C was selected. However, all other Koji mutants had lower t½ for proteases and lowest was for the M-120(2) strain, which was 14 min compared to control (Fig. 3; Table 3).

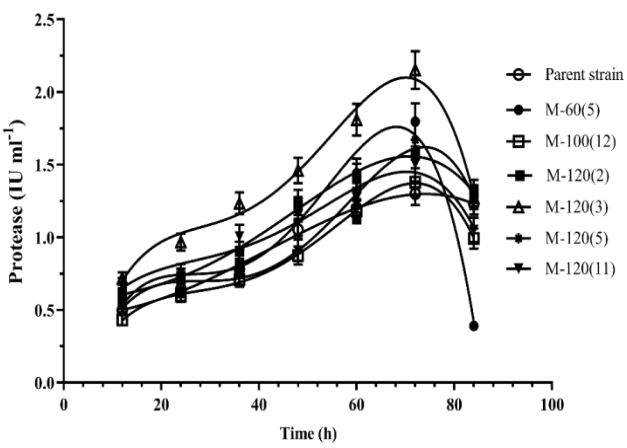

Fig. 2. Production of proteases by mutant Koji strains grown on skimmed milk under liquid state conditions at 30°C, pH 6.5.

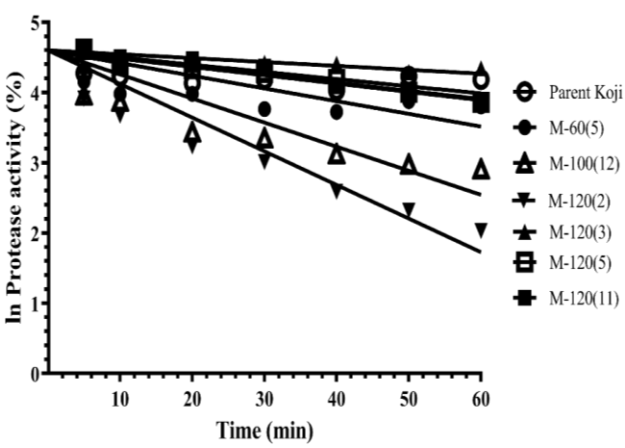

Fig. 3. Pseudo first-order plots to determine inactivation rate constants for irreversible thermostability of proteases at 65°C produced by parent and mutants of *A. oryzae.*

Growth kinetics of protease production: Enzyme production from any specific microorganism in industries requires a lot of considerations such as effects of temperature, pH, substrate, etc. on growth and production of the enzymes. Optimization of temperature and substrate concentration for the enhanced production of protease was carried out at laboratory scale in shake flasks.

Effect of temperature: Incubation temperature is an important parameter for the growth of microbes. Every organism has a specific optimum temperature at which its metabolic system is at its peak (Sharma *et al*., 2017). The potent mutant M-120(3) and control Koji strain were grown at temperatures: 26°C, 28°C, 30°C, 32°C and 34°C to investigate their optimum growth temperature. Increasing trend for cell mass formation of both strains at all temperatures was found, however, the mutant strain gave higher cell mass formation. The protease production for parent and M-120(3) strains was highest at 30°C and 32°C, respectively. The mutant strain was a hyper producer of proteases compared to parent strain. For the significance of results, kinetics parameters were involved such as specific growth rate (μ), biomass doubling time (t_d), product yield coefficient with respect to cell mass (q_p) and specific rate of product formation ($Y_{p/x} \times \mu$) at temperature ranges of 26-34°C (Table 4). Specific growth rate (μ), which reflects the growth of fungal population in the exponential phase was 0.036 (h^{-1}) for parent strain, while it was slightly decreased to 0.033 (h^{-1}) at 30°C for the mutant koji. It was observed that in most cases low specific growth rate was desirable for the enhanced production of the enzymes, which led to the higher product yield. Decrease in the specific growth rate affected the doubling time (t_d). The t_d of mutant strain was increased to 21.0 h as compared to parent strain which had 19.2 h.

The product yield coefficient ($Y_{p/x}$) focuses on protease enzyme production with respect to cell mass. At 30°C, $Y_{p/x}$ of parent strain was 6.0 (U g^{-1}) while for mutant strain it was 8.8 (U g^{-1}) confirming that the mutant cells produced more enzyme than parent strains. The specific rate of protease production (q_p) of parent strain was 0.21 ($Ug^{-1}h^{-1}$), whereas for mutant strain it was 0.29 ($Ug^{-1}h^{-1}$) at 30 °C, which was 1.38 folds higher than the parent strain. It is evident that the mutant strain has an improved efficiency of protease production by conversion of substrate (1% casein Hammerstein). Highest q_p was observed for the mutant strain i.e., 0.31 U $g^{-1}h^{-1}$ at 32°C, which was optimum temperature of the mutant strain.

(Gomi, 2014) suggested about the optimal temperature for growth of *A. oryzae* as 32-36°C (±1°C), while its optimal temperature for the enzyme production was different (Narahara *et al*., 1982). This was due to the different environmental conditions such as stress and temperature in fermentation processes. The optimal temperature for product formation depends upon the type of product, for example in fermentation the phenols, esters and alcohol were produced in temperature ranges of 15 to 45°C (Jiang *et al*., 2022). Lipase production by *A. oryzae* was found to be maximum at 28°C (Ohnishi *et al*., 1994). Its optimal growth temperature varies due to its strong metabolic adaptability to different temperatures (Jiang *et al*., 2022). In protease productions, *A. oryzae* showed maximum production mostly at 30°C (Chutmanop *et al*., 2008; Vishwanatha *et al*., 2010).

Effect of substrate: The influence of substrate on biomass production and product formation is a crucial factor in the scientific study of growth kinetics in submerged conditions. Microorganisms rely on a particular substrate concentration to facilitate the production of various products. The growth of microorganisms in submerged conditions follows various stages, namely the lag phase, log phase (exponential phase), stationary phase, and death (decline) phase. The occurrence of these phases is dependent upon the presence of a substrate that has the potential to influence both production and the formation of cell mass. When the substrate concentration is low, microbial growth experiences a rapid decline, resulting in reduced production and cell mass formation. The lack of profitability in industries is due to the inability to achieve high substrate utilization and low product formation. Hence, in order to achieve a favorable outcome within a specified timeframe while minimizing substrate usage, it is essential to optimize the quantity of substrate prior to scaling up to industrial production.

To evaluate kinetics parameters for the substrate concentration's effect, the parent and mutant M-120(3) strains were grown on various substrate concentrations (0.5, 1.0, 1.5, 2.0 and 4.0% w/v) i.e., skimmed milk. Maximum protease production i.e., 1.80 U ml^{-1} and 2.27 U ml^{-1} was obtained for parent and mutant strain, respectively at substrate concentrations of 1.5% and 2.0% and highest yield coefficient ($Y_{p/x}$) was also observed at these substrate concentrations. The mutant strain M-120(3) gave higher production compared to the parent strain on all substrate concentrations. Specific growth rate (μ) of parent and mutant strains was highest at 1.0% and 2.0% of substrate concentration, respectively (Table 5).

Kinetics and Thermodynamics of Casein Hydrolysis: The kinetics of substrate utilization for maximum cell mass formation (μ_{max}) and protease production (q_{pmax}) was determined by applying linear and non-linear regression (direct fit model). For linear regression the Eadie Hofstee plot was applied, which was drawn between μ & μ/S to determine the μ_{max} which represented the fastest rate at which a microorganism can grow under ideal conditions (Fig. 4a). The μ_{max} for parent and mutant strains was 0.024 (h^{-1}) and 0.054 (h^{-1}). Likewise, for q_{pmax}, which is the maximum specific rate of product formation by a microorganism, the plot was drawn between q_p & V/S (Fig. 4b). The q_{pmax} for the parent and mutant strains was 0.048 $Ug^{-1}h^{-1}$ and 0.261 $Ug^{-1}h^{-1}$, respectively.

The thermodynamics parameters were also determined to check the performance of mutant strain M-120(3) compared to parent strain. Thermodynamic values calculated in the Table 6 are based on non-linear regression (direct fit method) because direct fit method is considered to be more reliable because results are based on the actual data of interactions and it depends upon underlying relationship between the variables. The maximum specific rate of cell mass formation (μ_{max}) of mutant strain M-120(3) was 0.042 h^{-1}, which was slightly lower than parent strain (0.043 h^{-1}). The substrate saturation constant (K_{sx}) to achieve maximum growth of parent strain (0.218 % w/v) was nearly one and half fold greater than the mutant M-120(3) having value of 0.142% (w/v), which showed that the mutant required less substrate for the cell mass formation. The Eadie-Hofstee plot was also employed to compare the outcomes achieved through linear regression and it was observed that these outcomes concurred with those obtained through the direct fit method (Table 6).

Table 4. Temperature effect on kinetics of protease production by parent and mutated *A. oryzae* M-120(3) grown in submerged conditions at pH 6.5.

Temp (°C)	Koji strain	Protease (U ml^{-1})	Cell mass (g ml^{-1})	μ (h^{-1})	t_d (h)	$Y_{p/x}$ (U g^{-1})	q_p (U g^{-1} h^{-1})
26	P	1.38 ± 0.085	0.21 ± 0.011	0.04	17.3	6.5	0.26
	M	1.41 ± 0.081	0.22 ± 0.012	0.041	16.9	6.7	0.26
28	P	1.45 ± 0.075	0.23 ± 0.012	0.04	17.3	6.3	0.25
	M	1.73 ± 0.098	0.22 ± 0.011	0.034	20.4	7.9	0.27
30	P	1.73 ± 0.099	0.29 ± 0.016	0.036	19.2	6	0.21
	M	2.02 ± 0.113	0.23 ± 0.013	0.033	21.0	8.8	0.29
32	P	1.72 ± 0.089	0.15 ± 0.013	0.027	25.6	11.5	0.31
	M	2.04 ± 0.136	0.25 ± 0.014	0.037	18.7	8.2	0.31
34	P	1.35 ± 0.071	0.21 ± 0.014	0.04	17.3	6.4	0.25
	M	1.61 ± 0.091	0.22 ± 0.013	0.041	16.9	7.3	0.29

Where, P and M stands for parental and mutant M-120(3) strains. μ = Specific growth rate, t_d = Biomass doubling time = ln2/μ, $Y_{p/x}$ = Product yield coefficient with respect to cell mass and q_p = Specific rate of product formation =$Y_{p/x}\times \mu$. Data presented was average values of n = 3 experiment and ± was Standard deviation (SD)

Table 5. Substrate effect on kinetics of protease production by *A. oryzae* parent and mutant strains grown in submerged conditions.

Skim Milk % (w/v)	Strain	Protease (U ml^{-1})	Cell mass (g ml^{-1})	μ (h^{-1})	t_d (h)	$Y_{p/x}$ (U g^{-1})	q_p (U g^{-1} h^{-1})
0.5	P	1.47 ± 0.076	0.15 ± 0.011	0.043	16.1	9.8	0.42
	M	1.95 ± 0.101	0.15 ± 0.011	0.033	21.1	13.0	0.41
1.0	P	1.66 ± 0.085	0.14 ± 0.014	0.045	15.4	11.9	0.53
	M	2.02 ± 0.119	0.15 ± 0.010	0.034	20.4	13. 6	0.46
1.5	P	1.80 ± 0.093	0.15 ± 0.013	0.038	18.2	12	0.46
	M	2.15 ± 0.124	0.145 ± 0.013	0.028	24.8	14.7	0.32
2.0	P	1.62 ± 0.084	0.15 ± 0.012	0.039	17.8	10.8	0.43
	M	2.27 ± 0.118	0.150 ± 0.012	0.04	17.4	15.13	0.46
4.0	P	1.56 ± 0.081	0.13 ± 0.011	0.04	17.3	11	0.48
	M	1.89 ± 0.122	0.155 ± 0.014	0.036	19.3	12.1	0.41

Where, P and M stands for parental and mutant M-120(3) strains. μ = Specific growth rate, t_d = Biomass doubling time = ln2/μ, $Y_{p/x}$ = Product yield coefficient with respect to cell mass and q_p = Specific rate of product formation =$Y_{p/x}\times \mu$. Data presented was average values of n = 3 experiment and ± was Standard deviation (SD)

Table 6. Kinetics and thermodynamics of cell mass formation by *A. oryzae* parent and mutant M-120(3) strain grown on casein under submerged condition at 30°C, pH 6.5.

Description	Parameters	Parent strain	Mutant M-120(3)
Cell mass formation	μ_{max} (h^{-1})	0.043 0.044*	0.042 0.043*
	$Ks_{(x)}$ (% w/v)	0.218 0.281*	0.142 0.258*
	$Ea_{(x)}$ (kJ mol^{-1})	47.946	29.074
	$\Delta G^*_{(x)}$ (kJ mol^{-1})	105.212	105.296
	$\Delta H^*_{(x)}$ (kJ mol^{-1})	45.427	26.554
	$\Delta S^*_{(x)}$ (J mol^{-1} K^{-1})	-192	-254

Where: Maximum specific rate of cell mass formation (μ_{max}) and substrate saturation constant ($Ks_{(x)}$) for maximum cell mass formation were determined from Figure 5a, activation energy for cell mass formation (Ea_x) for parent and mutant strain M-120(3) were determined from Figure 6(a). Thermodynamic parameters for cell mass formation ($\Delta H^*_{(x)}$, $\Delta G^*_{(x)}$ and $\Delta S^*_{(x)}$) were calculated as described in materials and methods. The values denoted by * were determined by linear plot

Arrhenius plot was used to determine the activation energy for the biomass formation $E_{a(x)}$ of parent (47.94 kJ mol^{-1}) and mutant strain M-120(3) (29.07 kJ mol^{-1}). These activation energies showed that mutant M-120(3) required lesser amount of energy for the production of cell mass and mutant M-120(3) was growing at faster rate than the parental strain (Fig. 6a). Hence, the mutant M-120(3) required low energy to produce cell mass that benefits mutant strain M-120(3) to grow fast by using low energy. Parent strain used most of its energy in production of cell mass, that's why its enzyme production was comparatively low.

Thermodynamic parameters (Gibbs free energy (ΔG^*), enthalpy (ΔH^*) and entropy (ΔS^*) were calculated using equation 4 and 5 using the data from direct fit method (Table 6). The Gibbs free energy (ΔG^*) which is also known as functional energy and it describes the spontaneous occurrence of reaction in a thermodynamics system, the system with lower ΔG^* proceeds towards reaction more than the system with high ΔG^*. The determined $\Delta G^*_{(x)}$ for cell mass formation of mutant strain M-120(3) was 105.296 kJ mol^{-1}, which was almost equivalent to parent strain (105.212 kJ mol^{-1}). The enthalpy ΔH^* is another parameter of thermodynamics. Low ΔH^* means the system requires less energy to hydrolyze the substrate. The calculated enthalpy for cell mass formation $\Delta H^*_{(x)}$ of mutant strain M-120(3) was 26.554 kJ mol^{-1} and was lower than parent strain (45.427 kJ mol^{-1}). This indicator showed that mutant strain required less energy to produce the cell mass. The entropy ΔS^* was also determined to check the randomness in the production system comparatively. The determined value of entropy for cell mass formation $\Delta S^*_{(x)}$ in parent strain was higher (-192 $Jmol^{-1}$ K^{-1}) when compared with mutant strain M-120(3) (-254 $Jmol^{-1}$ K^{-1}) showing higher cell division.

The maximum specific rate of product formation q_{pmax} of mutant strain M-120(3) was 1.07 U g^{-1} h^{-1} and was nearly double than parental strain (0.632 U g^{-1} h^{-1}). Hence, highlighted that the mutant strain M-120(3) performed well by producing more product (protease). While substrate saturation constant for product formation (K_{sp}) of mutant strain M-120(3) was 0.81 % (w/v), greater than parent strain (0.53 % w/v) indicating that the mutant required lesser amount of substrate for the production of protease enzyme. Arrhenius plot between ln q_p and 1000/T was used to determine the activation energy for the protease production $E_{a(p)}$, slopes showed the activation energy values of protease production $E_{a(p)}$ to be 66.437 kJ mol^{-1} and 65.223 kJ $mol^{-1}$for parent and mutant strains, respectively which showed that mutant strain M-120(3) was more efficient in protease production and required less energy compared to the parent strain. ΔG^* for protease formation of mutant M-120(3) was 96.952 $kJ.mol^{-1}$ and was lower than parent strain (98.309 $kJ.mol^{-1}$) and required less energy to break the cells (Table 7). The ΔG^* proved that mutant strain was metabolically more active than parent strain comparatively in term of cell mass and product formation. The calculated enthalpy for the product formation $\Delta H^*_{(p)}$ of mutant strain M-120(3) (62.704 $kJ.mol^{-1}$) was also lower than parent strain (63.918 $kJ.mol^{-1}$). This indicator showed that mutant strain M-120(3) required less energy to produce the protease enzyme in the system. The determined value of entropy for product formation $\Delta S^*_{(p)}$ was found to be slightly lower in parent strain (-111 $Jmol^{-1}$ K^{-1}) when compared with mutant strain M-120(3) (-110 $Jmol^{-1}$ K^{-1}). The observed low entropy $\Delta S^*_{(p)}$ indicated a favorable condition, providing evidence that mutant strain 120(3) possessed a robust production system utilizing cell mass as a production factory. The results indicated that the metabolic pathways regulating the synthesis of protease in mutant M-120(3) exhibited a higher degree of thermodynamic favorability when compared to the parent strain.

Table 7. Kinetics and thermodynamics of protease production by *A. oryzae* parent and mutant M-120(3) strain grown on casein under submerged condition at 30°C, pH 6.5.

Description	Parameters	Parent strain	Mutant M-120(3)
Protease production	q_{pmax} (U g^{-1} h^{-1})	0.632 0.499*	1.07 1.163*
	$Ks_{(p)}$ (% w/v)	0.53 0.278*	0.81 1.04*
	$Ea_{(p)}$ ($kJ.mol^{-1}$)	66.437	65.223
	$\Delta G^*_{(p)}$ ($kJ.mol^{-1}$)	98.309	96.952
	$\Delta H^*_{(p)}$ ($kJ.mol^{-1}$)	63.918	62.704
	$\Delta S^*_{(p)}$ ($Jmol^{-1}$ K^{-1})	-111	-110

Where: Maximum specific rate of product formation (q_{pmax}) and substrate saturation constant ($Ks_{(p)}$) for maximum product (protease) production were calculated from Figure 5b. Activation energy for protease production (Ea_p) for control and mutant strain M-120(3) were determined from Figure 6b. Thermodynamic parameters for product (protease) production (ΔH^*_p, ΔG^*_p and ΔS^*_p) were calculated as described in materials and methods. The values denoted by * were determined by linear plot.

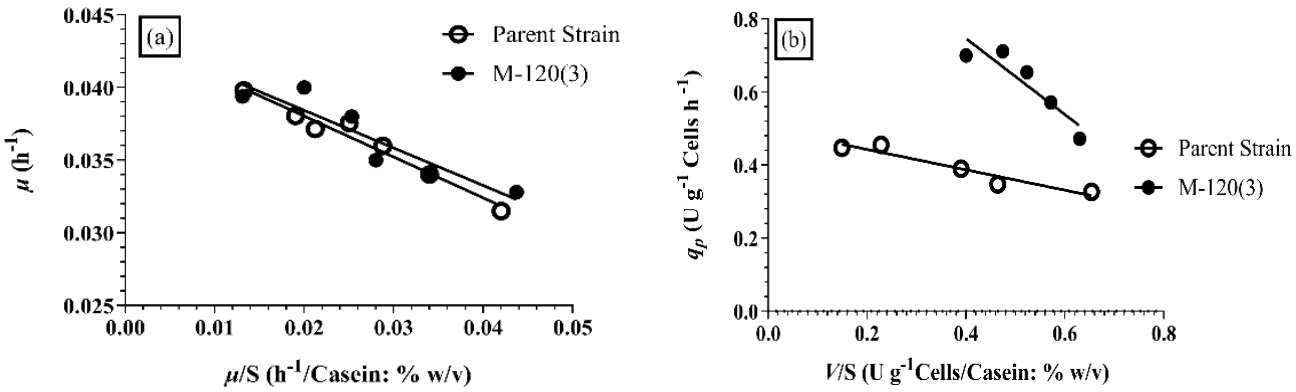

Fig. 4. Determination of Growth kinetic constants (μ_{max}, q_{pmax}, K_s) for protease production on casein as a substrate by parent and mutant M-120(3) strains of *A. oryzae* grown at 30°C and pH 6.5 by using Eadie-hofstee plot: (a) μ_{max} and (b) q_{pmax}

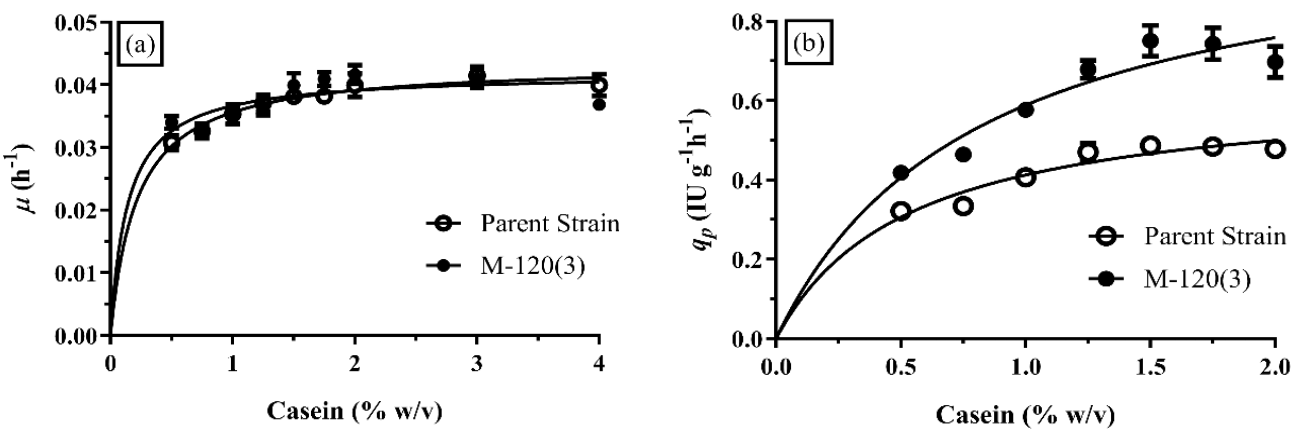

Fig. 5. Determination of Growth kinetic constants (μ_{max}, q_{pmax}, K_s) for protease production on casein as a substrate by parent and mutant M-120(3) strains of *A. oryzae* grown at 30°C and pH 6.5 by using direct fit model of graphpad prism version 8.4.3: (a) μ_{max} and (b) q_{pmax}

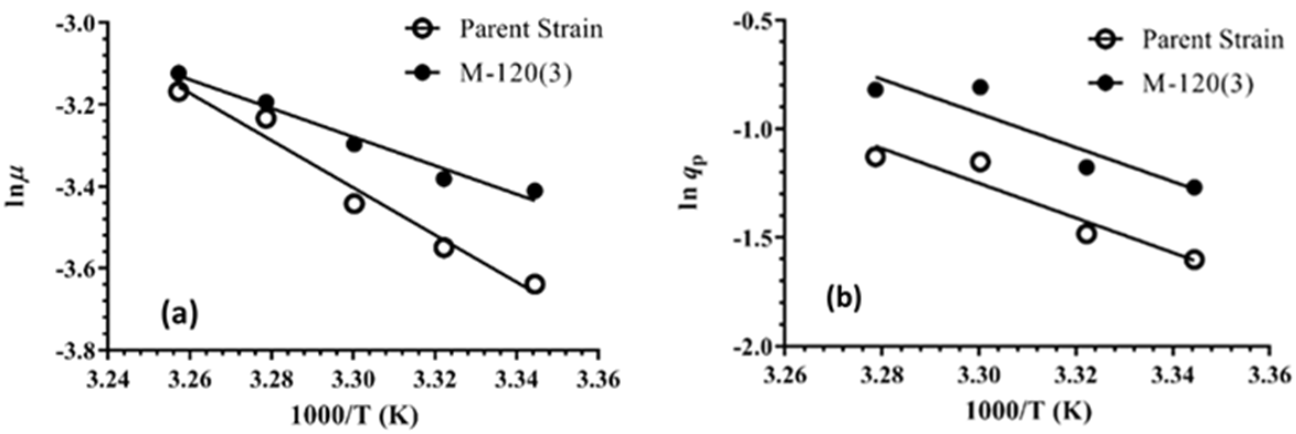

Fig. 6. Arrhenius plots to determine the activation energy: (**a**) biomass formation (Ea_x) and (**b**) protease production (Ea_p) by parent and mutated M-120(3) *A. oryzae.*

Conclusions

The M-120(3) mutant emerged as promising strain and exhibited enhanced protease production with improved thermostability (t½ = 125 min). These findings validate the most efficient production of thermostable proteases derived from *A. oryzae* through the application of γ-ray mutagenesis. The kinetics and thermodynamics parameters (K_s, ΔG^*, ΔH^*, and ΔS^*) supported the potential of mutant M-120(3) strain as a hyper producer of thermostable protease. Higher thermostability of M-120(3) protease signifies its application in various industrial processes. Hence, the possible local production of mutated protease will certainly help to reduce the import burden on Pakistan's economy.

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