KINETIC STUDIES OF PARTIALLY PURIFIED KERATINASE FROM *Streptomyces albogriseolous* NGP 2

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Abstract
This study was focused on partial purification and characterization of keratinase from *Streptomyces albogriseolous* NGP 2, isolated from marine sediment of south Indian coastal region. In purification steps, 1.56 fold purification was achieved after 85% ammonium precipitation of the with 8.03% recovery. In further purification steps, 8.34 fold purified keratinase was recovered by Sephadex G-100 chromatography with 1.79% of recovery. The specific activity of purified enzyme was 1045.83 U/mg. Zymogram of crude enzyme on native-PAGE presented bands with keratinase activity of molecular weight and Isoelectric point were 40 kDa and 8.3. These findings suggested that the keratinase may have much industrial applications.

IndexTerms **-** Marine sediment, Molecular weight, Isoelectric point, Zymogram

I. Introduction

Poultry feathers contain more than 90% of crude protein in keratin form, found as wastes or by-products at poultry processing plants. Increasing quantities of feathers leads to drastic environmental pollution (Rajput and Gupta, 2013). The crude protein content in feather wastes should have a amazing capability nutrient cost and can have a few advantage as a protein resources for alternative from greater expensive dietary components for animal feed which include fowl and ruminant animal (Xie et al, 2010).Global commercial poultry processing generates five hundreds of thousands of lots off eathers in step with 12 months, which can be presently converted to feather meal through steam pressure and chemical treat ment(Freeman et al, 2009).In Indonesia, rooster industries are growing faster evaluating to the other cattle industry due to the high call for of poultry meat as cheap and high excellent protein assets for human intake. Furthermore, chemical remedy procedure which includes alkali hydrolysis and steam strain cooking, both are excessive cost tactics and negative to certain amino acids from feathers which include methionine, lysine, and tryptophan (Tork et al., 2013).

Currently, keratinases have been also successfully applied in several business and biotechnological tactics, which include production of sluggish-release nitrogen fertilizers in the agricultural enterprise, detergent industry, medication, cosmetics, the utility of enzymatic de-hairing in the leather-based industry, and synthesis of biodegradable films and coatings inside the biomedical enterprise (Habbeche et al, 2014). keratins which have insoluble structures are normally observed in feathers, hair, wool, skin, and nails. Evaluating to other soluble proteins, keratins are widely recognized to reveal high mechanical balance, degraded slowly in nature, and resistance to the action of proteolytic enzymes which include pepsin,trypsin, and papain (Sahoo et al, 2012).

Microbial keratinases are mostly extracellular enzymes which are inducible in nature but some are membrane linked (cell bound) and intracellular. Keratinases are by and large serine or metalloproteases which are capable of degrading the structural keratinous protein (Gupta and Ramnani, 2006). 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 (Abdel-Naby et al, 2017).

The current study presents sequential optimization strategy to improve keratinase production using *Streptomyces albogriseolous* NGP 2. Partial purification and characterization of the partially purified keratinase were investigated and these parameters are necessary to use the enzyme efficiently in all industrial applications.

II. METHODS

Isolation of actinomycete

The marine sediment was collected from the coastal region of Tuticorin, Tamilnadu, India at 2-3m depth by using grab sampler. The collected sediment was subjected for enrichment prior to serial dilution. One gram of enriched sample serially diluted using sterile distilled water and spread over starch casein Agar (SCA) plates and incubated at 28 + 2^0 C for 7 days (Basha et al, 2010).

Keratinase Screening

Actinomycete isolates were screened for the production of keratinase by inoculating them in modified basal liquid medium. A volume of 20 ml of modified basal liquid medium was taken in boiling tubes. In the tube, one medium sized chicken feather was added. Selected isolates were inoculated and incubated at room temperature for two weeks and the tubes were examined every two days (Saber *et al*., 2010). The keratinase activity was assayed by the modified method of Gradisar et al. (2005).

Genetic identification

The genetic level identification of potential actinomycete isolates were carried out. Genomic DNA was isolated according to the method of marmur (1961). Phylogenetic relationships with closely related species were determined by using MEGA version 4.0. Distance matrices were determined by Kimura (1980) and were used to elaborate a dendrogram by the neighbor-joining method (Saitou and Nei, 1987).

Growth kinetics and enzyme production

To determine the optimum culture conditions for keratinase enzyme production, the actinomycete isolates were grown in basal liquid medium (Wawrzkiewicz et al, 1991) in an orbital shaker at120 rpm.

Ammonium sulphate Precipitation

The culture filtrate was collected by filtration aseptically in laminar air flow chamber (HEPA filter - 0.3 µm) through whatmann No. 1 filter paper and the filtrate was centrifuged at 4000 rpm for 5 min. The enzyme was precipitated from the supernatant by the gradual addition of ammonium sulphate with gentle stirring to 85.0 per cent saturation, allowed to stand for 2 hrs and centrifuged at 10,000 rpm for 30 min. The precipitate was dissolved in tris-HCl buffer (25 mM; pH 7.8), dialyzed against 10 mM of the same buffer for 24 hrs and concentrated by lyophilization.

An aliquot of the lyophilized sample (1 ml) was loaded on to a sephadex G-100 (45 X 1.5 cm) column equilibrated and eluted with 10 mM trisHCl buffer (pH 7.8) and NaCl (0 to 0.5 M) respectively. A total of 30 fractions were collected at a flow rate of 12 ml/h. All the steps were performed at 4ºC and every fractions enzyme and protein activity were calculated (Anbu *et al*., 2008; Paula *et al*., 2010).

Optimum substrate concentration

The optimum substrate concentration for the maximum activity of the enzyme determined in terms of maximum reaction velocity (V_{max}) and michaelis constant (Km at which reaction velocity is half maximum). For this, various concentrations of specific substrates were prepared and incubated with purified enzyme preparations. For L-asparaginase activity, L-asparagine (0 - 2.5 mM)

in tris-HCl buffer (0.05 M; pH 8.4) was used as substrate (Senthilkumar and Selvam, 2011) and for keratinase activity, different concentrations of keratin from 5.0 to 50.0 mg (w/v) in tris-acetate buffer (0.2 M; pH 7.0) was used as substrate. V_{max} and Km were estimated graphically by plotting substrate concentration in µM on X axis against enzyme activity U/mg protein on the Y axis. The accurate values of V_{max} and Km were obtained by double reciprocal Line Weaver-Burk plot and Eadie-Hofstee plot. The protein content was estimated by the method of Lowry *et al.* (1951 and the molecular weight of the sample was determined by SDS-PAGE (Laemmli, 1970).

Determination of pI values

The pI values of each enzyme fraction were determined by Iso electric focusing (IEF) technique (O" Farrell, 1975).

III. Results and Discussion

Screening and Identification

The actinomycetes growth occured on the SCA plate was subjected for keratinase screening in the basal liquid medium and it produced maximum enzyme of about 32.0 U/ml. The genetic level analysis of the 16S rRNA gene is the most important tool for correct identification of microbial species. The isolate was identified as *Streptomyces albogriseolous* and the sequence was submitted to Gen-Bank (JX843531). A phylogenetic tree constructed by MEGA 4 software based on 16srRNA partial sequence. Similarly, According to Nithya et al. (2012) 16SrRNA phylogenetic analysis of actinomycetes isolated from Eastern Ghats was carried out neighbor-joining algorithm.

Growth kinetics and enzyme production

The results showed that the culture filtrate of *S. albogriseolus* NGP 2 had a keratinase total activity of 17565 U with a protein content of 0.28 mg/ml; the specific activity was 125.46 U/mg protein. When concentrated by ammonium sulphate, the specific activity was increased to 196.0 U/mg protein with a purification factor of 1.56. The protein content was decreased to 0.24 mg/ml. The enzyme recovery was 8.03 per cent. When passed through sephadex G 100 column, the fraction 14 exhibited keratinase activity. In fraction 14, the protein content was 0.06 mg/ml; specific activity was 1045.83 U/mg protein, the purification fold and recovery yield were 8.34 and 1.79 per cent (Fig. 1 and Table 1). Besides, the V_{max} and Km of purified keratinase from *Bacillus thuringiensis* (TS2) was 265.3 U/mg and 5.979 ± 0.17 mM (Sivakumar et al, 2012a). Similarly, Km of the keratinase from halophilic bacteria was16.67 mM (Nigsm et al, 2013).

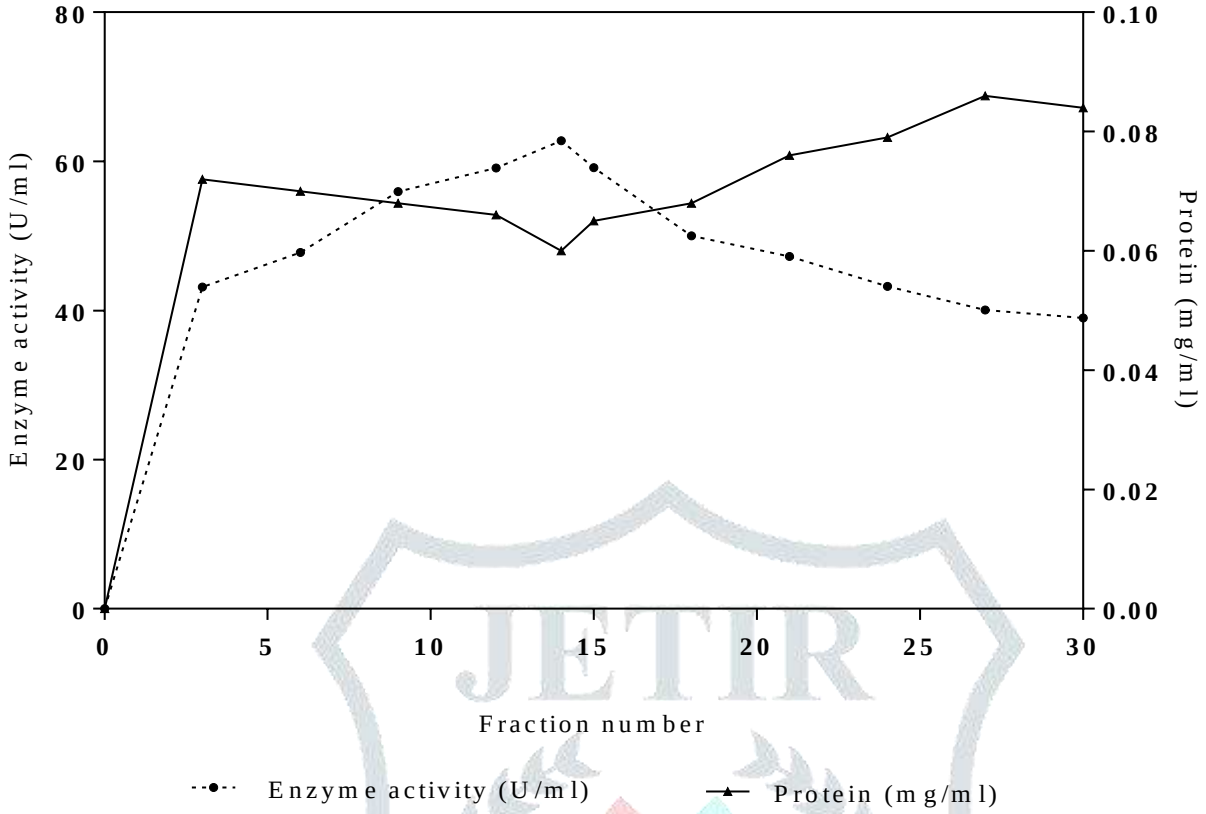


Figure 1: Purification of keratinase of *S. albogriseolous* NGP 2 on Sephadex G 100 column

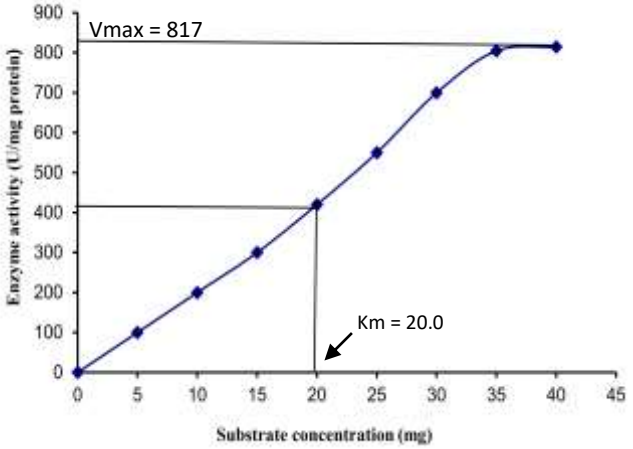
Sample		Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Total Activity (U)	Specific Activity (U/mg)	Recovery Yield (%)	Purification factor
Culture filtrate		500	35.13	0.28	17565	125.46	100	1.00
Ammonium sulphate precipitation		30	47.04	0.24	1411.20	196.00	8.03	1.56
Column Chromatography Sephadex G -100								
Fraction	14	5	62.75	0.06	313.75	1045.83	1.79	8.34

Table 1: Purification of keratinase from the culture filtrates of *S. albogriseolous* NGP 2

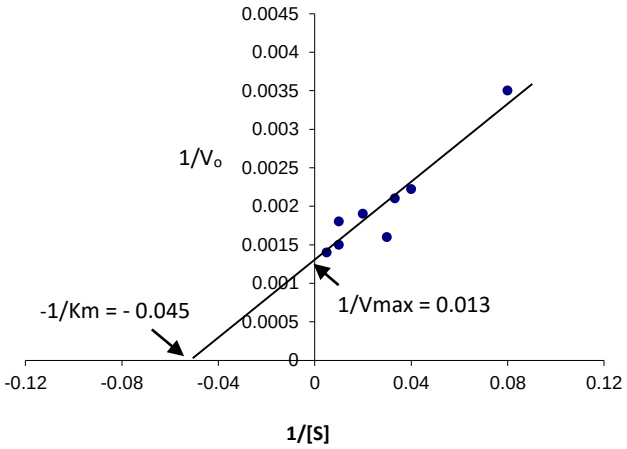
In keratinase, Vmax and Km values were estimated by using keratin in tris-acetate buffer (0.2 M; pH 7.0) as

substrate; Vmax and Km for *S. albogriseolous* NGP 2 keratinase were 817 U/mg protein and 20.0 mg respectively (Fig. 2). According to Anitha and Palanivelu, (2013), the enzyme hydrolyzed the substrate azocasein and the Vmax and Km of the purified keratinase from *Aspergillus parasiticus* were found to be 3463.34 U/mg protein and 1.04 mM respectively.

V max



Double reciprocal plot



Eadie-Hofstee plot

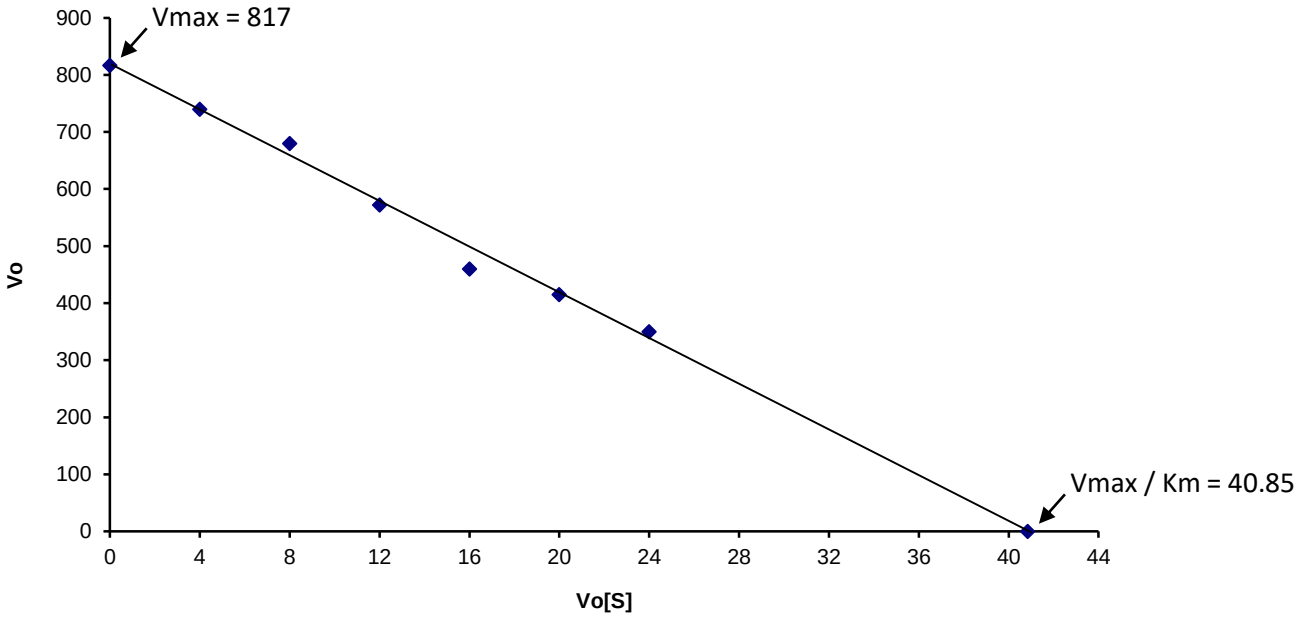
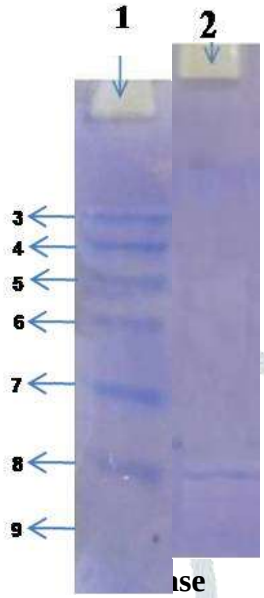


Figure 2: Kinetics of *S. albogriseolus* NGP 2 keratinase

pI and Molecular weight

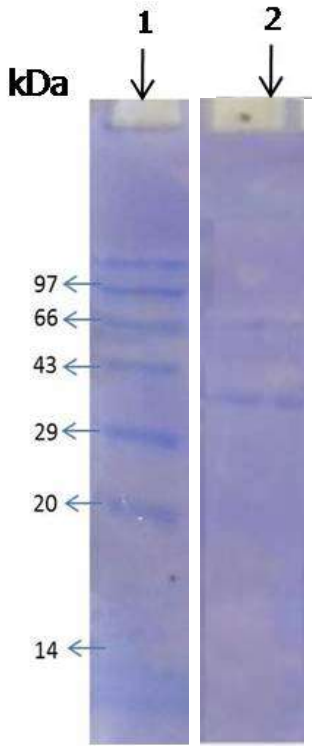
The enzyme keratinase of actinomycete NGP 2 exhibited single fraction of pI. The pI value was found to be 8.3 (Plate 1). The molecular weight of the the enzyme keratinasewere determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). A single band was exhibited by Keratinase *S. albogriseolus* NGP 2 had a molecular weight of 40kDa (Plate 2).



Lane 1: Molecular weight markers

Lane 2:

Plate 1: pI val ase



Lane 1: Molecular weight markers Lane 2: Keratinase

Plate 2: Molecular Weight of keratinase

Similarly, the isoelectric point and molecular weight were determined of the keratinase from *Doratomyces microsporus* were 9 and 33.0 kDa (Gradisar *et al*., 2000). Similarly, the molecular weight of the *Bacillus megaterium* was 41±1 kDa (Saibabu *et al.*, 2013).

IV. Conclusion

The present study revealed the kinetic parameters of the keratinase produced from actinomycetes, isolated from the South Indian coastal region. According to the kinetic studies, the enzyme will be optimized efficiently to carry out the industrial applications.

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