A Keratin-specific enzyme from bacterial species isolated from post tsunami soil samples

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Abstract

Release of enzymes by bacterial species is done for the organisms to gain energy needed for their growth, repair, reproduction, and other biological functions needed for survival. It has been established that, these unicellular organisms, change their habitat according to the environment and are able to release enzymes to live satisfactorily. Thus, delivery of enzymes by microorganisms, purely depends on the environment where it endures. Recent Tsunami made human life miserable and the post tsunami conditions may jeopardize human welfare due to unidentified microbial species. In the present study, an assessment on whether, tsunami affected or changed the enzyme profile of bacterial species was made. Marine soil samples collected from pre-tsunami and post-tsunami conditions were subjected for isolation and characterization of bacterial species. Interestingly, it has been found, compared to bacterial isolate from pre-tsunami soil samples, the enzyme expression of post – tsunami soil bacterial isolates were completely different for the same bacterial species. It has been identified that, pre-tsunami samples expresses only protease and catalase enzymes that too with very minimum activity, whereas, the same bacterial species from post-tsunami soil samples expresses enzymes of different classes, viz., protease, collagenase, urease, hydrolases, catalase, peroxidases, cellulase, pectinase and lipase. Among the twenty two bacterial isolates of post-tsunami soil samples, only two bacterial isolates namely GSPOT 001 and GSPOT 005 expresses the above mentioned enzymes extracellularly with high activities. Characterization of the enzyme of the isolate GSPOT 001 reveals that it is highly keratin –specific and releases considerable quantities of amino acids (Phenyl alanine, Tyrosine, Tryptophane, Iso-lecuine, Valine, Cystein), ammonia, sulfite as end products reveals the keratinolytic and sulfitolytic pathway of hydrolysis exhibited by the isolate within 16 hours when keratin is used as substrate. The quantity of the enzyme required (Units) for the dehairing process will be discussed.

Key words: Keratinases, dehairing, *Bacillus polyfermenticus*, enzymes

Objectives

1. To obtain keratin –specific proteases from bacterial sources especially marine sources
2. To assess whether any natural calamities will affect the genetic/enzymatic profile of the native flora/fauna of marine source
3. To assess whether the protease activity expressed by the new isolate is induced or inherent.

Introduction

In order to grow and remain alive microorganisms (whatever may be their source) must carry out a tremendous number of enzyme catalyzed reactions. Despite their constant genotypes, microbes are amazingly flexible in their ability to alter their composition and metabolism in response to environmental changes. The environment doesnot change the genetic makeup of the cell but markedly affects the phenotypic expression of the genes.

A new paradigm shift in the conversion of putresciable collagen to non-putresciable stable material opens up a few more avenues for the enzymes. Inspite of availability of number of reports on role of proteases in leather processing, still, intensive research is required to have the requisite properties of leather with very minimum pollution load. Moreover, only a scanty reports are available on kertain-specific proteases. Based on this concept, in the present study, an attempt was made to have bacterial isolate of marine origin, because, the ecological communities of marine sources are highly different and not explored well. The first sample collection from marine source, was carried out, with out any intention during on 10th - 15th December 2004. But, when the major disaster, tsunami on 26th December 2004

was realized, immediately, the second sample collection was done on 28th – 30th December 2004. Keeping the said objectives in mind, studies were carried out on pre-tsunami and post-tsunami soil samples for evaluation of biodiversities profile and enzyme profile of the highly stable and purified isolate.

Materials and Methods

All analytical reagents and media components were purchased from Hi-Media (Bombay, India) and Sigma Chemicals (St. Louis, USA).

Biodiversities profile

To understand the complete biodiversities profile of the marine samples of pre and post tsunmai, general method of isolation, screening was performed. Soil samples of sandy (75%) – clay character with pale black colour, collected from coastal area (10 m from the sea) (Kovalam) at 0.5 -1.0 m depth was collected in the pre-sterilized polyethylene bags and screened to remove the pebbles and other coarse materials and subjected to isolation by serial dilution technique immediately after brought into laboratory conditions. Biochemical characterizations and identification upto genus level was done according to the general methods followed.

Isolation and cultivation of microorganisms exhibiting Keratinase activity

To have bacterial isolate exhibiting keratinase activity, a special culture medium containing Keratin substrate was provided. Marine soil samples (1g) was diluted in 5ml of sterile saline solution. The aliquots of diluted samples were plated onto Keratin - yeast extract-peptone (KYP) agar plates containing (g/l): casein, 10; peptone, 5; yeast

extract, 1; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.1; and agar, 15; pH 7.5. Plates were incubated at 37°C for 24 h. A clear zone of Keratin hydrolysis gave an indication of Keratinase producing organisms. Individual colonies were transferred to fresh agar plates, purified through repeated streaking and stored on agar slants. All isolates, which formed a clear zone on KYP medium, were grown in basal medium which consists of (g/l) NH_4Cl, 0.5; NaCl, 0.5; K_2HPO_4, 0.3; $MgCl.6H_2O$, 0.1; yeast extract, 0.1% keratin (obtained from chicken feather); pH 7.5. The cultures were incubated at 37°C with rotary shaking and solubilisation of the Keratin was observed.

Taxonomical studies

Bacterial identification was conducted based on morphological and biochemical tests. Morphological and physiological characteristics of the isolated bacterium was compared with data from Bergey's Manual of systematic Bacteriology. The 16S rRNA gene was sequenced after genomic DNA extraction and PCR amplification as described by Thys et al (2004). The BLAST algorithm was used to search for homologous sequences in Genbank. Phylogenetic tree constructed using the software PHYLO DRAW , Ver.0.8.

Protein assay

Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin (BSA) as the standard. The concentration of protein during purification studies was calculated from the absorbance at 280 nm.

Protease activity assay

Neutral Protease activity was determined according to the method of keay and Wildi (1970). One unit (U) enzyme activity was defined as the amount of enzyme that liberates 1 μmol tyrosine from substrate (casein) per minute per ml of enzyme under assay conditions.

Keratinase activity assay

Keratinase activity was assayed with azokeratin as a substrate as described by Thys et al (2004). One unit of enzyme activity was the amount of enzyme that caused a change of absorbance of 0.01 at 420 nm for 30 min. at 45 C.

Cultural conditions for optimum keratinase production

Effect of Temperature:

To study the effect of incubation temperature, the strain GSPOT 001 was grown in basal medium containing 0.5 % feather from 30, 37, 40, and 50, 60°C.

Effect of pH

Strain was grown in basal medium containing 0.5 % feather and the medium pH was adjusted to 4, 5, and 6,7,8,9,10 with diluted HCl or NaOH solution and incubated at optimum temperature for 72 h.

Effect of Carbon Source:

Strain was grown in basal medium containing 0.5 % Keratin and in the presence of various kinds of carbon sources at 0.1%(w/v).

Effect of Nitrogen Source

Strain was grown in basal medium containing 0.5 % Keratin and in the presence of various kinds of nitrogen sources at 0.1%(w/v). In all experiments, the initial cell number was maintained at 5 X 10^7 cells/ml. Samples were removed at different time intervals for 72 h and assayed for the growth, final pH, protein content and keratinase activities as described above.

Purification of Keratinase

Purification was carried out at 4°C. Enzymes in the cell free supernatant portion of the culture were precipitated by ammonium sulphate (up to 80% saturation). The precipitate was dissolved in 0.2 M potassium phosphate buffer (pH 7.0) and dialyzed against 0.02 M potassium phosphate buffer (pH 7.0). The dialyzate was applied to a column of Sephadex G-50 (1.5 X 30 cm) equilibrated with 0.2 M potassium phosphate buffer (pH 7.0). Fractions (3.0 ml each) were collected at a flow rate of 20 ml h^{-1}. The active fractions were combined, dialyzed against 0.02 M potassium phosphate buffer (pH 7.0). Three millilitre fractions (19–28) corresponding to protease activity were collected and the purity checked on native PAGE. The purified enzyme was subsequently used for characterization work.

Hair removal studies

Bovine hair removal studies using the enzyme keratinase were carried out using 30 X 30 cm salted buffalo skin. The skin pieces were initially exposed to conventional processing such as desalting, soaking (both soak I and soak II) followed by enzyme soaking for different time intervals both with and without lime and sulphide at 0, 25 and , 50% concentrations. For control experiments, the soaked skins were subjected to

conventional liming processing. Skins were removed from the enzyme solution and the hair free skin samples were subjected to sections followed by staining (H & E). The slides were examined under microscope at 40 X to observe how far the

Results and Discussion

Figure 1 emphasizes the biodiversity profile of marine samples before and after Tsunami. It has been observed that the percentage of occurrence of Pseudomonad and Bacillus species are comparatively higher than other classes in the pre-tsunami samples. However, followed by tsunami, there has been a significant change in the diversity profile and higher numbers of bacterial species grouped under enterobacteria was encountered. Table 1 illustrated the bacterial isolates exhibiting protease activity. Interestingly, it has been found that, bacterial isolates obtained from post tsunami samples, exhibiting high proteolytic activity compared to the samples of pre-tsunami samples. During tsunami, there must be a continuous stress to the existing bacterial species and only some of the species responded to the stress and ability to thrive at critical conditions by excreting proteoytic enzymes extracellularly. In addition, the source, where the isolates have been isolated under pre-tsunami condition, has got adulterated with wastes of different characteristics. Thus, completely inhibit the growth of existing species, but in turn accelerate the growth of the new comers. Biochemical properties of the bacterial isolate exhibiting high proteolytic activity was shown in Table 2 and confirmed that it belongs to Bacillus genera. Further genomic and plasmid profile of the isolate as shown in figure 2 demonstrated that the isolate contain both plasmid and genomic dna and based on the 16s rDNA sequence, the isolate has been identified as

Bacillus polyfermenticus. Table 3 demonstrated the antibiotic sensitivity test for *Bacillus polyfermenticus* and found that the species has shown resistant towards penicillin, Cefixme, and Tricarcilin. Figure 3 illustrated the purification of keratinase enzyme and the fractions exhibiting protease and keratinase activities. Effect of various environmental parameters on growth of the isolates showed that the bacterial isolate is moderately temperature stable and growth was observed even at 50^0 C (Figure 4). Interestingly, this bacterial isolate has shown higher growth only at alkaline condition, ie., pH 10.0 (Figure 5). Thus evidently proves that the protease expression of this isolate must be of alkaline nature. Figure 6 demonstrated the keratinolytic behaviour of the enzyme obtained from *Bacillus polyfermenticus*. The hydrolysis of keratin as shown in the zymogram pattern of SDS-PAGE electrophoresis emphasized that the two types of keratinases, ie., Kertinase I and Keratinase II having molecular mass of 23 and 73 Kda was expressed by the new isolate.

Further application of keratinase enzyme on dehairing studies (Figure 7 & 8) reveals that the enzyme keratinase is capable of hydrolyzing the keratin part of the bovine hair further leads to opening up of fibres which facilitates the hair removal followed by tanning process with out lime and sulphide.

CONCLUSION

The biodiversity profile clearly illustrates that, the majority of the heterotrophic microbial species of marine sources are found to exhibit varied phenotypic expressions of genes with accordance with the environmental alterations. The taxonomical studies and 16s rDNA sequencing reveals that the keratinase producing isolate is *Bacillus*

amyloliquifaciens The protease activity exhibited by the *Bacillus amyloliquifaciens* is keratin –specific (two types Kr1 & Kr2) as evidenced through keratin hydrolysis activities compared with other proteases (Commerical Proteases as well as Proteinase K (Sigma)). The optimum cultural conditions for keratinase production is 1. pH 10.0; Temperature 37 C; Lactose and peptone as carbon and nitrogen sources. The molecular weight of the purified keratinase is found to be 28 Kda (Kr2) and 73 Kda (Kr1). On comparing the Phenotypic expression of genes of the same isolate of Pre-tsunami and post –tsunami samples, the keratinase activity of the isolate may be of induced one. Further studies on the hair removal pattern by the extracellular enzyme of Bacillus polyfermenticus, showed that the enzyme is capable of removing the hair and opens the fibre with out lime and sulphide. Ongoing pilot and large scale studies provide the new avenue for the complete enzymatic system of pretanning operations, which inturn reduces the pollution load considerably in the pretanning operations.

Table 1: Isolates exhibiting Protease activity from marine soil samples (Pre and Post tsunami)

S.No	Isolates	Keratinase activity
Pre- Tsunami		
1	GSPRET 006	Nil
2	GSPRET 011	Nil
3	GSPRET 014	Nil
4	GSPRET 015	Nil
5	GSPRET 018	Nil
Post - Tsunami		
1	GSPOT 001	++ Ve
2	GSPOT 005	+Ve

Table 2: Complete Characterisitics of GSPOT 001

S.No	Tests	Observations
1	Gram Staining	+ve
2	Shape	Rod
3	Colony Morphology	Cream white flat colonies
4	Endospore Stain	-ve
5	Flagella Stain	-ve
6	Motility	+ve
7	Indole Production	-ve
8	MR	-ve
9	VP	-ve
10	Catalase	+ve
11	Oxidase	+ve
12	Citrate	-ve
13	Urease	+ve
14	H_2S production	-ve
15	TSI Bud Slant Gas	 Acid Alkali +ve
16	Arginine dihydolase	-ve
17	Starch hydrolysis	+ve
18	Casein hydrolysis	+ve
19	Gelatin hydrolysis	+ve
20	Keratin hydrolysis	+ve
21	Lipid hydrolysis	+ve
22	Pectinase	+ve
23	Cellulase	+ve
24	Chitinase	+ve
25	Collagenase	+ve
26	Dnase	-ve
27	O/F Test	Not Determined
28	Nitrite Reduction	+ve
29	Pigment Production	-ve
30	Haemolysis test in Blood agar	-ve
	Carbohydate Fermentation Glucose Lactose Sucrose Maltose Galactose Arabinose Raffinose Mannitol Sorbitol Inositol	 -ve -ve -ve -ve -ve -ve -ve +ve -ve -ve

Table 3: Antibiotic Senstivity test

S.No	Antibiotics	Observations
1	Penicillin	No Zone (Resistant
2	Chloramphenical	29
3	Erythromycin	19
4	Polymicin	10
5	Rifampin	16
6	Amaxycillin	No Zone (Resistant
7	Nalidixic acid	24
8	Lincomycin	10
9	Aminkacin	21
10	Neomycin	20
11	Cephalexin	22
12	Netillin	22
13	Nitrofuran	16
14	Cefixme	No Zone (Resistant
15	Tricarcilin	No Zone (Resistant)

Figure Legends

Figure 1: Biodiversity profile of marine soil samples under pre-tsunami and post-tsunami conditions.

Figure 2: Molecular profile (Both genomic and plasmid) of *Bacillus polyfermenticus* isolated from post –tsunami soil samples.

Figure 3: Purification of enzyme keratinase using Sephadex G-50 and the fractions exhibiting protease as well as keratinase acitivities

Figure 4: Effect of temperature on growth profile of GSPOT 001

Figure 5: Effect of pH on growth profile of GSPOT 001

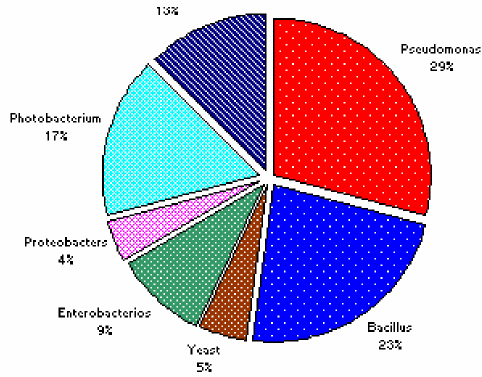
Figure 6: Zymography of Keratinase activity of purified enzyme of GSPOT 001

Figure 7: Bovine skin treatment with *B. polyfermenticus* GR 01 culture supernatant. Arrow indicates the outer epithelial root sheath, which disintegrated almost completely in the presence of culture supernatant. Sections of the root sheaths incubated in (**A**) Control, water-treated skin; (**B**) Treated skin with culture supernatant – 24 h incubation; (**C**) 36 h incubation; (**D**) 48 h incubation are shown. Incubation conditions: 37°C, pH 8.5, 400 rpm. Hematoxylin and eosin staining; 40X.

Figure 8: Bovine skin treatment with *B. polyfermenticus* GR 01 culture supernatant. Arrow indicates the epidermal zone, which disintegrated almost completely in the presence of culture supernatant. Sections of the root sheaths incubated in (**A**) Control, water-treated skin; (**B**) Treated skin with culture supernatant – 24 h incubation; (**C**) 36 h incubation; (**D**) 48 h incubation are shown. Incubation conditions: 37°C, pH 8.5, 400 rpm. Hematoxylin and eosin staining; 40X.

Figure 1

Biodiversities - Pre-Tsunami



Biodiversities- Post -Tsunami

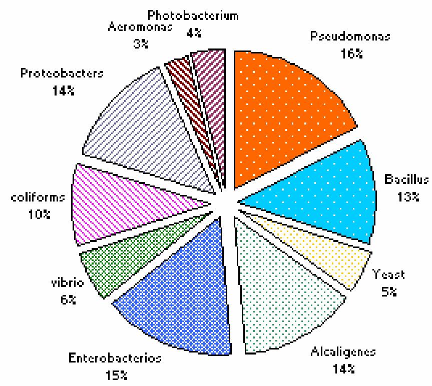
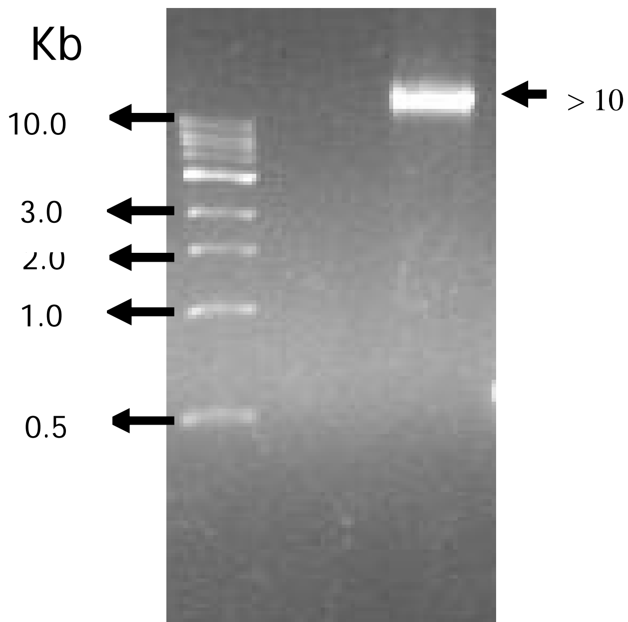


Figure 2: Molecular Characterization

Genomic DNA profile



Plasmid profile

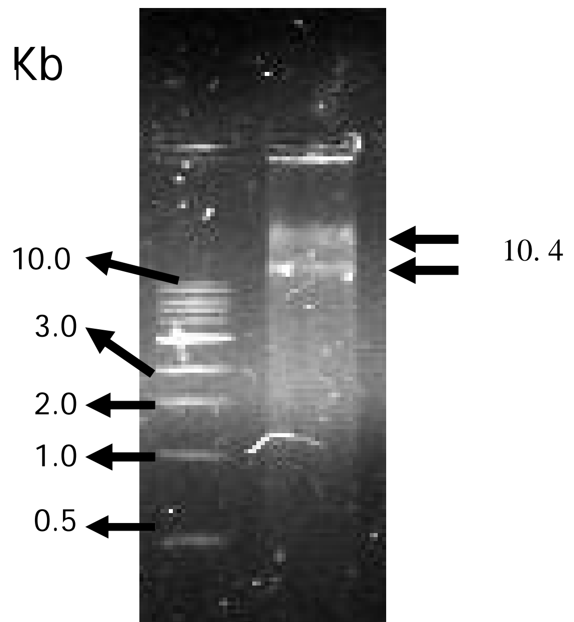


Figure 3: Enzyme Purification

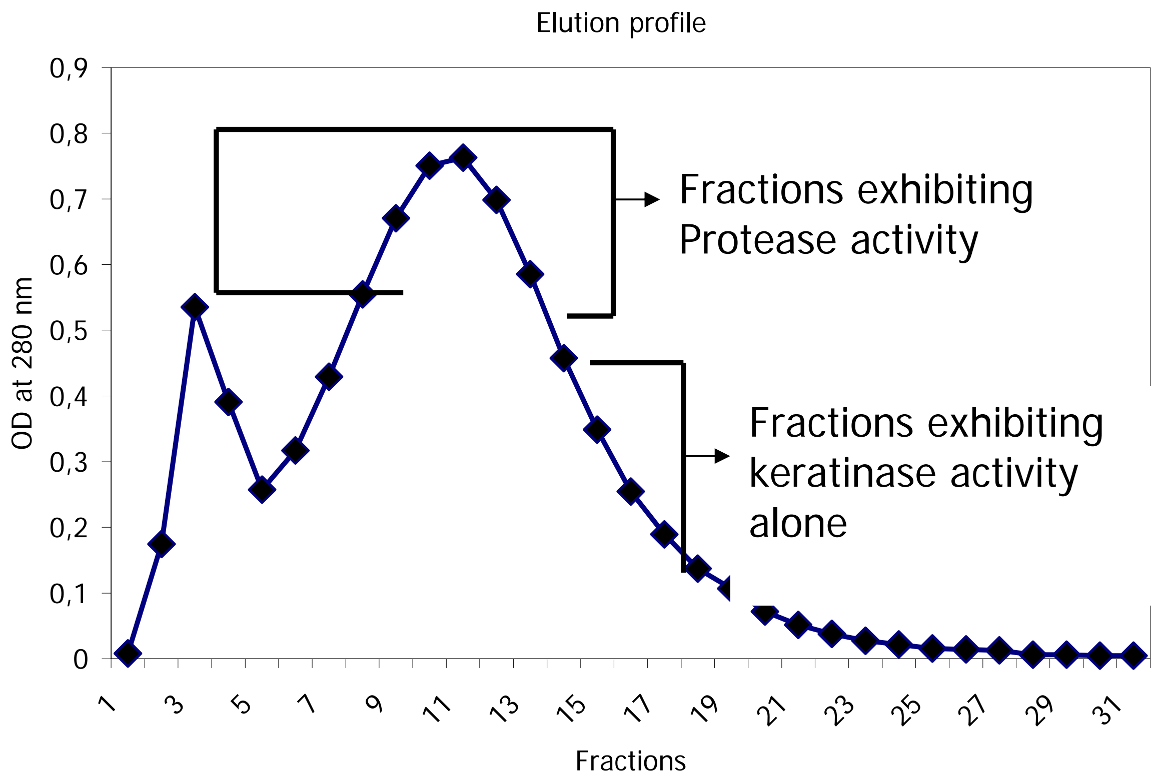


Figure 4: Effect of temperature on growth profile of GSPOT 001

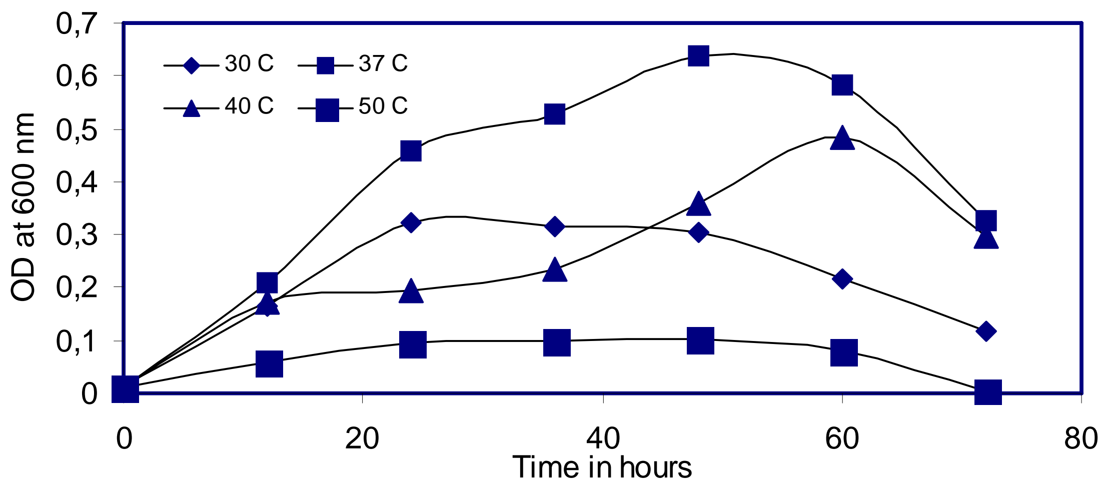


Figure 5: Effect of pH on growth of GSPOT 001

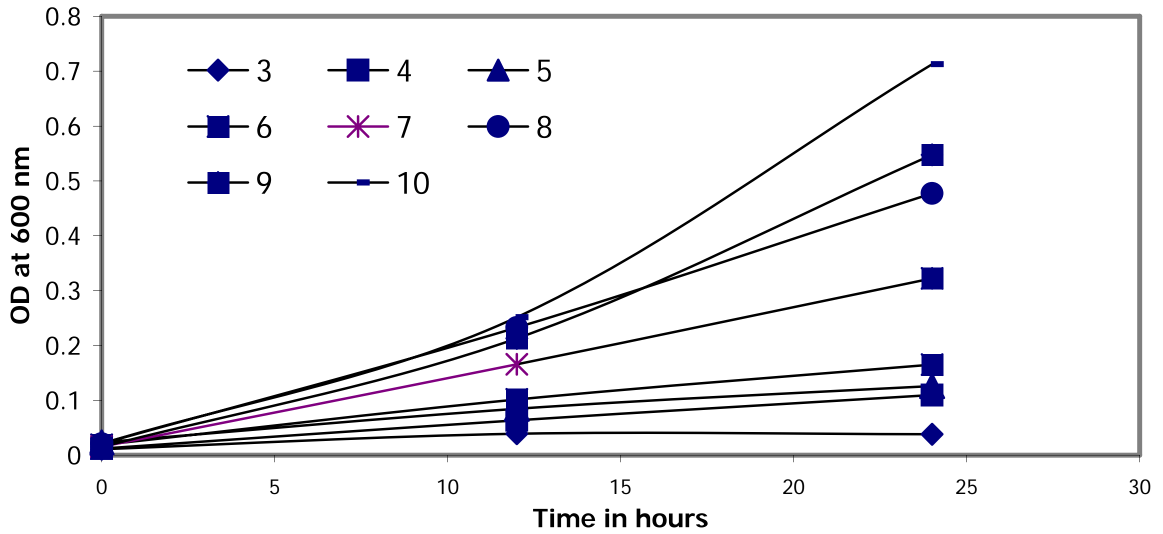
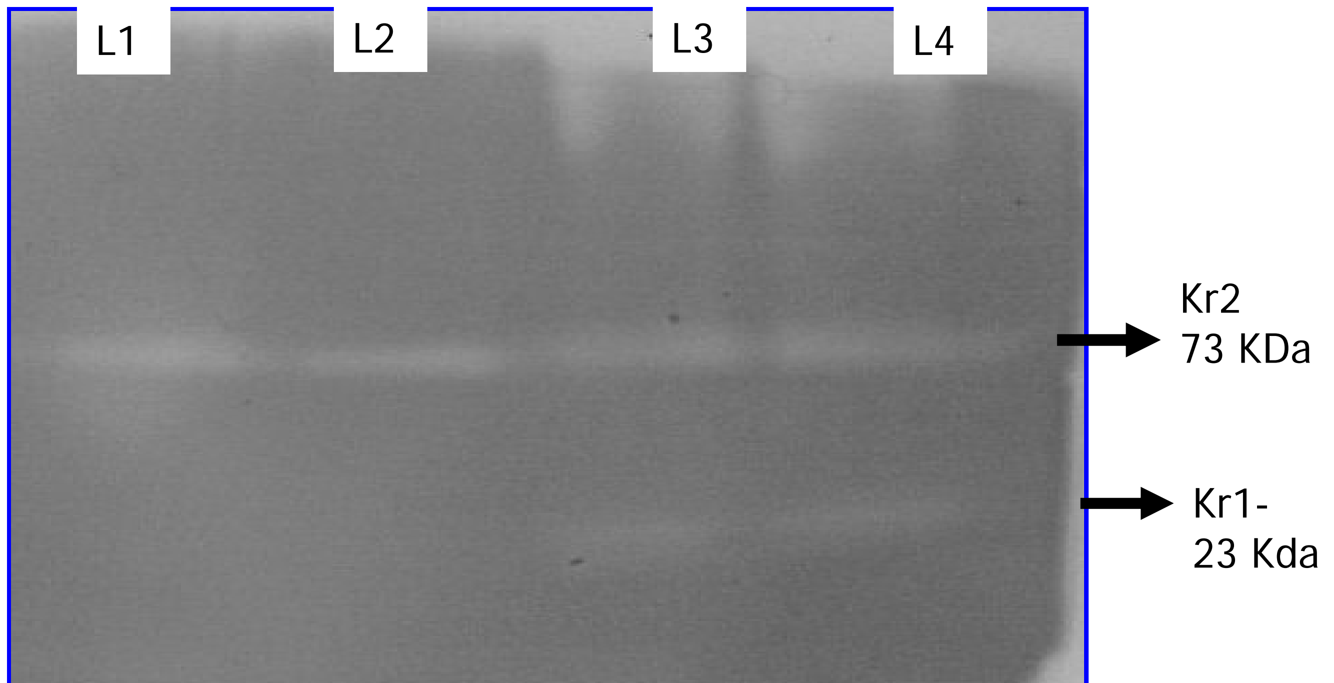


Figure 6: Zymography of Keratinase activity of purified enzyme of GSPOT 001



L1 & L2 – Culture supernatant (40 μg)
L3 & L4 - Purified keratinase (40μg)