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Optimization, Characterization and Thermodynamic Studies on *B. licheniformis* ALW1 Keratinase

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OPTIMIZATION of *B. licheniformis* ALW1 keratinase was investigated by using a Plackett – Burman design (PBD) and Central Composite Design (CCD). PBD showed that galactose, inoculum size and corn steep liquor were the most effective variables played a role in improving the enzyme productivity (87.65U/mL). CCD results recorded an increase in enzyme productivity to about1.4-fold compared to the basal medium (99.1 U/mL). The optimum activity for the partial purified enzyme was obtained at pH 8.5 and 70˚C. The activation and deactivation energy were calculated to be 25.37 kJmol^{-1} and 73.38 kJmol^{-1} respectively. The half-life time was 1380,690,530, and383 min. at 50˚C,55˚C,60˚C and 65˚C respectively. Also, D values were 4600,2300,1769, 1277min. at the same degree respectively. $\Delta G°$ (kJmol^{-1}) kept relatively constant between 50-60˚C (191.49 kJmol^{-1}-193.31 kJmol^{-1}) and noticeably increase at 65˚C (212.86 kJmol^{-1}). $\Delta H°$ (kJmol^{-1}) recorded minor decrease by the increase of temperature. Approximately, most of the tested metals ions have stimulation effect in enzyme activity and $MgSO_4.H_2O$ was the best (146%). Among all the tested detergents tween 80 retained 97% of original enzyme activity. DMSO increased the enzyme activity by about 11%, while propanol and acetonitrile reduced the enzyme activity to about 14% and 10% respectively. All the reducing agents had a stimulating effect on enzyme activity with variable degrees. The enzyme (980 U) had the ability to hydrolyze 74% of the feather to nutritional valuable protein.

Keywords: Keratinase, *B. licheniformis*, Partial purification, Thermodynamic, Kinetics.

Introduction

One of the most popular environmental pollution problems was the slaughterhouse keratinous wastes accumulation. The feather produced from poultry processing industries was common. Utilization of feathers by microbial keratinases is an efficient method for production of valued products (Fig. 1) with green and inexpensive method [1].

Around 60 % of industrial enzymes were recorded to proteases [2,3]. Keratinases as an example of proteases are used widely in cosmetics, prion degradation, detergents and leather industry [4,5].

Production of microbial enzymes was greatly influenced by the medium component and environmental conditions. Optimization of these factors by the classical method (one variable at a time) consumed a lot of time and effort and gave no date for the interaction between variables. Usage of statistical design could effectively increase the enzyme productivity [6, 7].

Feather meal is a product of feather improvement by keratinase. It was composed of carbon, nitrogen, sulfer and hydrogen with a percent of 44, 14, 3.2 and 1.4 respectively and the protein content of feather meal was 87% [8]. It is also contained rare amino acids such as serine, cysteine, arginine, threonine and proline, which makes feather meal suitable source for using as organic fertilizers, feedstuffs or feed supplement [9, 10].

Keratinase was produced from a diverse group of microorganisms including fungi, actinomycetes and bacteria. Keratinolytic enzymes from bacteria largely belonged to serine proteases. The metalloprotease was rarely

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found and associated mainly with Gram-negative bacteria [11, 5, 12].

Designing of efficient and economic bioprocess of enzymes was dependant on the enzyme stability. The thermodynamic study is considered as one of the essential keys to understand the thermal stability of the enzyme and to judge its capability to be used in industrial field [13, 14].

The current study presents sequential optimization strategy to improve keratinase production using *Bacillus licheniformis* ALW1. Partial purification and characterization of the partial pure keratinase were investigated. As there are a few studies about thermodynamic parameters affecting keratin hydrolysis, the study of these parameters is necessary to use the enzyme efficiently in all industrial applications.

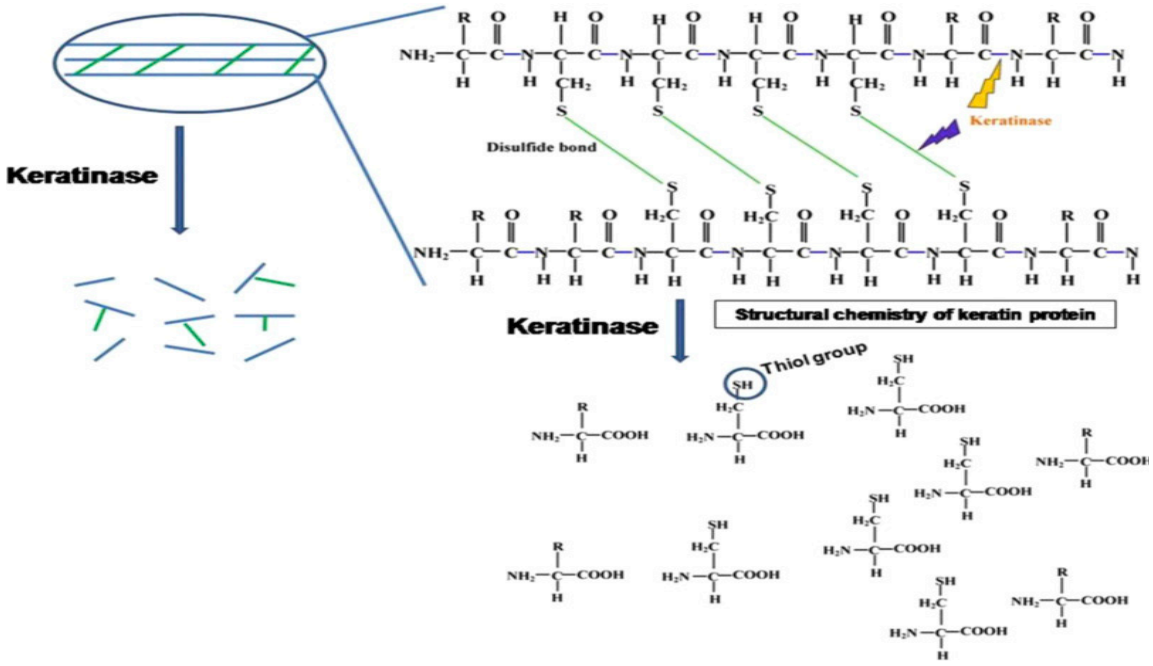

Fig. 1. Schematic representation of keratin and mode of action of keratinolytic enzymes: Source (Paul et al., 2016).

Materials and Methods

Chemicals

Turkey feathers were collected from local farm in Kafr Ghataty, Al-Haram, Giza, Egypt. Corn steep liquor (CSL) was obtained from Egyptian Starch and Glucose Company, Mostorod, Qalubia, Egypt. All other chemicals were of analytical grade.

Microorganism

Bacillus licheniformis ALW1 was identified based on 16s rDNA sequence and Transmission Electron Microscopy examination and It was registered in Gene bank with accession no. **LC315920.**

Feather and soluble keratin preparation

Turkey feathers were prepared for the experiment according to Saber et al. [15]. Soluble keratin was prepared according to the method of Mazotto et al. [5] to be used as the substrate in keratinase assay.

Inoculum preparation

Ten milliliters of sterilized medium composed of (g/L): glucose 10.0; peptone 10.0; yeast extract 3.0; $CaCl_2.2H_2O$ 2.0; pH 7.0 ± 0.2 [16] were added to each slant and scratched with sterile needle. This suspension was transferred to 250 mL Erlenmeyer flasks containing 40 mL of sterilized medium, incubated for 48h at 37˚C in shaking incubator at 180rpm.

Fermentation medium

The production medium contained 1% native feather which was submerged in 50 mL medium contained (g/L): NaCl 0.5; KH_2PO_4 0.7; K_2HPO_4 1.4; $MgSO_4.7H_2O$ 0.1; Galactose 1.0; CSL 9.2; pH 6.0 ± 0.2. The medium was prepared in 250 mL Erlenmeyer

flasks, inoculated with 5 % of inoculum medium and incubated at 42˚C under static condition for four days. At the end of the fermentation period the fermented medium was centrifuged at 5000 rpm for 15 min; the clear supernatant was used for determination of keratinase activity and protein content.

Keratinase assay

The keratinase activity was assayed by using soluble keratin as substrate according to the method described by Cai et al.[17]**.** One unit of keratinolytic activity was defined as an increase of 0.01/min in absorbance at 280 nm against the blank, under the reaction condition.

Protein determination

The protein content was determined by the method of Lowry et al. [18] using bovine serum albumin as standard.

Statistical optimization of keratinase production

The optimization study was performed in two steps started by determination of the effective factors significantly influence keratinase production by using PBD [19, 20]. In the second step RSM of CCD was performed for prediction of the optimal point of keratinase production. The optimal point was reached by determination the level of three variables significantly increase keratinase production [21, 22].

Plackett – Burman experimental design

The production of keratinase by *B. licheniformis* ALW1 in submerged fermentation was done where seven independent variables were screened in nine combinations organized according to the PBD matrix. For each variable, a high (+) and low (-) level was tested as described in Table 1. PBD was based on the first order linear model (Eq.1):

$$Y= B_0 +\Sigma B_i X_i \quad (1)$$

Where Y is the response (keratinase production), B_0 is the model intercept and B_i is the linear coefficient and X_i is the level of the independent variable. The main effect of each variable was determined by Eq. (2):

$$E_{(Xi)} = 2(\Sigma M_{i+} - M_{i-})/N \quad (2)$$

Where $E_{(Xi)}$ was the effect of the tested variable. M_{i+} and M_{i-} represented keratinase production from the trials where the independent variable (Xi) measured was present at high and low levels respectively and N is the number of trials. The significance level (*p*-value) of each tested variable was determined using student's t-test.

TABLE 1. PBD for evaluating factors influencing keratinase production by *B. licheniformis* ALW1 and the observed response.

Trial	Independent variables							Response			
	Galactose (g/L)	CSL (g/L)	Inoculum size (ml)	NaCl (g/L)	Incubation period (h)	pH	K_2HPO_4 (g/L)	Protein[a] (mg/ml)		Keratinase[a] (U/ml)	
1	0.8(-1)	8(-1)	3 (+1)	0.6(+1)	108(+1)	5.5(-1)	1.2(-1)	2.94	±0.04	80.20	±2.66
2	1.2(+1)	8(-1)	2 (-1)	0.4(-1)	108(+1)	6.5(+1)	1.6(+1)	3.31	±0.01	84.66	±1.66
3	0.8(-1)	10(+1)	2 (-1)	0.6(+1)	84(-1)	6.5(+1)	1.6(+1)	3.64	±0.04	75.85	±4.11
4	1.2(+1)	10(+1)	3 (+1)	0.4(-1)	84(-1)	5.5(-1)	1.2(-1)	2.88	±0.09	87.65	±2.56
5	0.8(-1)	8(-1)	3 (+1)	0.4(-1)	84(-1)	6.5(+1)	1.6(+1)	3.30	±0.05	86.05	±5.08
6	1.2(+1)	8(-1)	2 (-1)	0.6(+1)	84(-1)	5.5(-1)	1.2(-1)	2.64	±0.06	81.16	±1.68
7	0.8(-1)	10(+1)	2 (-1)	0.4(-1)	108(+1)	5.5(-1)	1.2(-1)	3.03	±0.04	70.24	±7.61
8	1.2(+1)	10(+1)	3 (+1)	0.6(+1)	108(+1)	6.5(+1)	1.6(+1)	3.55	±0.06	75.27	±1.70
9*	1(0)	9.19(0)	2.5 (0)	0.5(0)	96(0)	6(0)	1.4(0)	2.83	±0.11	71.91	±0.73
10*	1(0)	9.19(0)	2.5 (0)	0.5(0)	96(0)	6(0)	1.4(0)	2.76	±0.07	72.11	±2.09
11*	1(0)	9.19(0)	2.5 (0)	0.5(0)	96(0)	6(0)	1.4(0)	2.81	±0.09	72.16	±3.15

*Design control, (a) Results are mean ± SD of three determinations, Numbers in the brackets indicate the code for real values of variables outside the brackets.

TABLE 2. Statistical analysis of PBD showed the coefficient values, *t* and *P* values for each variable on keratinase production.

Variables	Keratinase analysis			
	Coefficient	*t*-statistics	*P*-value	Confidence level (%)
Intercept	114.661	-	-	-
Galactose (g/L)	10.25	2.328	0.028	97.20
Corn steep (g/L)	-3.274	-3.012	0.006	99.40
Inoculum size (ml)	4.315	1.975	0.059	94.10
NaCl conc. (g/L)	-20.125	-1.842	0.077	92.30
Incubation period (h)	-0.212	-1.877	0.072	92.80
K_2HPO_4 (g/L)	1.617	.296	0.770	23.00

Central Composite Design

Three variables (Galactose, CSL and Inoculum size) were selected for RSM of CCD. The experimental design consisted of 20 runs and the independent variables were studied at five different levels as shown in Table 3. The second-order polynomial function in Eq. (3) was fitted to correlate the relationship between the independent variables and the predicted response Y.

$$Y_{Activity}=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3 \quad (3)$$

$Y_{Activity}$ is the predicted keratinase production (U/mL), β_0 was the model constant, X_1, X_2, and X_3 (Galactose, CSL and Inoculum size respectively) were the independent variables, β_1, β_2, and β_3 are the linear coefficients, β_{12}, β_{13}, and β_{23} are the cross-product coefficients, and β_{11}, β_{22}, and β_{33} are the quadratic coefficients. 'SPSS' Version 15.0 was used for the regression analysis of the experimental data obtained. The fit quality of the polynomial model equation was expressed by a coefficient of determination, R^2. The experiments were performed in triplicate and the mean values were recorded.

The quadratic models were represented as 3D response surface curves generated by using MATLAB version 14 after using Newton method to find the location of the equation maximum.

Partial purification and enzyme protein detection by SDS-PAGE

The culture filtrate was centrifuged (5000 rpm at 4˚C for 10 min.) and fractionated with acetone (20-90%). The precipitate was collected by centrifugation at 10,000 rpm for 10 min. and dissolved in distilled water.

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis was performed according to Laemmli [23] for the crude and partial pure keratinase along with pre-stained molecular weight protein marker (11-250 kDa - FERMENTAS). Electrophoresis was applied to 12% (w/v) SDS-polyacrylamide gel at 25 mA in 1X Tris/glycine-SDS-running buffer. The gel was fixed and stained with Coomassie brilliant blueR-250. After destaining, the gel was dried on gel drier for 2 hrs and photographed.

Properties of partial pure keratinase
Effect of temperature and pH

The optimum temperature of partial pure keratinase was investigated under standard keratinase assay condition by incubating the reaction mixture at a temperature range from 40˚C to 80˚C for 15 min. The optimum pH was determined by incubating the reaction mixture at different pH values using 0.1 N tris - HCl buffer in the range of 7.0 to 9.0 for 15 min. at optimum temperature.

The activation energy (Ea) of catalysis was determined from the slope of the Arrhenius plot [log V (logarithm of % residual activity) versus reciprocal of absolute temperature in Kelvin (1000/T)], which is given by the following equation (Eq. 4) where R is the gas constant (8.314 $mol^{-1}k^{-1}$).

$$\text{Slope} = -\text{Ea R} \quad (4)$$

TABLE 3. Examined concentration of the key variables and results of CCD experiment.

Trials	Independent variable			Observed response				Predicted Keratinase (U/ml)
	X_1 Galactose (g/L)	X2 Corn steep (g/L)	X_3 Inoculum size (ml)	Protein[(a)] (mg/ml)		Keratinase[(a)] (U/ml)		
1	1.1 (-1)	8 (-1)	2.5 (-1)	2.87	± 0.13	82.52	± 2.27	80.81
2	1.3 (+1)	8 (-1)	2.5 (-1)	2.86	± 0.20	79.61	± 2.06	83.09
3	1.1 (-1)	12 (+1)	2.5 (-1)	3.00	± 0.16	75.66	± 3.33	79.32
4	1.3 (+1)	12 (+1)	2.5 (-1)	3.12	± 0.05	76.77	± 3.03	68.64
5	1.1 (-1)	8 (-1)	3.5 (+1)	2.73	± 0.04	75.27	± 3.54	79.54
6	1.3 (+1)	8 (-1)	3.5 (+1)	2.87	± 0.08	99.11	± 2.61	91.60
7	1.1 (-1)	12 (+1)	3.5 (+1)	2.97	± 0.03	84.66	± 2.94	77.33
8	1.3 (+1)	12 (+1)	3.5 (+1)	3.12	± 0.05	85.58	± 2.08	83.45
9	1.0 (-2)	10 (0)	3 (0)	2.89	± 0.03	83.41	± 3.83	82.00
10	1.4 (+2)	10 (0)	3 (0)	2.75	± 0.17	84.43	± 3.61	89.80
11	1.2 (0)	6 (-2)	3 (0)	2.92	± 0.15	76.83	± 1.29	75.60
12	1.2 (0)	14 (+2)	3 (0)	3.07	± 0.11	60.01	± 2.98	65.19
13	1.2 (0)	10 (0)	2 (-2)	3.05	± 0.08	74.75	± 2.29	74.17
14	1.2 (0)	10 (0)	4 (+2)	2.84	± 0.07	81.84	± 2.27	86.39
15*	1.2 (0)	10 (0)	3 (0)	3.07	± 0.20	87.47	± 0.79	88.28
16*	1.2 (0)	10 (0)	3 (0)	3.14	± 0.12	86.75	± 0.83	88.27
17*	1.2 (0)	10 (0)	3 (0)	3.12	± 0.04	86.27	± 1.40	88.28
18*	1.2 (0)	10 (0)	3 (0)	3.07	± 0.20	87.10	± 0.29	88.28
19*	1.2 (0)	10 (0)	3 (0)	3.14	± 0.12	86.75	± 0.83	88.29
20*	1.2 (0)	10 (0)	3 (0)	3.12	± 0.04	86.27	± 1.40	88.28

*Design control, (a) Results are mean ± SD of three determinations, Numbers in the brackets indicate the code for real values of variables outside the brackets.

Effect of substrate concentration

The enzyme activity with different soluble keratin concentrations (0.4, 0.5, 0.6, 0.7 and 0.8 %) was estimated at the optimal pH and temperature of the tested partial pure enzyme. The maximum reaction velocity (V_{max}), Michaelis–Menten constant (K_m), specificity constant (V_{max}/K_m) were calculated from Lineweaver Bulk plot equation (Eq. 5). The reaction velocity (V) is the specific activity represented in U/mg protein, (S) is thesoluble keratin concentrations (mg/mL).

$$\frac{1}{V} = \frac{Km}{Vmax} * \frac{1}{S} + \frac{1}{Vmax} \quad (5)$$

Determination of thermal stability

Thermal stability of the partial pure keratinase was carried out by preheating the enzyme in the absence of substrate at different temperature (50-65˚C) for different time intervals (15, 30, 45, 60, 90, 120 min.). The residual activities of heat exposed enzymes were then measured under the optimum conditions.

Thermodynamic studies

The k_d was estimated by regression plot of log relative activity (%) versus time (min). The $t_{1/2}$ (the time it takes for the activity to reduce to a half of the original activity) and D-value (the time need to reduce 90% of enzyme activity) for the *Bacillus licheniformis* ALW1 keratinase was determined from the Eq.(6, 7).

$$t_{\frac{1}{2}} = \frac{ln2}{k_d} \quad (6)$$

$$D - \text{value} = \frac{ln10}{k_d} \quad (7)$$

The activation energy (E_d) for *Bacillus licheniformis* ALW1 keratinase denaturation was determined by a plot of log denaturation rate constants (lnk_d) versus reciprocal of the absolute temperature (K) using the Eq. (8)

$$Slope = -\frac{E_d}{R} \quad (8)$$

The change in enthalpy ($\Delta H°$, kJ mol^{-1}), free energy ($\Delta G°$, kJ mol^{-1}) and entropy ($\Delta S°$, J mol^{-1} K^{-1}) for thermal denaturation of *Bacillus licheniformis*ALW1 keratinase was determined using the following Eq. (9, 10 and 11)

$$\Delta H^{\circ} = E_a - RT \quad (9)$$

$$\Delta G^{\circ} = -RT\ln\left(\frac{k_d.h}{k_B.T}\right) \quad (10)$$

$$\Delta S^{\circ} = \frac{\Delta H^{\circ} - \Delta G^{\circ}}{T} \quad (11)$$

Where T is the corresponding absolute temperature (K), R is the gas constant (8.314 J mol^{-1} K^{-1}), h is the Planck constant (11.04×10^{-36} J min), and k_B is the Boltzman constant (1.38×10^{-23} J K^{-1}).

Effect of various inhibitors, reducing agents, detergents, organic solvents and metal ions on keratinase activity

The effect of inhibitors [phenyl methyl sulfonyl fluoride (PMSF) as serine protease inhibitor and ethylene diamine tetraacetic acid (EDTA) as metallo protease inhibitor], reducing agents [2-mercaptoethanol (2-ME), cysteine and sodium sulfite], organic solvents [propanol, dimethyl sulfoxide and acetonitrile], various monovalent metal ions [NaCl,KCl and $LiSO_4.H_2O$] and divalent metal ions [$ZnCl_2$, $MgSO_4.H_2O$, $CuCl_2.2H_2O$, $CoCl_2.6H_2O$, $CaCl_2$, $BaCl_2.2H_2O$, $HgCl_2$ and $MnCl_2.2H_2O$] at 5 mM were investigated by pre-incubating the partial pure enzyme for 30 min at room temperature with each item. Keratinase activity was carried out under standard assay conditions and the activity measured as residual activity of the control (100%) which is measured in the absence of any tested agents.

Feather degradation by cell free crude keratinase:

Feather (1.0 g) was autoclaved at 15 psi, 121˚C for 15 min in a 100 mL flask containing 15 mL, 0.1 M phosphate buffer pH 8.0. Volume was completed to 20 mL with crude enzyme to final concentrations of 180, 280, 380, 480, 580, 680, 780, 880 and 980 U/ flask). Flasks were kept at 150 rpm and 50˚C for 24h. At the end of the degradation period, feather meal was filtered through 2 mm sieve and residual feather was dried at 80˚C until constant weight. Degradation percent was calculated on the basis of dry weight of residual feather. Feather meal suspension was centrifuged at 5000 ×g for 15 min and the remaining keratinase activity (%) and total protein (mg/g feather) were calculated [8]. Experiments were set up in triplicate and the results were represented as mean of three independent experiments.

Results and Discussion

Enzyme production and statistical optimization using PBD

Among large number of culture conditions and medium composition, variables largely affecting keratinase production were determined using PBD. Nine combination matrix of seven independent variables for keratinase production were presented in Table 1. The keratinase production yield increased from 70.24 to 87.65 U/mL and the optimum yield was obtained by using combination number 4 in Table 1. Variables main effect was shown in Fig.2 and indicated that galactose, inoculum size, pH and K_2HPO_4 had positive main effect while CSL, NaCl and incubation period had negative main effect.

The regression coefficients, t-test and p-value of seven independent variables in Table 2 indicated *p*-values of 0.006, 0.028 and 0.059 for CSL, galactose and inoculum size respectively. These results make CSL, galactose and inoculum size the most significant variables affecting the keratinase production. Similar data were reported by Sivakumar et al. [24]; they found that galactose had a fundamental role in increasing the *Chryseobacterium* sp. keratinase productivity. CSL is a rich source of proteins, vitamins and minerals and expected to be a good medium for production of *Bacillus Licheniformis* ZJUEL31410 keratinase[25]. Jani et al. [26] reported that inoculum size was an important factor in *Bacillus* sp. B13 keratinase production.

The correlation between independent variables and keratinase production could be presented by first order model Eq. (12):

$$Y_{Activity} = 114.661 + 10.25\,X_1 - 3.274\,X_2 + 4.315\,X_3 - 20.125\,X_4 - 0.212X_5 + 1.617X_6 \quad (12)$$

Central Composite Design

Statistical and mathematical analysis of multivariable data obtained from RSM of CCD was very important in improving and optimization of keratinase production by *B. licheniformis* ALW1. The medium composed of (g/L): feather 10; NaCl 0.4; KH_2PO_4 0.7; K_2HPO_4 1.2; $MgSO_4.7H_2O$ 0.1; Galactose 1.2; CSL10.0; pH 5.5 ± 0.2; inoculated with 6 % of inoculum medium and incubated at 42° C under static condition was used as the central point of the CCD. Twenty trials matrix of CCD with three factors obtained from PBD (galactose, CSL and inoculum size) and five levels (-2, -1, 0,

+1 and +2) including six replicates at the central point were represented in Table 3. A second order polynomial equation (Eq. 13) was obtained by multiple regression analysis of observed data and the mathematical optimal point of equation was determined by Newton method using MATLAB version 14.

$$Y_{Activity} = -104.974 + 102.499X_1 + 33.48X_2 - 30.624X_3 - 70.015X_1^2 - 1.144X_2^2 - 8.422X_3^2 - 11.813X_1X_2 + 66.433X_1X_3 + 0.697X_2X_3 \quad (13)$$

Where response($Y_{Activity}$) was predicted keratinase production and (X_1, X_2 and X_3) were the codes of most effective variables galactose, CSL and inoculum size respectively. The mathematical optimal point of the equation was 101.14 U/mL at 1.4, 8.6 gm/L of galactose and CSL respectively with 4 mL/flask inoculum size. Our result was superior than that of Ni et al. [25]. They used RSM to optimize *B. licheniformis* ZJUEL31410 keratinase production under submerged fermentation to reach 54.9 U/mL under the optimal fermentation condition. Three dimensional graphs of the regression equation (Fig. 3A–C) explained main and interaction effects of galactose and CSL; galactose and inoculum size; CSL and inoculum size on keratinase production by *B. licheniformis* ALW1 respectively, when the third variable was kept at the value which gave the optimum point of the equation.

The *P*-value was used as a tool to check significant of each coefficient which in turn indicate the pattern of the interaction between the variables [27]. The statistical analysis of data (Table 4) indicated high significant effect of terms with smaller *P*-values ($P< 0.05$) on keratinase production.

Validation of the model

The validity of the proposed model was estimated by prediction of *B. licheniformis* ALW1 keratinase production for each trial of the matrix. The experimental results in Table 3 showed that the maximum observed keratinase production (99.1 U/mL) was very close to the predicted value (91.6 U/mL). The model *P* and F-values were equal to 0.0001 and 20.923 respectively and R^2 (determination of coefficient) was found to be 79% indicated that the model equation could explain the response variability. Thus, the second-order polynomial equation (Eq. 13) could be used to identify the relation between the three variables (Galactose, CSL, Inoculum size) and the keratinase production. The optimum keratinase production reached 99.1 U/mL which represented 1.4 fold increases with respect to the basal medium.

Partial purification and enzyme protein detection by SDS-PAGE

The *B. licheniformis* ALW1 crude keratinase was partially purified at 60–70 % acetone fraction with a specific activity of 785.6 U/mg with 24.9 fold of purification. Saibabu et al. [28] and Tiwary and Gupta [29] mentioned acetone as the best solvent for the partial purification of keratinase from *Bacillus* sp.

SDS-PAGE results (Fig.4) indicated multiple protein bands in crude and partial pure culture filtrate of *B. licheniformis* ALW1. Partial purification processes resulted on remove of some protein bands and increase the intensity of some other bands. Bands at Rfs 0.423, 0.576 and 0.696 (Mwts 59.2, 27.4 and 17.2 kDa, respectively) appeared in partial pure keratinase lane. However, the majority of keratinases presented molecular masses varying from 30-50 kDa [30]. On the other hand a small molecular mass (18 kDa) was also reported for *Streptomyces albidoflavus* [31].

TABLE 4. Analysis of CCD for keratinase production by *B. licheniformis* ALW1.

Term	Regression coefficient	Standard error	*t*- test	*P*-value
Intercept	-104.974	110.091	-0.954	0.345
X_1	102.499	127.783	0.802	0.426
X_2	33.48	5.951	5.626	0.000
X_3	-30.624	23.97	-1.278	0.207
X_1^2	-70.015	46.071	-1.520	0.135
X_2^2	-1.144	0.115	-9.931	0.000
X_3^2	-8.422	1.843	-4.570	0.000
X_1X_2	-11.813	4.084	-2.893	0.006
X_1X_3	66.433	16.335	4.067	0.000
X_2X_3	0.697	0.817	0.853	0.398

F value = 20.923; $P>F= 0.0001$; $R^2=0.790$; R =0 .889; Adjusted $R^2=0.752$

Properties of partial pure B. licheniformisALW1 keratinase

Effect of pH

The results in Fig. 5A showed that the partial pure *B. licheniformis* ALW1 keratinase was optimum at pH 8.5. Lateef et al. [32] reported that most of keratinases are active in neutral to alkaline conditions ranged from pH 7.0 to pH 9.5. On the other hand Mitsuiki et al. [33] reported that some keratinases were optimum at pH 12-13. However, the alkaline conditions lead to breaking of disulfide bonds for rapid keratin degradation [9].

Effect of temperature

The effect of the reaction temperature was investigated in a temperature range from 40 to 80 (Fig. 5A). The optimum temperature was found to be 70°C with 50% increase of activity with respect to control. This result was higher than that reported by Abdel-Naby et al. [13]. Also some authors reported an optimum temperature over 90˚C [34].

Within the temperature range 50-65˚C the activation energy (Ea) of *Bacillus licheniformis* ALW1 keratinase recorded 25.37 kjmol^{-1} (Fig. 5B). This result was lower than that obtained by Abdel Naby et al.[13] (24 kJmol^{-1}), Hernández-Martínez et al.[35] (62 kJ mol^{-1}). The low value of Ea means low energy needs to make enzyme substrate binding (ES), accordingly the previous result is urgently demanded in industrial application due to cost reduction.

Effect of substrate concentration

The effect of the substrate concentrations on the rate of its hydrolysis by partial pure keratinase at optimum conditions (pH 8.5 and 70˚C) was investigated and indicated that the optimum activity was at 0.7 % substrate concentration. To apply Lineweaver Bulk plot equation, the reciprocal of substrate concentration and specific activity were plotted (Fig. 6). The value of K_m was calculated to be 2.45 mg/ mL which is lower than the reported values of *B. pumilus* FH9, 5.55 mg/ml and that for halophilic bacteria, 16.6 mg/ml[13]. The low k_m means high affinity of substrate to make enzyme - substrate complex. The value of V_{max} was calculated to be 2272.7 U/mg protein which was known as the maximum velocity which theoretically suggested that the enzyme amount participated in the reaction. Also, the value of V_{max}/K_m (925.9 U mg protein^{-1}/ mg ml^{-1}) was higher than the observed ratio (562.79) for *A. niger* protease [36].

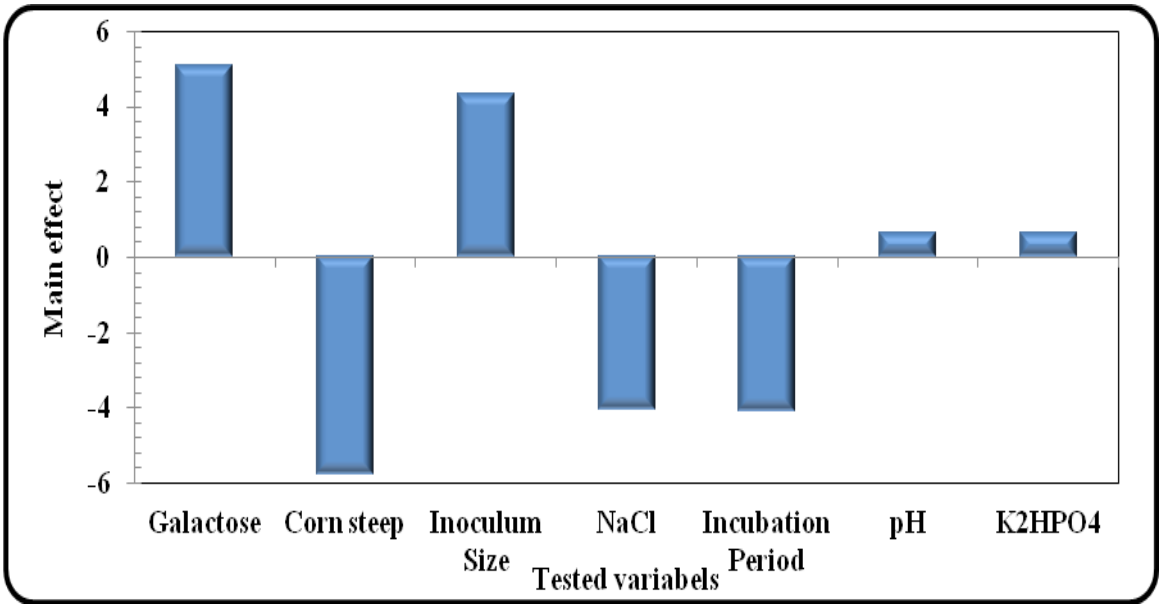

Fig. 2. Main effects of independent variables on Keratinase producution by *Bacillus licheniformis* ALW1 according to the results of the PBD.

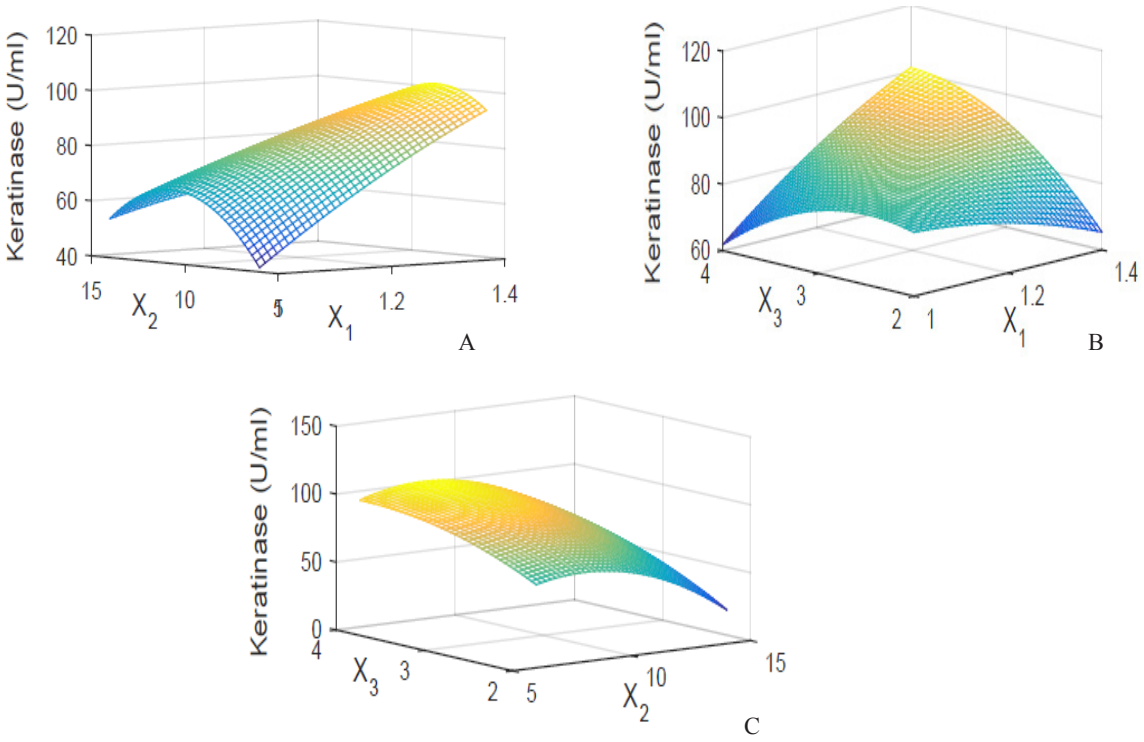

Fig. 3. Response surface plot showing the interactive effects of different concentrations of galactose and CSL at inoculum size = 4mL (A), inoculum size and different concentrations of galactose at CSL = 8.6 g/L (B) and inoculum size and different concentrations of CSL at galactose = 1.4 g/L (C) on keratinase production by *B. licheniformis* ALW1.

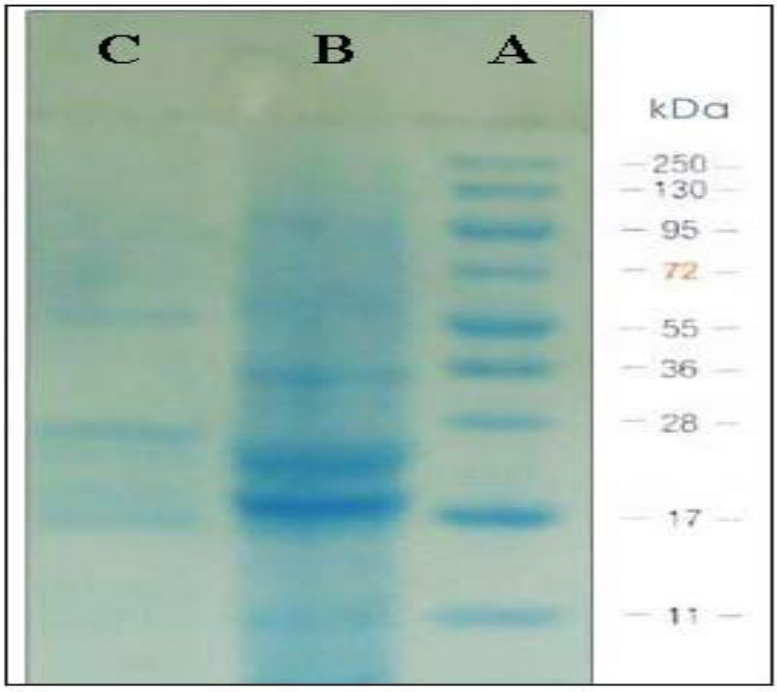

Fig. 4. SDS-PAGE of *B. licheniformis* ALW1 keratinase. Lane A: MW marker, lane B: crude keratinase (specific activity: 31.5 U/mg) and lane C: partially purified keratinase by 60 – 70 % acetone (specific activity: 785.6 U/mg).

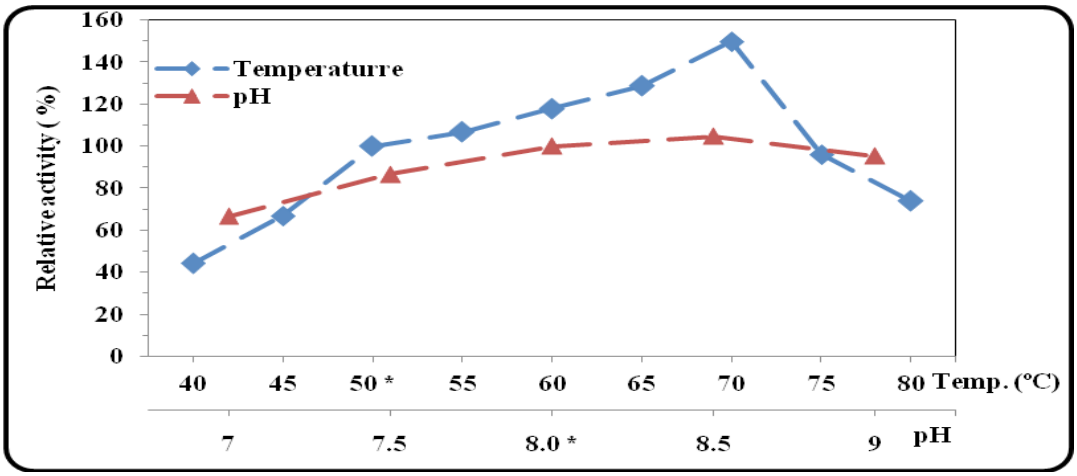

Fig. 5A. Effect of reaction pH and temperature on the *B. licheniformis* ALW1 P.P. keratinase activity.

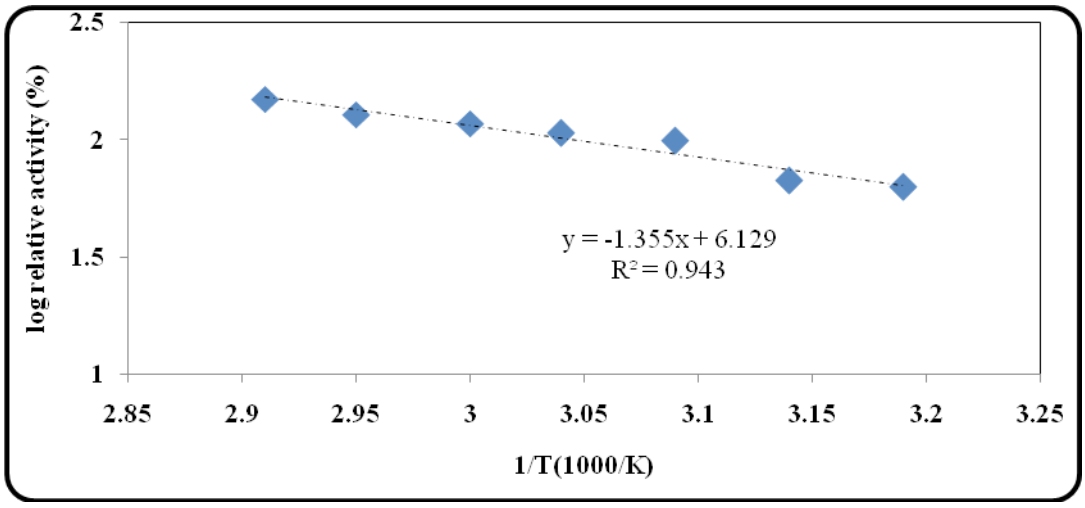

Fig. 5B. Arrhenius plot to calculate the activation energy of the *B. licheniformis* ALW1 P.P. keratinase

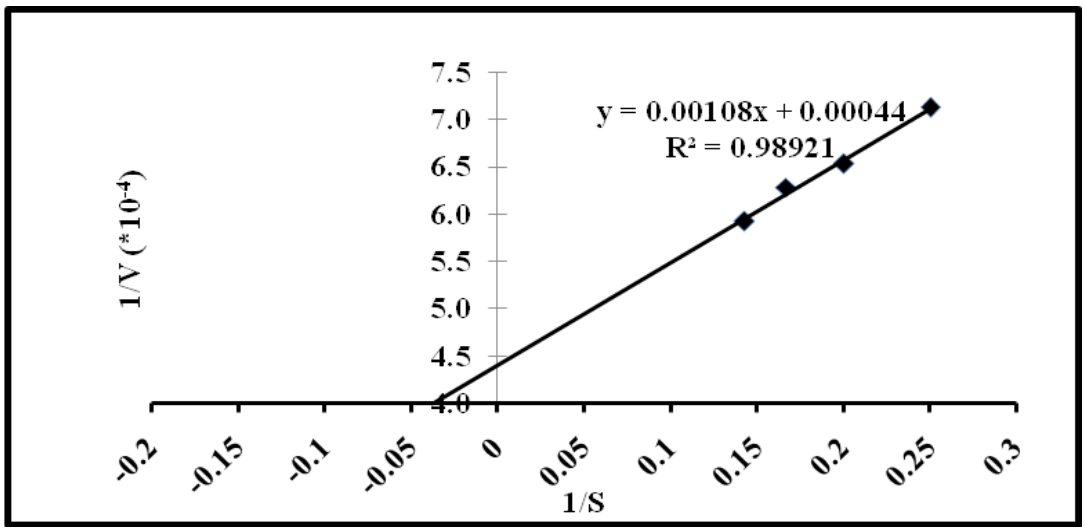

Fig. 6. Lineweaver Bulk plots to measure Michaelis–Menten constant (Km) and maximum reaction rate (Vmax) and specificity constant (Vmax / Km) values of partial pure keratinase.

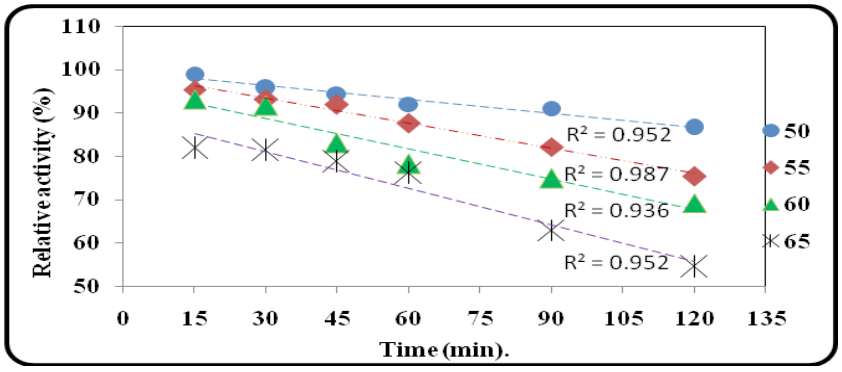

Fig. 7A. Thermal stability profile of *B. licheniformis* ALW1 P.P.keratinase.

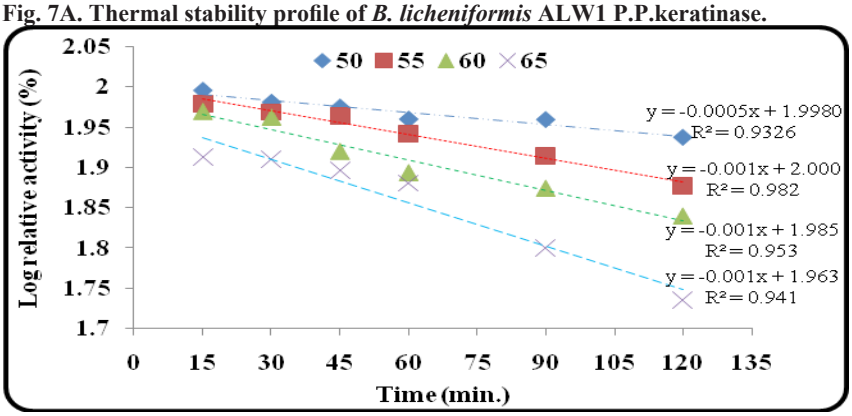

Fig. 7B. First order of thermal deactivation of the *B. licheniformis* ALW1 keratinase

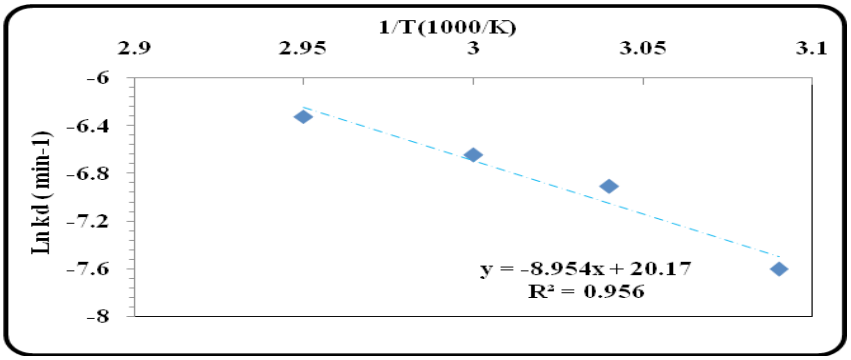

Fig. 7C. Arrhenius plot to calculate activation energy for denaturation (Ed).

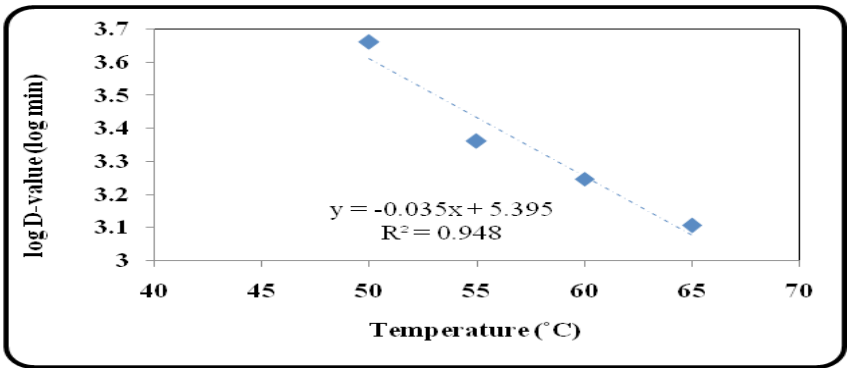

Fig. 7D. Temperature dependence of the decimal reduction of the enzyme.

Determination of thermal stability

The thermal stability means the enzyme ability to protect their molecules against the harsh condition. The thermal stability profile (Fig. 7) was studied in the absence of substrate, aiming to evaluate the enzyme denaturation starting point. The results referred to the importance of the substrate in enzyme stability. Although the enzyme achieved its maximum activity at 70˚C as mentioned above, its original activity reduced about 18% and 45% at 65˚C after 15 and 120 minutes of incubation respectively. The energy of activation for irreversible thermal inactivation (E_d) was calculated to be 73kJ mol^{-1}. This result was near to *B. pumilus* FH9 keratinase E_d (73.38 kJ mol^{-1}) [13]. The half-life ($t_{1/2}$ min.) was calculated to be 1380, 690, 530, 383 at 50, 55, 60, 65˚C. The enzyme long age is one of the important criteria demanded in industrial applications. Also, *D* values recorded 4600, 2300, 1769, 1277 min. at 50˚C, 55˚C, 60˚C, 65˚C respectively. Accordingly, the study of thermodynamic and kinetic parameters was an essential step to evaluate the efficiency of ***B. licheniformis*** ALW1 keratinase.

Thermodynamic studies

The study of thermodynamic parameters considers as an important step to evaluate the enzyme capability for application. Within this context, different thermodynamic parameters were determined. Such as, the changes in Gibbs free energy *(*$\Delta G°$), enthalpy ($\Delta H°$*)* and entropy ($\Delta S°$*)* of activation during the thermal enzyme denaturation. In this study the enthalpy results were 70.21 $kJmol^{-1}$, 70.17 $kJmol^{-1}$, 70.13 $kJmol^{-1}$ and 69.71 $kJmol^{-1}$ at 50, 55, 60 and 65˚C respectively. This result was about 3 fold higher than *B. pumilus* keratinase FH9 enthalpy [13]. The high value of enthalpy $\Delta H°$ means more energy was required for enzyme denaturation [37]. Also, the results pointed to a slight decrease in $\Delta H°$ by the temperature elevation and accordingly, margin less energy was required for enzyme unfolding. $\Delta G°$ Gibbs free energy recorded approximately, no change between 50-55˚C (191.49, 191.84$kJmol^{-1}$ respectively) (Table 5). This result could interpret the gradual loss in activity from (50- 55˚C at different time intervals) then the $\Delta G°$ was elevated to 193.31 and 212.86 ($kJmol^{-1}$) at 60 and 65˚C respectively. The increase in $\Delta G°$ with the increase in temperature worked as barrier in limiting the denaturation process [38]. Also, the negative entropy $\Delta S°$ means that the enzyme was in more order state. Where, the enzyme structure unfolding was accompanied by an increase in entropy[37].

Effect of various inhibitors, reducing agents, detergents, organic solvents and metal ions on keratinase activity

The effect of PMSF and EDTA on the activity of the partially pure enzyme was investigated. The results indicated that the enzyme activity was partially inhibited by PMSF to 40.6% and EDTA to 65.5% (Table 6) which indicated that partial pure keratinase could be a mixture of serine and metalloproteases. Other researchers also indicated the presence of keratinolytic activity produced from combined action of serine and metalloproteases [39, 40, 41].

Reducing agents, such as 2-Mercaptoethanol, Na_2SO_3 and cysteine were used and observed to have significant positive effect on keratinase activity. The action of reducing agents was done by facilitating the enzyme work on the substrate (after cleavage of cysteine bridges in the substrate) rather than direct effect as keratinase activator [42, 43].

Exposure of enzymes to some types of solvents, surfactants or salts could lead to enzyme inactivation as a result of denaturation, aggregation, coagulation or autolysis of the enzyme [44].

The effects of several metal ions on the partial pure keratinase indicated that almost all assayed metal ions were significantly increase keratinolytic activity. The highest two salts caused significant increases of the relative activity were $CaCl_2$ and $MgSO_4$. The inhibitory effect of metal ions was only recorded for $HgCl_2$, $CoCl_2.6H_2O$ and $ZnCl_2$ with relative activity of 5.8, 70.9 and 59.2 % respectively with respect to control. Our results agree with what was reported by Benkiar et al. [45] and Jaouadi et al. [46]. Where the keratinase was inhibited strongly by 5 mM of Hg^{2+} and moderately by Zn^{2+} ions while Ca^{2+} and Mg^{2+} ions were totally activated the enzyme.

The inhibitory effect of Zn^{2+} on some metalloproteases may be a result of bridges formation with the catalytic zinc ions at the active site of the enzyme [47]. Also Hg^{2+} was classified as a strong enzyme inhibitor because it had high affinity to react with tryptophan residues, carboxyl or thiol groups at or near the active site of the enzyme [48].

Bridges formed by Ca^{2+} and Mg^{2+} ions played an important role to maintain the specific active conformation of the enzyme, which could protect

the enzyme against thermal denaturation and stimulate the enzyme activity [49].

Among all used detergents and organic solvents, DMSO was the best solvent. It increased the keratinolytic activity by 11.4 % over the control. The activating effect could be attributed to the increase of the substrate accessibility to the enzyme [50].

Feather degradation by cell free crude keratinase

This trial was conducted to improve feather properties by keratinase to be used as organic fertilizer, feedstuff or feed supplement. The results in Fig. 8 indicated that the feather degradation process was a linear function of keratinase units. The percent of hydrolyzing was increased from 48 % to 68.2 % by using 180 U and 680 U respectively. Slightly increase in the hydrolyzing percent was observed by increasing units to about 980 U to get only 74 % degradation percent. Soluble protein released in the degradation broth was frequently increased by increasing keratinase units as a result of feather degradation. This trial represented an ideal method to produce feather meal by crude keratinase as reported by Tiwary and Gupta [8] without need to use reducing agents or reduction- oxidation system of the cells.

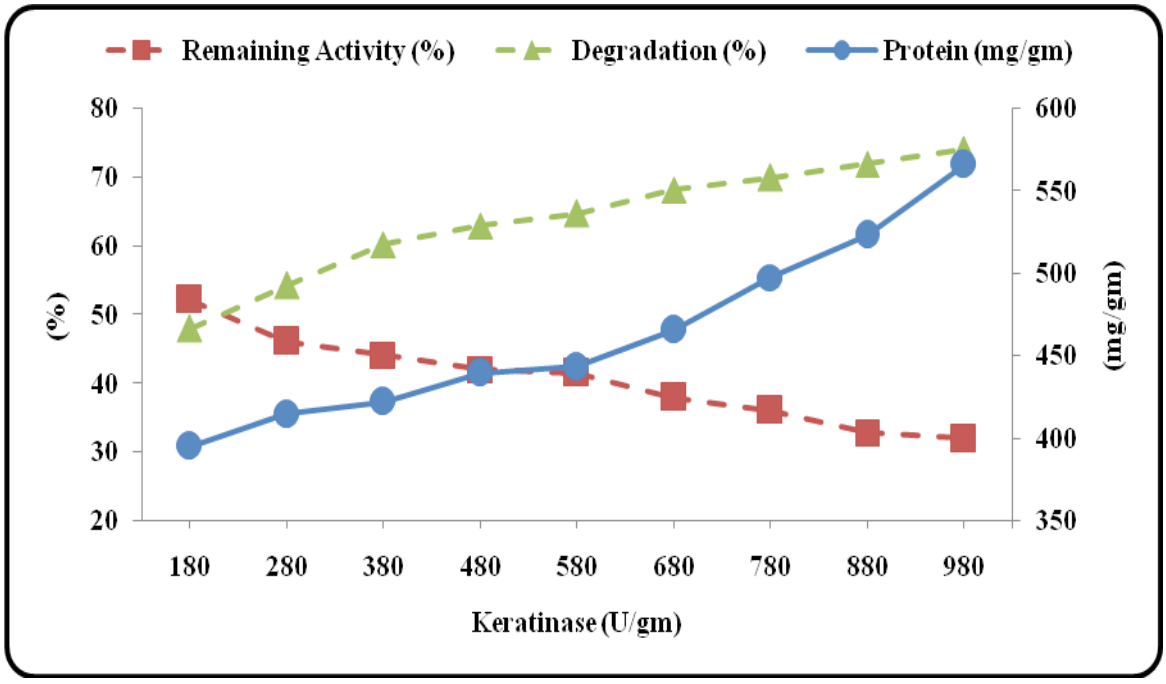

Fig. 8. Feather degradation by cell free crude keratinase

TABLE 5. Thermodynamic parameters for thermal inactivation of *B. licheniformis* ALW1 keratinase.

Temp °C	50 °C	55 °C	60 °C	65 °C
k_d (min^{-1})	0.0005	0.001	0.0013	0.0018
$t_{1/2}$ (min)	1380	690	530	383
D-value (min)	4600	2300	1769	1277
$\Delta H°$ (kJ mol^{-1})	70.21	70.17	70.13	69.71
$\Delta G°$ (kJmol^{-1})	191.49	191.84	193.31	212.86
$\Delta S°$ (kJmol^{-1})	-375.48	-370.94	-369.9	-373.76

TABLE 6. Effect of various additives on the activity of the P.P. keratinase.

5 mM	Relative activity (%)	
Control	100.0	± 3.2
$MnCl_2.2H_2O$	107.6	± 1.3
$HgCl_2$	5.8*	± 2.3
$BaCl_2.2H_2O$	111.7**	± 4.4
$CaCl_2$	132.5**	± 2.2
$CoCl_2.6H_2O$	70.9*	± 2.1
$CuCl_2.2H_2O$	125.4**	± 2.2
$MgSO_4.H_2O$	146.0**	± 1.0
$ZnCl_2$	59.2*	± 3.4
NaCl	122.0**	± 2.4
KCl	118.6**	± 0.6
$LiSO_4.H_2O$	110.8**	± 5.0
Na_2EDTA	65.5*	± 1.7
PMSF	40.6*	± 1.9
SDS	34.2*	± 1.5
Tween 80	97.0	± 4.9
Glycerin	78.5*	± 2.2
Propanol	86.4*	± 3.1
DMSO	111.4**	± 0.9
Acetonitrile	90.1*	± 1.8
2-Mercaptoethanol	117.9**	± 2.4
Na_2SO_3	201.5**	± 4.3
Cystein	112.9**	± 1.8

(**) Significant increase, (*) Significant decrease when compared to control at 95 % confidence level.

Conclusion

This work tries to produce and optimize *B. licheniformis* ALW1 keratinase through a Plackett – Burman design (PBD) and Central Composite Design (CCD). The results recorded 1.4-fold increase in the enzyme activity compared to the basal medium. The partially purified enzyme was characterized and a correlation between the thermal stability results and the thermodynamics parameters was done. The study pointed to the rigidity and high thermal stability of the enzyme. Also, the effects of metals ions, detergents, organic solvents, and reducing agent in the enzyme activity were tested. The results gave us explanation about the enzyme structure and the role of the tested substances in activating and inhibiting of the enzyme. Finally, the enzyme had efficiency in hydrolyzing the poultry feather. From the previous results it could be concluded that *B. licheniformis* ALW1 keratinase could be used in different industrial aspects.

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تحسين وتوصيف ودراسات ديناميكية حرارية علي الكيراتينيز المنتج من العزلة البكتيرية *Bacillus licheniformis* ALW1

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تهدف الدراسة الي إستخدام النماذج الإحصائيةPlackett – Burman design و Central Composite Design لتعظيم إنتاجية إنزيم الكيراتينيز من السلالة البكتيرية *B. licheniformis* ALW1. وقد لوحظ بتطبيق هذه النماذج أن العوامل الأكثر تأثيرا في إنتاج إنزيم الكيراتينيز هي حجم الحقنة وتركيز كلا من ماء نقيع الذرة وسكر الجلاكتوز في بيئة الانتاج مما أدي إلي زيادة إنتاج الإنزيم بنسبة ٤٠٪ لتصبح ٩٩.١ وحدة/مليلتر. وبالتنقية الجزئية للإنزيم المنتج بواسطة الأسيتون ٦٠-٧٠% تبين أن الظروف المثلي لنشاط الأنزيم هي عند درجة حرارة ٧٠° س وأس هيدروجيني ٨.٥ . وبحساب الطاقة اللازمة لتنشيط وتثبيط الإنزيم تبين أنها علي التوالي تساوي ٢٥.٣٧ و ٧٣.٣٨ كيلو جول / مول. كما تم حساب قيم فترة عمر النصف والفترة اللازمة للإبقاء علي ١٠٪ من نشاط الإنزيم عند درجات حرارة ٥٠ و ٥٥ و ٦٠ و ٦٥° س لتكون علي التوالي (١٣٨٠ و ٦٩٠ و ٥٣٠ و ٣٨٣ دقيقة) و (٤٦٠٠ و ٢٣٠٠ و ١٧٦٩ و ١٢٧٧ دقيقة). ومع رصد قيم التغير في الطاقة الحرة (GΔ°) وجدت أنها تقريبا في حالة ثبات عند قيمة ١٩١.٤٩ كيلو جول / مول ما بين درجات الحرارة من ٥٠ -٦٠° س ولكن مع زيادة الحرارة إلي ٦٥° س حدثت زيادة بشكل ملحوظ في قيمة GΔ° لتصل إلي ٢١٦.٨٦ كيلو جول / مول بينما أدت الزيادة في درجة الحرارة إلي نقص طفيف في المحتوي الحراري للإنزيم (HΔ°). وكما أظهرت النتائج التي تمت لدراسة تأثير بعض الفلزات علي نشاطية الإنزيم أن معظم تلك الفلزات كان لها تأثير إيجابي وأن التأثير الأعظم لهذة الفلزات كان لكبريتات الماغنسيوم بزيادة ٤٦٪ مقارنة بالمعاملة القياسية. وعلي نفس المنوال كان لعوامل الإختزال تأثيرا إيجابي علي نشاط الإنزيم بينما أدي إستخدام عوامل تثبيط إنزيمات هضم البروتين مثل PMSF , EDTA إلي تثبيط جزئي للإنزيم مما يدل علي وجود مزيج من إنزيمات هضم البروتينات المعدنية والسيرينية. وكمحاولة لحل مشكلة تراكم الريش وتوفير مصدر بروتيني رخيص يصلح للإستخدام في مجالات عدة تم التوصل الي٧٤٪ تحلل بمعاملة الجرام الواحد من الريش ب ٩٨٠ وحدة من الإنزيم المنتج مما قد يوفر حلا فاعلا لهذه المشكلة.