Purification and characterization of Keratinase enzyme from *Streptomyces* species JRS 19.

T. Jayalakshmi 1, P. Krishnamoorthy 1, G.Ramesh kumar 2, P.Sivamani 3

1 Dept of Bioinformatics, Bharath University, Chennai,Tamilnadu - 600073, India.

2 Dept of Bioinformatics, MIT Campus, Anna University, Chennai, Tamilnadu-600044, India.

3 Microlabs, Vellore, Tamilnadu-632521, India.

jayamaniraaja07@gmail.com

Abstract: A keratinase producing enzyme bacterial culture JRS 19 was isolated from Soil samples were collected from 5 different districts of Tamil Nadu and in addition, soil samples were also collected from prawn shell decomposing area at Redhills, Chennai , India. It was related to *Streptomyces* sp. on the basis of biochemical properties and Screening for sensitivity and resistant to antibiotics. Determination of cellwall amino acid and cell wall sugar techniques applied to identify the chemotaxonamy of actinomycetes. The purification of keratinase present in the culture medium was grown in a fermenter containing optimized production medium for eight days and showed an optimal activity at 4ºC for 15 minutes. The concentrated crude enzymes were analyzed extracellular protein profile and Keratinase hydrolytic activity using SDS-PAGE and Native-PAGE. Keratinase activity of each fraction was determined Diethyl Amino Ethyl Cellulose (DEAE) column chromatography Sephadex G-100 gel filtration column chromatography Polyacrylamide gel electrophoresis of the Keratinase was carried out to determine the protein profile of the enzyme and make this keratinase extremely useful for biotechnological process involving the hydrolysis of poultry feathers and de-hairing of bovine pelts.

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1. Introduction:

Proteolytic enzymes are largely used in the industry for biotechnological applications involving the hydrolysis of protein substrates. 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). 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) and hydrolysis of feather keratin (Lin et al. 1995), which is a by-product generated in huge amounts by the poultry industry. Discarded feathers are currently used to produce feather meal through thermal processing, resulting in a low nutritional value product (Wang and Parsons, 1997). Feather hydrolysates produced by bacterial keratinases have been used as additives for animal feed (Williams et al. 1991). In addition, keratin hydrolysates have potential use as organic fertilizers, production of edible films and rare amino acids (Dalev and Neitchev, 1991; Choi and Nelson, 1996). Cultivation conditions are essential in successful production of an enzyme, and optimization of parameters such as pH, temperature and media composition is 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). This is even more the case for keratinases of Gram-negative bacteria (Sangali and Brandelli, 2000). The *Chryseobacterium* sp. strain kr6 was isolated from waste of a poultry industry and was capable to completely degrade chicken feathers. Its extracellular keratinase is a metalloprotease with great potential for biotechnological applications (Riffel et al. 2003a). This work describes the effect of temperature, initial pH and substrates on keratinase production by *Chryseobacterium* sp. kr6 during growth on native feathers. Moreover, Feather waste represents a potential protein alternative to more expensive dietary ingredients for animal field (El-Refai et al., 2005; Onifade et al., 1998). Worldwide, commercial poultry processing generates million tons of feathers per year which are currently converted to feather meal through steam pressure and chemical treatment (Shih, 1993).

2. Materials and Methods:

Preparation of enzyme for purification

Initially the *Streptomyces* sp. JRS19 was grown in a fermenter containing optimized production medium for eight days and then, the filtrate was collected through Whatman No.1 filter paper, and centrifuged at 10,000xg for 15 minutes at 4ºC. The cell free culture filtrate was precipitated with using 80% ammonium sulphate. The precipitated proteins was centrifuged at (10,000xg) at 4ºC and dissolved in 50 mM sodium acetate buffer at pH 5.2, and dialyzed against 5 mM sodium acetate buffer at pH 5.2 with several changes of buffer. The dialyzed proteins was concentrated by lyophilization and used for purification. The concentrated crude enzyme were analyzed extracellular protein profile and Keratinase hydrolytic activity using SDS-PAGE and Native-PAGE.

Diethyl Amino Ethyl Cellulose (DEAE) column chromatography

Fifty mg of total protein was loaded on the column with out disturbing the surface of column. Elution was started with sodium acetate buffer 50 mM (pH 5.2) followed by the same buffer with linear gradient of NaCl (0 to 0.5 M). Fractions of 3 mL were collected for every 12 minutes by an automatic fraction collector (LKB Broma 7000 ultra fraction collector) at the flow rate of 1 mL/4 minutes. The protein content of each fraction was read at 280 nm. Keratinase activity of each fraction was determined and the Keratinase positive fractions were pooled together, dialyzed, concentrated and its was subjected to gel filtration chromatography.

Sephadex G-100 gel filtration column chromatography

Sephadex G-100 (15 g) (Sigma Chemical Co. USA) was allowed to swell overnight in sterilized glass distilled water. The floating gel beads were removed and the gel slurry was packed into a glass column (100 x 2 cm), which contained glass wool packed at bottom. While packing, care was taken to avoid any air bubbles. The packed gel column was equilibrated with sodium acetate buffer (50 mM) at pH 5.2. The concentrated enzyme of above sample was loaded onto a Sephadex G-100 column. Protein was eluted with 50 mM sodium acetate buffer (pH 5.2) at the flow rate of 3 mL/16 minutes. Absorbance of the fractions were read at 280 nm for protein and 585 nm for Keratinase activity using colloidal Keratin as a substrate. The purified Keratinase was stored at (-20ºC) until further use.

Electrophoretic studies

Native Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel elecrophoresis of the Keratinase was carried out to determine the protein profile of the enzyme. The following stocks solutions were prepared, filtered, stored in amber bottles and refrigerated.

Reagents

Solution A		Solution B	
1N HCl	48.0 mL	Acrylamide	30.0 g
Tris	36.6 g	N,N' – methyl bisacrylamide	0.8 g
Glass distilled water	100 mL	Glass distilled water	100 mL
pH 8.8 for separating gel pH 6.8 for staking gel			

Solution C		Solution D
Ammonium persulphate	0.14 g	N,N,N,N'–tetramethyl Ethylene diamine (TEMED)
Glass distilled water	100 mL	

Preparation of gel

Separating gel [10% (w/v)]		Stacking gel [5% (w/v)]	
SolutionA	0.74 mL	Solution A	0.35 mL
Solution B	2.0 mL	Solution B	0.50 Ml
Solution C	0.3 mL	Solution C	0.15 mL
Glass distilled water	2.89 mL	Glass distilled water	2.0 mL
Solution D	0.005 mL	Solution D	0.005 mL
Tank buffer (pH 8.3)			
Tris			6.0 g
Glycine			28.8 g
Glass distilled water			1000 mL

Tank buffer stock solution, 100 mL was made up to 1 L with glass distilled water, adjusted the pH 8.3 and used as tank buffer. Slab gel electrophoresis was carried out on glass plates of 10.5x10.5 cm.

Procedure

Polymerization of separating gel was carried out on the glass plates. Stacking gel was polymerized over the separating gel, after inserting a Perspex comb. The known amount of enzyme sample, mixed with glycerol and layered in each well. A few drops of aqueous bromophenol blue (0.01% w/v) was added to the upper tank buffer and the power supply was connected, with cathode in the upper tank and anode in the lower tank reservoir. Electrophoresis was carried out at 4ºC with constant voltage and 20 mA current supply for 2 h or until the tracer dye reached 0.5 cm above the lower end.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970)

SDS-PAGE was performed on slab gel with separating and stacking gels (10 and 5% w/v) according to the method of Laemmli (1970).

Reagents

The following stock solutions were prepared.

Solution A	1.5 M Tris HCl buffer (pH 8.8) with 0.4% (w/v) SDS
Solution B	0.5 M Tris HCl buffer (pH 6.8) with 0.4% (w/v) SDS
Solution C	30% (w/v) acrylamide with 0.8% bisacrylamide
Solution D	1.4% ammonium sulphate
Solution E	1% SDS
Solution F	N,N,N,N' tetramethyl ethylene diamine (TEMED)

Preparation of gel

Separating gel [10% (w/v)]		Stcking gel [5% (w/v)]	
Solution A	0.75 mL	Solution B	0.38 mL
Solution C	2.0 mL	Solution C	0.5 mL
Solution D	0.3 mL	Solution D	0.15 mL
Solution E	0.6 mL	Solution E	0.3 mL
Glass distilled water	2.6 mL	Glass distilled water	1.98 mL
Solution F	0.005 mL	Solution F	0.005 Ml

SDS-Sample buffer

Glycerol	2.0 Ml
-mercaptoethanol	1.0 mL
10% SDS (w/v)	4.0 mL
Solution B	1.7 mL
Bromophenol blue (aqueous)	0.2 mL
Glass distilled water	0.6 mL

Tank buffer (pH 8.3)

Tris	3.0 g
Glycine	14.4 g
SDS	1.0 g
Glass distilled water	1.0 L

Procedure

The solution was mixed with an equal volume of SDS - sample buffer and boiled in a water bath for 3 minutes, cooled and added to the wells.

Staining with separated proteins

At the end of electrophoresis, gel was removed and stained by silver staining. After staining, the gels were stored in 7% (v/v) acetic acid.

Determination of molecular mass

The molecular mass of the purified Keratinase was determined by SDS-PAGE. Purified protein samples were run on SDS-PAGE with concurrent run of standard protein markers consisting of Phosphorylase B (97,400 Da), Bovine serum albumin (66,000 Da), Ovalbumin (43,000 Da), Carbonic anhydrase (29,000 Da), Soybean trypsin inhibitor (20,100 Da) and Lysozyme (14,300 Da) obtained from Genei, Bangalore, India. After separation, the gel was stained with silver nitrate.

Zymogram analysis

The purified Keratinase sample was subjected to electrophoresis under Native condition. After electrophoresis the gel was equilibrated with sodium acetate buffer (50 mM, pH 5.2) for 5 minutes. This gel was overlayed onto a 7.5% (w/v) polyacrylamide gel containing 0.01% (w/v) glycol Keratin. The entire set up was kept under moist condition for 3 h at 37°C. The glycol Keratin containing gel was then stained with freshly prepared 0.01% (w/v) calcoflour white M2R (Sigma Chemical Co., USA) in Tris-HCl buffer (50 mM, pH 6.8). After 5 minutes the brightner solution was removed and the gels were incubated in glass distilled water for 1 h at room temperature. Enzyme activity zone was observed by placing the gels on fotodyne transilluminator (USA) and photographed.

3. Results and discussion:

Purification and characterization of *Streptomyces* SP. JRS19

Detection of extracellular protein profile on SDS-PAGE

Extracellular protein content from the cell free culture filtrate of *Streptomyces* sp. JRS19 was treated with 80% ammonium sulphate precipitated and separated by denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% of gel. After protein separation, the gel was stained with silver nitrate. The lane 1 represented standard protein markers and lane 2 represented protein profile of *Streptomyces* sp. JRS19 (Fig. 1).

Detection of extracellular keratinase activity on Native-PAGE

The ammonium sulphate precipitated keratinase (10 U) was separated on Native-PAGE. After separation this gel was over laid 7.5% poly acrylamide containing (w/v) of glycol KERATIN (0.01%). Then it was stained with Calcoflour M2R white and observed under fotodyne transilluminator. The keratinase hydrolytic activity showed isoforms of Chi I and Chi II were detected .

Purification of Keratinase

The cell free culture filtrate of *Streptomyces* sp. JRS19 which is shown in table 1was collected after 8 days of incubation and its proteins were precipitated by ammonium sulphate (80%). The crude proteins preparation was dialyzed, concentrated by lyophilization and used for further analysis. The flow chart of the purification of keratinase is presented .

DEAE cellulose column chromatography

The two peaks of keratinase activity were showed the major peak I keratinase activity was recorded from fractions 19 to 38 . The enzyme active fractions were pooled, concentrated by lyophilization, dialyzed against sodium acetate buffer (5 mM, pH 5.2) and used for further purification.

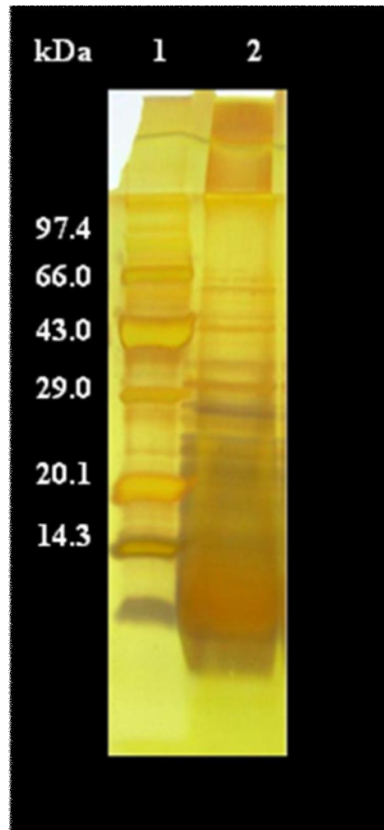

Fig.1 shows the extracellularprotein profile of *Streptomyces* sp. JRS 19 on SDS-PAGE

Lane 1; Standard protein markers

Lane 2; Ammonium sulphate precipitated protein (80%)

Table 1 shows keratinase production of *Streptomyces* spp.

Isolates	Results
Streptomyces sp. JRS1	+ +
Streptomyces sp. JRS2	+ + + +
Streptomyces sp. JRS3	+
Streptomyces sp. JRS4	+ + + +
Streptomyces sp. JRS5	+
Streptomyces sp. JRS6	-
Streptomyces sp. JRS7	+ +
Streptomyces sp. JRS8	+ + + +
Streptomyces sp. JRS9	-
Streptomyces sp. JRS10	+
Streptomyces sp. JRS11	+ +
Streptomyces sp. JRS12	+
Streptomyces sp. JRS13	-
Streptomyces sp. JRS14	+ + + +
Streptomyces sp. JRS15	+ +
Streptomyces sp. JRS16	+
Streptomyces sp. JRS17	+
Streptomyces sp. JRS18	+ + + +
Streptomyces sp. JRS19	+ + + + + +
Streptomyces sp. JRS20	+

High activity ++++++ **Moderate activity** ++++ **Low activity** ++ **Poor activity** + No **activity** –

SephadexG-100 column chromatography

A total of 110 fractions (3 mL) were collected. The fractions from 30 to 55 showed a single peak of keratinase activity (Fig. 18b). They were pooled, dialyzed, freeze dried and used for characterization. The purification of keratinase from the culture filtrate was summarized in Table 12. The enzyme was purified up to 4.82 folds, with the recovery of 18 %. The specific activity of purified keratinase was 854.02 U/mg of protein.

Molecular mass determination of purified keratinase by SDS-PAGE

The purified keratinase (6 µg) was analyzed on SDS-PAGE (10% w/v) and stained with silver nitrate. On sodium dodecyl sulfate poly acrylamide gel electrophoresis the purified keratinase showed a single band indicated that it was electrophorotically homogeneous. The molecular mass of the purified keratinase was determined as 31.1 kDa by comparing with relative mobility of the molecular mass of the protein markers.

Purified keratinase activity on Native PAGE

The activity of purified keratinase (5 U) was determined on Native PAGE using glycol KERATIN as a substrate. The hydrolytic region appeared under the fotodyne transilluminator .

4. SUMMARY AND CONCLUDING REMARKS

A new *Streptomyces* species JRS 19 isolate producing keratinase was isolated and purified. According to Radhika tateni *et al.,* 2007 keratinases from microbial sources have considerable potential in biotreatments particularly for dehairing and bating, and removing substances like feathers that causes environmental pollution. The production of keratinase on simple media with feathers as a sole source allowing its production from a cheap substrate and a commercial production with low production cost.Furher work is focused on application of pure keratinase on keratinous tissue to identify the keratinolytic activity.

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6. Corresponding Author:

T. Jayalakshmi

Dept of Bioinformatics, Bharath University,

Chennai,Tamilnadu - 600073, India.

E-mail: jayamaniraaja07@gmail.com

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