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Research Article

Enhanced production, purification and characterization of alkaline keratinase from *Streptomyces minutiscleroticus* DNA38

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Abstract

A thermotolerant, feather-degrading, newly isolated actinobacterial strain *Streptomyces minutiscleroticus* DNA38 was investigated for its ability to produce keratinase. Maximum production (283.4 IU) of keratinase by *Streptomyces minutiscleroticus* DNA38 in starch chicken feathers medium under submerged bioprocess was observed at optimized conditions of pH 9.0 of the medium and 45 °C incubation temperature. Further, an enhanced production (435.8 IU)of keratinase was achieved employing response surface methodology. Combined interactive effect of starch (7.50 g/L), yeast extract (0.74 g/L) and chicken feathers (7.50 g/L) were found to be the critical process variables for enhanced production under central composite design. Chicken feathers showed a direct action and addition of starch and yeast extract to the medium proved effective for a significant increase in the production of keratinase. The purified keratinase was monomeric and had a molecular mass of 29 kDa. The enzyme activity was significantly inhibited after pH 9.0 and temperature 50 °C.

Keywords: *Streptomyces minutiscleroticus;* Response Surface Methodology; keratinase; purification; characterization.

Introduction

Chicken feather mainly contains keratin, which is an insoluble protein with high stability and indigestible by common proteases (Goddard and Michaels L, **1934;**Papadopoulos, **1986**]. Actinomycetes have the ability to breakdown many different varieties of organic compounds and are crucial in the mineralization of organic matter (Ryckeboer et al., 2003). Proteases constitute an important fraction of the global enzyme sales and a relevant part of this market is accounted by bacterial proteases (Rao et al., 1998). Keratinases are projected to generate a potential worldwide market similar to other proteases. Bacterial keratinases are of particular interest because of their action on insoluble keratin substrates and generally on a broad range of protein substrates (Lin et al., 1995).These enzymes have been studied for de-hairing processes in the leather industry (Raju et al., 1996).Cultivation conditions are essential in successful production of enzyme, and optimization of parameters such as pH, temperature and media composition are important in developing the cultivation process. Despite all the work that has been done on production of proteolytic enzymes, relatively little information is available on keratinases (Wang and Shih, 1999). Upto now, a limited number of reports are available on the isolation of thermophiles, in particular thermophilic Streptomyces with the ability to hydrolyse wool and other keratinous wastes (Ignatova et al., 1999; Kabadjova et al., 1996).

Diverse groups of microorganisms are reported to produce keratinase (Han et al., 2012; Deivasigamani and Alagappan, 2008; Mukhopadhyay and Chandra, 1993; Nam et al., 2002; Suneetha and Lakshmi, 2005). However, the full commercial potential of keratinases is yet to be realized. At present, the major focus in this field is still rests in identifying novel isolates with high keratinase activity and improving the yield using

conventional and r-DNA approaches, in addition to optimizing the physical and nutritional parameters to maximize keratinase yield. Microbial alkaline proteases for industrial uses are produced and studied mainly from Bacillus and Streptomyces. Little is known about proteases from other actinomycetes, Actinomycetes produce extracellular enzymes like proteases, chitinases, amylases etc. Actinomycetes, particularly Streptomycetes are known to secrete multiple proteases in culture medium (Tatineni et al., 2008). With comparison to Bacillus spp, actinomycetes have been less explored for proteases. Using response surface methodology (RSM) for optimized production is an efficient way to design and predict the role of each ingredients in the resultant product.

Many keratinase were well characterized and reported. However, purification and characterization of keratinase obtained from different sources required to be carried out from the point of any novel properties or applications. Purification of an enzyme is generally a multi-step process. The techniques selected for enzyme purification should be moderate and native conformation of the enzyme protein should not change as a result of purification (Kumar *et al.*, 2012). Commonly, the first step in enzyme purification is based on fractionation of proteins on the basis of solubility. Various chromatographic and electrophoretic techniques are known to be employed for the purification of enzymes. Most commonly used electrophoretic technique is Polyacrylamide Gel Electrophoresis (PAGE). Characterization of any enzyme or protein deals with physical properties like native molecular weight determination. It also deals with the study of chemical properties like optimum pH, optimum temperature, energy of activation and kinetic properties. The activities optimal for pH of various enzymes vary. The present study reports the enhanced production of keratinase using RSM by Streptomyces isolated from the soil of poultry farm. Thus obtained keratinase was purified and also characterized.

Materials and Methods

Optimization of production of keratinase

Keratinase was produced in a submerged bioprocess by *Streptomyces minutiscleroticus* DNA38 (a potential isolate for keratinase isolated and preserved in our A- DBT Research laboratory), at laboratory scale, employing 100ml starch chicken feathers broth (Dastager et al., 2009). Optimization of physicochemical and nutritional variables, employing one at a time experimental protocol (Liuan, et al., 2010) was followed for the production of keratinase. Initial pH (7.0 to 9.0 with an increment of 0.5), inoculum size ($1X10^5$ to $1X10^9$ with an increment of $1X10^1$), incubation temperature (30 to 50°C with an increment of 5 ºC), agitation speed (150 to 250 rpm with an increment of 25rpm), carbon sources (Starch, glucose, sucrose, maltose andarabinose, from 0.2 to 1.0 % with an increment of 0.2%), nitrogen sources(beef extract, casein, yeast extract and chicken feathers from 0.2 to 1.0 % with an increment of 0.2%) and mineral salts ($CuSO_4$, $MgSO_4$, $FeSO_4$, $MnSO_4$ and K_2HPO_4 from 0.01 to 0.05% with an increment of 0.01%) were optimized manually for the production of keratinase.

The keratinase activity of the fermented broth was estimated by modified method of Cheng *et al*. (1995) using keratin as a substrate. The reaction mixture containing 1ml of 1% keratin in phosphate buffer (pH 8.0) and 0.5 ml of enzyme (fermented broth) was incubated at 30 ºC for 30 min. After incubation, the reaction was terminated by adding 2 ml of 10% trichloroacetic acid (TCA). After the separation of untreated keratin as pellet by centrifugation, 1ml of clear supernatant was mixed with 5ml of 0.4 M Na $2CO_3$ and 0.5 ml of Folin-Ciocalteau's phenol reagent. The absorbance was measured at 660 nm against blank after 30 min. All assays were carried out in triplicate. One unit of keratinase activity was defined as the amount of enzyme that released one microgram of tyrosine per minute under the standard assay conditions.

Enhanced production of keratinase

The identified critical process variables namely Starch, yeast extract and chicken feathers, under response surface method were evaluated (Annapurna et al., 2009) with central composite design for their optimum combined effect on the production of keratinase in submerged bioprocess. The CCD of 33 runs was set using the Design Expert Software, USA (Version 7.0). All the experiments were carried out in duplicate and average of keratinase production obtained was considered as the dependent variables or responses (Y). The predicted response was calculated from the

second degree polynomial equation, $\mathbf{Y= \beta_0+\sum \beta_iX_i+\sum \beta_{ii}X_i^2+\sum \beta_{ij}X_iX_j;}$ where Y stands for the response variable, β_0 is the intercept coefficient; β_i represents the coefficient of the linear effect, β_{ii} the coefficient of quadratic effect and β_{ij} the ij^{th} interaction coefficient effect. X_iX_j are input variables which influence the response variable Y, β_i is the i^{th} linear coefficient. The statistical and numerical analysis of the model was performed with the analysis of variance (ANOVA). The statistical significance of the model was analyzed by the Fisher's F-test, its associated probability P (F), correlation coefficient R and determination coefficient R^2. The quadratic models representing for each variable as contour plots (three-dimensional response surface curves) were created and the model was validated.

Purification of keratinase

The purification of keratinase produced by an efficient isolate *Streptomyces minutiscleroticus* DNA38 was carried out using crude enzyme extract as follows.

Ammonium sulphate precipitation: About 500 ml of culture filtrate was centrifuged at 10,000 rpm for 10 min at 4 ºC (Wenk and Fernandis, 2007). Finely powdered ammonium sulphate was added to the clear supernantent. The keratinase activity was associated with the fraction precipitated at 60 - 90% saturation. This was carried out with continuous stirring in an ice bath and it was kept at 4 ºC for overnight. The precipitate was collected by centrifugation at 10,000 rpm for 10 min. The supernatant was decanted; the precipitate was dissolved in minimum volume of 10 mM phosphate buffer pH 7.0. Protein estimation (Lowry *et al*., 1951) was done with Folin-Phenol reagent using BSA (200 µg/ml) as a standard.

Dialysis: Activation of cellulose membrane was performed as follows (Wenk and Fernandis, 2007). 100 ml distilled water was kept for boiling. Dialysis membrane was placed in water for 10 min. Then 2% sodium carbonate was added and further kept for boiling for 10 min. Finally the membrane was transferred to fresh water. The precipitate obtained after ammonium sulphate treatment was dialyzed against 50 mM phosphate buffer, pH 7.0 with occasional changes of buffer. Protein estimation (Lowry *et al*., 1951) was done with Folin-Phenol reagent using BSA (200 µg/ml) as a standard.

Ion exchange chromatography: The dialyzed ammonium sulphate fraction was applied (Wenk and Fernandis, 2007) to a column of DEAE cellulose. 2 g of DEAE cellulose was suspended in 10 Mm Tris-Cl-NaCl pH-7.0 and kept at 4 °C overnight. The column was equilibrated with 0.05 M Tris-Cl, pH 7.0 Swollen DEAE cellulose was loaded into a chromatographic column and allowed to settle. The sample was loaded to top of the column. The unbound protein was eluted and the bound protein eluted with linear gradient of NaCl. Each fraction was analyzed for enzyme activity and protein. The keratinase activity was assayed by following modified method of Cheng et al., (1995) using a substrate keratin as described earlier. Protein estimation was done with Folin-Phenol reagent using BSA (200 µg/ml) as a standard (Lowry *et al*., 1951).

Gel filtration chromatography: The concentrated enzyme solution was applied (Wenk and Fernandis, 2007) to a Sephadex G-200 column (39x9 cm) and the sample was subjected to GPC using SEPHADEX G-75 (SIGMA ALDRICH). 2 g of SEPHADEX was suspended in 0.05M Tri-HCl (pH 7.0) and kept at 4ºC overnight. Before loading, the column was well equilibrated with 0.05 M Tris-HCl. Swollen sephadex was loaded into a chromatographic column and allowed to settle. The 3 ml of the sample was loaded to top of the column. The enzyme was eluted using 0.05 M Tris HCl and flow rate was adjusted to 0.5 ml/min. Different fractions were collected and each fraction was assayed for protein by taking absorbance at 280 nm. Protein estimation (Lowry *et al*., 1951) was done with Folin-Phenol reagent using BSA (200 µg/ml) as a standard.

Electrophoresis: SDS – PAGE was performed according to the modified method of Laemmli (1970), with a separating acrylamide gel of 10% and stacking gel 5% containing 0.1% SDS. The gel was stained with coomassie brilliant blue R-250 and destained.

Characterization of keratinase

The purified keratinase was subjected to characterization by studying the influence of pH and temperature as physicochemical factors, by following the standard procedures as mentioned below. Enzyme activity was recorded under the influence of varied conditions.

Influence of pH: The activity of keratinase was evaluated at different range of pH (3.5-10.0). Purified enzyme was incubated with 1% keratin and 0.05 M phosphate buffer of pH 4-10, with an increment of pH 0.5, under assay conditions and the enzyme activity was determined as described earlier. The different buffers used were 0.1M acetate buffer (pH 3.0-6.5), 0.1 MTris-HCl (pH 7.0-8.0) and 0.1M glycine NaOH (pH 8.0-10).

Influence of temperature: The activity of enzyme was determined by incubating the assay mixture at temperatures ranging from 10 to 100 ^{o}C with an increment of 10^{o}C. The reactions were performed at optimum pH value of the enzyme (Liu *et al.,* 2001).

Results and Discussion

Enhanced production of Keratinase

An amount of 283.4 IU of keratinase was produced by *Streptomyces minutiscleroticus* DNA38 in starch feathers medium under submerged bioprocess, at optimized physicochemical and nutritional variables, considering manually one variable at a time and keeping others constant. pH 9.0 and temperature 45 ^{o}C were showed a constant influence on the production of keratinase. Starch, yeast extract and chicken feathers were identified as most critical variables, keeping optimized pH, temperature and other media components as constant, for the enhanced production of keratinase under response surface method with central composite design. The critical process variables are those, influence theenzyme activity on minimum variationin concentration of the process variables. The run 24 showed (Table 1) a maximum response on the production of keratinase with an actual value of 435.8 IU against predicted value of 441.83 IU. The closer similarity between actual and predicted values could reflect the accuracy and applicability of response surface method with CCD to optimize the process. A polynomial equation derived from the multiple regression analysis for the analysis of critical variables was as follows. Keratinase production (Y) = $409.38+14.72X_1+0.40X_2+53.05X_3-4.81X_1^2+14.23X_2^2-40.96X_3^2+6.54X_1X_2+2.92X_1X_3-0.52X_2X_3$, where, X was the response variable with X_1, X_2 and X_3 as coded values for starch, yeast extract and chicken feathers respectively.

The model characteristic response for the production of keratinase employing ANOVA (Table 2) showed a high coefficient R^2 value of 0.8513. If R^2 value is closer to 1 (should be between 0 and 1 and >0.75), the model would be stronger and it predicts the better response (Haaland, 1989). Thus, the statistical model could explain 97.09% of variability in the response. An F-value 14.63and Lack of Fit value 31.38 were found to be significant. Adeq Precision ratio greater than 4 is desirable and in this investigation the ratio was 13.875. Three-dimensional response surface plots (Fig. 2) with a response at 120h of incubation for keratinase production were on Z axis against any two independent variables, while keeping the other independent variable at their central level. Three response surfaces with all the possible combinations of critical variables starch (7.5 g/L), yeast extract (0.74 g/L) andchicken feathers (7.5 g/L) given the maximum (435.8 IU) production of keratinase. However, a varied range of keratinase activities were reported by several researchers using different substrates. The recent literature reveals the production of 16.53 U/ml Streptomyces keratinase(Jayalakshmi et al., 2011).Shilpa Ashok Jani et al. (2014) reported 92.810 Units/mlStreptomyceteskeratinase activity and Ana Maria Mazotto (2011) obtained 463 u/mL of keratinase activity from *B. subtilis*. In the present investigation an impressive level (435.8 IU/ml) production of alkaline keratinase was achieved, which can be explored further for competitive commercial viability.

Purification of keratinase

Protein purification is a series of processes intended to isolate a single type of protein from a complex mixture and is also vital for the characterization of the function, structure and interactions of the protein of interest. The starting material is usually a biological tissue or a microbial culture. In the present investigation, purification of keratinase was carried out by four steps as shown in Table 2, with a final yield of 3.8 % and a purification fold of 113.45. The total protein decreased from 2846 mg to 0.72 mg and the specific activity increased from 30.56 to 58.85 IU/mg in the ammonium sulfate precipitation, which leads to a 2.68 fold purification of the keratinase. In the second step, the enzyme was subjected to dialysis. The specific activity of keratinase increased from 58.82 to 330.82 IU/mg leads to 15.10 fold purification of keratinase by dialysis. In the next two consecutive stages, ion exchange and gel exclusion chromatography, specific activity of keratinase was increased from 330.82 to 1171.6 IU/mg with 53.44 fold purification and 2486.9 IU/mg with 113.45 fold purification respectively. SDS-PAGE analysis of the purified enzyme showed a single band as represented in Figure 2. The apparent molecular weight of the enzyme was determined to be 29 kDa by using different standard protein markers.

Table 1: Optimization of critical process variables for the production of keratinase by *Streptomyces minutiscleroticus* DNA38 under response surface method with central composite design

Run	Critical process variables			Keratinase Production (IU)	
	X_1:A: Starchg/L	X_2:B: Yeast extract g/L	X_3: C: Chicken feathersg/L	Actual Value	Predicted value
1	10.0	1.0	05.0	340.1	329.13
2	05.0	2.0	10.0	380.6	406.59
3	05.0	1.0	10.0	402.7	419.91
4	7.50	1.50	7.50	414.7	409.38
5	3.69	1.50	7.50	341.7	375.75
6	7.50	1.50	3.69	171.3	233.29
7	7.50	1.50	7.50	413.2	409.38
8	7.50	0.74	7.50	429.6	441.83
9	7.50	1.50	7.50	415.1	409.38
10	5.0	1.0	5.00	360.3	318.50
11	7.50	1.50	11.31	425.2	395.05
12	11.31	1.50	7.50	422.7	420.65
13	7.50	1.50	3.69	179.7	233.29
14	11.31	1.50	7.50	418.2	420.65
15	7.50	2.26	7.50	425.7	443.06
16	7.50	1.50	7.50	416.1	409.38
17	7.50	2.26	7.50	426.8	443.06
18	10.0	2.00	5.00	362.1	344.06
19	7.50	1.50	7.50	415.9	409.38
20	5.0	2.00	5.00	338.2	307.37
21	10.0	2.00	5.00	370.6	344.06
22	10.0	2.00	10.00	426.9	454.95
23	5.0	2.00	5.00	336.3	307.37
24	7.50	0.74	7.50	435.8	441.83
25	10.0	1.00	10.00	429.1	442.11
26	10.0	1.00	5.00	352.6	329.13
27	5.0	1.00	5.00	335.1	318.60
28	7.50	1.50	7.50	416.3	409.38
29	5.0	2.00	10.00	428.3	406.59
30	10.0	1.00	10.00	425.5	442.11
31	3.69	1.50	7.5	358.3	375.75
32	7.50	1.50	11.31	428.6	395.05
33	5.00	1.00	10.00	422.7	419.91

Table 2: Analysis of variance (ANOVA) for the assessment of the model

Source		Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model		1.109E+005	9	12324.10	14.63	<0.0001
A-Starch	0.2	*5169.37*	1	5169.37	6.14	<0.0210
B-Yeast Extract		*3.86*	1	3.86	4.585E-003	0.9466
C-Chicken feathers		*67109.73*	1	67109.73	79.69	<0.0001
AB		*624.36*	1	624.36	0.74	0.3981
AC		124.06	1	124.06	0.15	0.7046
BC		3.98	1	3.98	4.723E-003	0.9458
A^2		407.14	1	407.14	0.48	<0.4938
B^2		3564.48	1	3564.48	4.23	<0.0512
C^2		29536.35	1	29536.35	35.07	<0.0001
Residual		9368.78	23	842.12		

Figure 1: Three dimensional response surface curves showing the interactive effect of critical process variables on the production of keratinase by *Streptomyces minutiscleroticus* DNA38

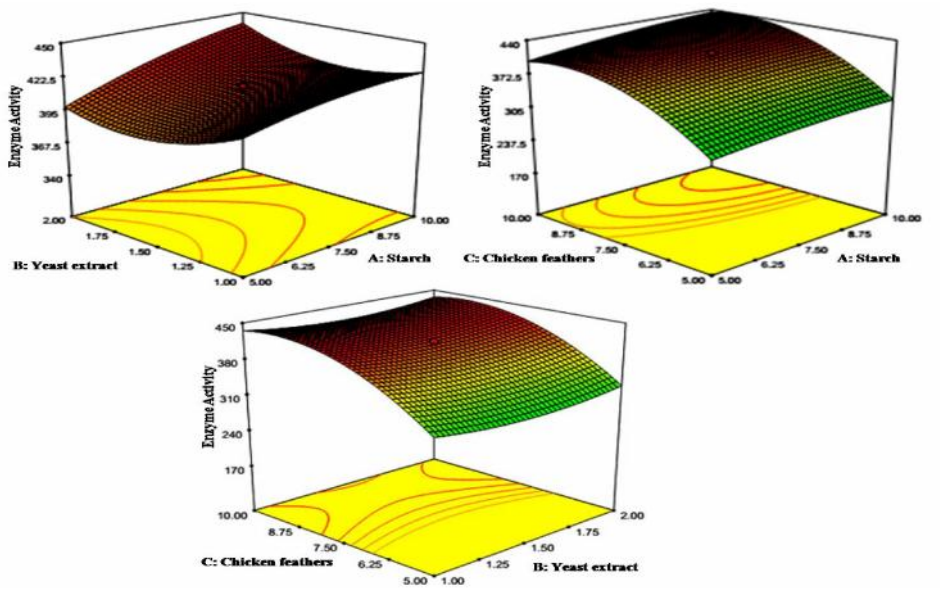


Table 3: Purification profile of keratinase produced by *Streptomyces minutiscleroticus* DNA38

Purification stage	Volume (mL)	Enzyme activity (IU/mL)	Total activity	Protein (mg/mL)	Total Protein	Specific activity (IU/mg)	Fold purification	% yield
Crude fraction	200	435.00	46800	14.23	2846	30.56	1	100
Ammonium sulphate precipitation	30	480.27	14408.1	8.16	244.8	58.85	2.68	30.7
Dialysis/desalting	12	516.09	6193.08	1.56	18.72	330.82	15.10	12.2
Ion exchange chromatography	7	562.38	3936.66	0.48	3.36	1171.62	53.44	8.4
Gel filtration/permeation	3	596.86	1790.58	0.24	0.72	2486.91	113.45	3.8

Unlike most eukaryotic keratinases, the active form of the *Pseudomonas* sp. protein is a monomer, without tendency of concentration dependent aggregation as shown by analytical ultracentrifugation. The enzyme has a molecular mass of 39,000 Da in SDS-PAGE**.** A keratinase from a *pseudomonas aeruginosa* strain has been purified by a simple steps purification by Liu-Jung et al., (2006). This keratinase has one of the lowest (29 KDa) molecular masses among the known keratinases from different sources (Xiang lin et al., 1992; Brigitte et al., 1995; Cheng Gang et al., 2008). About 66 KDa keratinase was reported, produced by Bacillus sp. JB99 was found to the largest sized keratinase (Pushpalatha and Naik, 2010). In average, the size of the keratinase obtained by many researchers was in between the size of 35 to 45 KDa (Dastager et al., 2009; Venkata saibabu et al., 2013; Srinivas et al., 2013). The molecular weight of the enzyme in the present study seems to be in the range with the data of other researchers as mentioned above.

Figure 3: SDS page profile of purified keratinase (E) compared with standard markers (M)

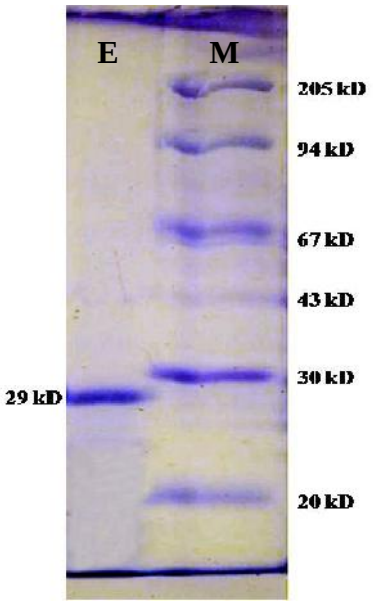
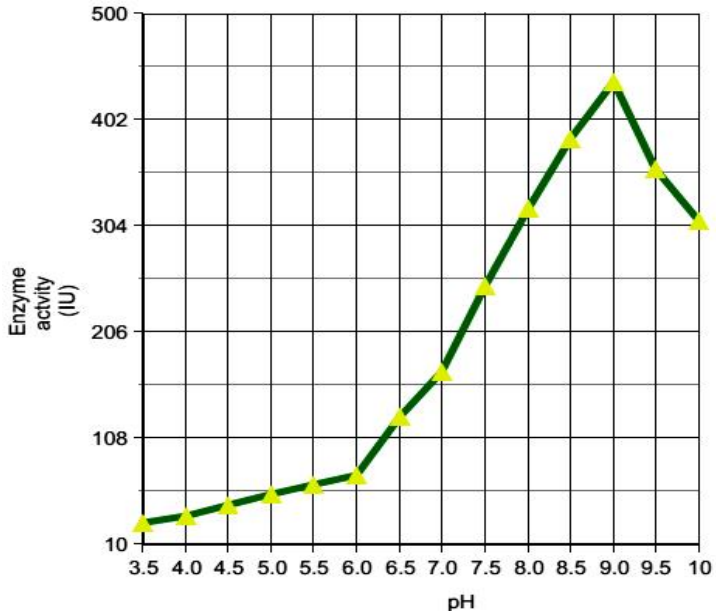


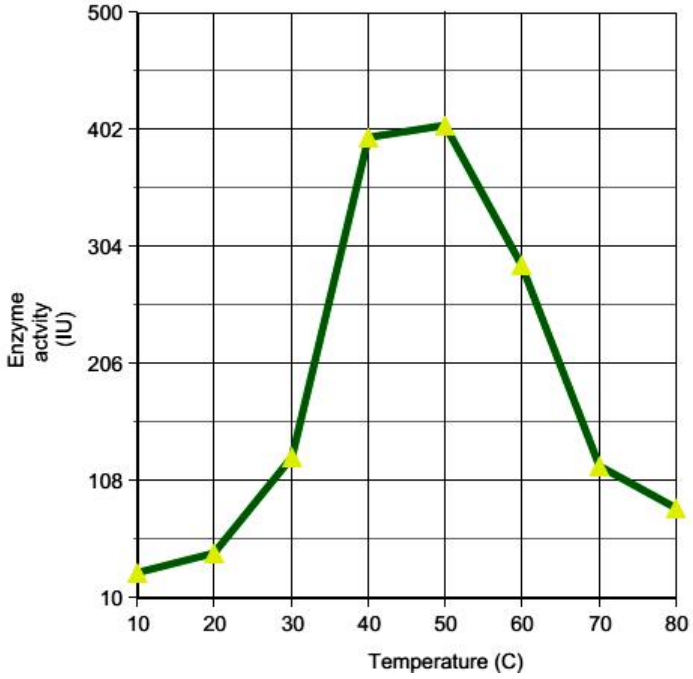
Figure 4: Influence of pH on activity of keratinase



Characterization of keratinase

Biochemical and enzyme kinetic properties of enzymes normally vary with the microbial sources. Properties of keratinase obtained from different microbial sources were recorded to be influenced by variations in pH and temperature.In the present study, a range of pH (5.0–10.0) was employed to evaluate the optimal pH for keratinase activity. The activity of keratinase was increased from pH 3.5 to 8.5. The optimal pH for keratinase activity (437.2 IU) was observed at pH 9. Figure 4 depicts the effect of pH on keratinase activity. Temperature is an important parameter that governs the enzyme activity. A range of temperature (10-80 ºC) was employed in present investigation to evaluate the optimal temperature for keratinase activity (Figure 5). The activity of keratinase consecutively increased with a simultaneous increase with temperature in the range from 10°C – 50 °C and the optimal temperature for keratinase activity (406.0 IU) was observed at 50 ºC. Figure 5 depicts the effect of temperature on keratinase activity.

Figure 5: Influence of temperature on activity of keratinase



The purified enzyme could able to show a stable and broad activity in presence different buffers. The purified keratinase showed maximum activity in phosphate buffer (pH 9.0) followed by 0.1 M acetate buffer (pH 8.0) and 0.1 M glycine NaoH (pH 8.5) and least activity was obsereved in the presence of 0.1 M Tris-HCl (pH 9.0). Production of an alkaline keratinase by *Streptomyces minutiscleroticus* DNA38, with a tolerance of temperature up to 50 ºC is an important feature.

Conclusion

A successful production of alkaline keratinase from an isolate of Streptomyces is a significant observation of the present investigation. An enhanced production (435.8 IU) of keratinase, in starch chicken feathers medium under submerged bioprocess was achieved by response surface methodology. The isolate had shown a greater consistency towards higher conditions of temperature (45 ºC) and pH (9.0) for the maximum production of keratinase, using simple nutritional ingredients such as starch, yeast extract and chicken feathers. The purified keratinase revealed an activity of 596.86 IU with specific activity of 2486.91 indicating 113.45 fold purification by gel filtration. SDS page profile reveals a size of 29 KDa of keratinase. Activity of purified keratinase was tolerant to a wide range of pH and temperature. However, pH 9.0 and temperature of 50 °C were optimum for the maximum activity of keratinase. The results obtained showed that, newly isolated *Streptomyces minutiscleroticus* DNA38 could be a useful strain in biotechnology in terms of valorization of keratin containing wastes.

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