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RESEARCH ARTICLE

Isolation, Identification, Characterization of *Bacillus subtilis* producing the Keratinase Enzyme under Optimization, Purification and immobilization method

R.MANJU
Ph.D Research Scholar, Bharathiar University, Coimbatore.

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Abstract

the keratinase producing bacteria from poultry farm and slaughter house. In that, feather dumping soil (100 samples) was collected from several area includes the Coimbatore, Pollachi, and Erode. Among these samples, the eighteen isolate were subjected for primary screening using Milk casein agar plate. From these isolates, the *Bacillus subtilis* showing a highest keratinase activity in secondary screening using Casein agar medium & it was confirmed by 16s rRNA molecular sequence. The specific organism subjected to keratinase activity assay and identified by the spectrometric using the keratin azure as a substrate. Thus, this organism was optimized by Response enzyme methodology (RSM) using the production medium. The optimum level of glucose for about 5.61g/l; enzyme activity was around 13.280 U /ml. In same way, the keratinase production was favored in the presence of ammonium tartarate as the nitrogen source; the enzyme activity in yeast extract (nitrogen source) as 9.8±1.3.The optimized enzyme were undergone with the Purification process, its includes the ammonium sulphate precipitation, DEAE Sephadex K-50, Biogel G-200.In this, the Bio-gel (Gel-200) specific activity 2860 U/ml, purification fold 34.30 and its yielding for about 7.5%. The molecular mass of specific protein is 40KDa.Again these enzymes were produced in 5L Bio-reactor. It includes Solid state fermentation (1456.10 KU/ml for 8 gram of dry weight of chicken feather) and in submerged fermentation (1800 KU/ml for 10 gram of dry chicken feather). The keratinase enzyme can be immobilized by using calcium alginate.5µg of keratin beads can able to produce 490KU/µg.

The aim of this present study was to isolate

Introduction

Feathers are generated in bulk quantities as a by-product in the poultry industry globally. It's a very rich source of protein with β-keratin (Mabrack MEM, 2008). Typically, each birds has up to 125 gram of feather and with more than 400 million Chicken being processed every week worldwide, the daily accumulation of feather waste reaches 5 million tons(Hans *et al*., 2012). In mature chicken feathers accounts up to 5-7% of the live weight (Swetlana Nagai *et al*., 2010). Basically, keratin occurs in nature mainly in the form of hair, feather, wool, horn, horn, and nail (Jones *et al*., 1999). Goat hair, sheep wool and buffalo horn showed lower response towards Keratinolytic hydrolysis (Saber *et al*., 2010). Keratin containing materials are abundant in nature, but have limited uses in practices since they are insoluble and resistant to degradation by the common proteolytic enzyme keratinase wastes represent a source of valuable protein and amino acid (Jayalakshmi *et al*., 2010).Feather waste was generated in large quantities as a byproduct occurring in commercial poultry plant. Feathers are considered as a waste product annually, which consist of approximately 90% keratin.

Keratinase was first coined by Kuhne in 1878 and it was analyzed by Pesox in 1883. Basically, there are keratin are observed in the hair, hooves of mammals, horn, claw and Beta helix keratin are present in the nail, scale and claw of reptile . Alpha helix keratin are packed as folded with cysteine .Based on the percentage of sulphur content, the hard and soft keratin are classified. Hard keratin characteristic include the 5% sulphur with low lipid and stability/thermal nature is high Vis versa the soft keratin having 1% sulphur, high lipid stability is low.

Keratin is insoluble in water, aqueous solution of neutral salt, organic solvents, resistant to degradation by common. Proteolytic enzyme trypsin, papain because of their high degree of cross liking by disulphide bound, hydrogen bound and hydrophobic interaction.

However, much current research is centered on the potential use of keratinase of bacterial origin for the industrial compounds (Vigneshwaran *et al*., 2010). Keratinase material is important in medical, agricultural and biological (Shih 1993), biodegradation films (Schrooyen *et al*., 2001), prion protein (Caughey 2001), conversion of keratin waste into bio-hydrogen (Balint *et al*., 2005), woolen textile industry (Sausa *et al*., 2007), detergent application (Gupta and Ramani, 2006). In order to utilizing the chicken feather waste for the production of keratin and it was used in worldwide and also more important to controlling the environmental pollution.

1. Isolation of Microorganism:-

100 gm of Soil sample (for about 1000 numbers) were taken from the natural composting waste in the poultry shop at different places includes Coimbatore, Pollachi, and Erode place. Serial dilution from each sample was prepared by adding 1 gm of the soil sample to 100ml distilled water. The distilled water makes up to 10^{-9} dilution. All the dilution were placed on nutrient agar medium and incubated at 37^0C for 24 hrs.

(i) spread plate method

(ii) Nutrient agar medium

1.1. Identification of Microorganism:-

From the isolated colonies, the organism is again inoculated into the casein agar medium. Organism producing keratinase activity was confirmed by zone of hydrolysis in casein agar medium.

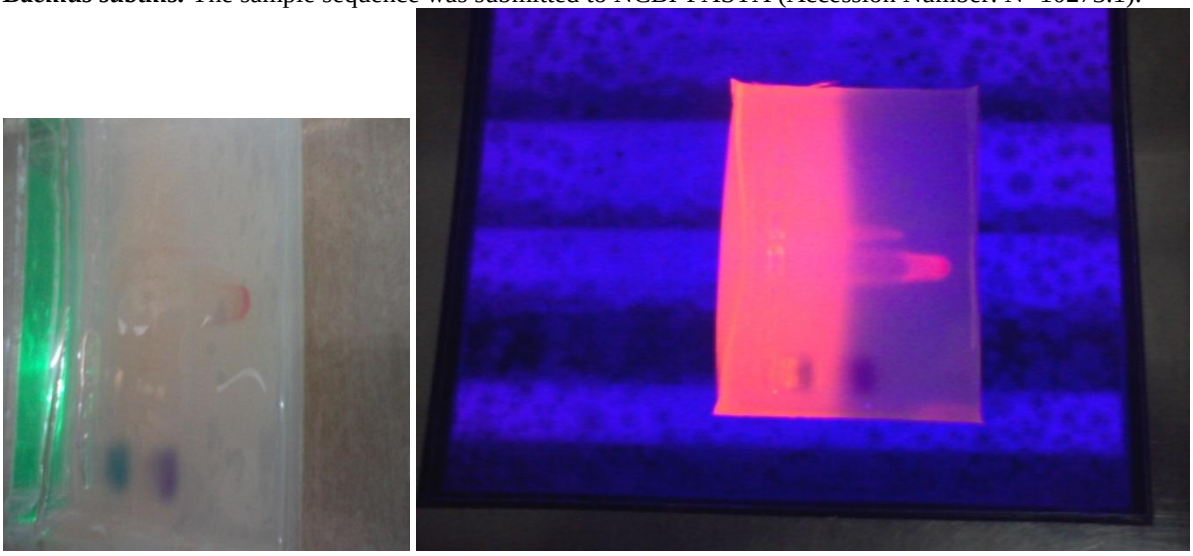
(iii) Casein agar medium

1.2. Characterization of Microorganism:-

The specific organism is confirmed by its Bio-chemical characteristic is as follows' (Table 1):-

S.no	Bio-Chemical Test	Result
1.	Gram staining	Gram positive
2.	Shape	Rod shape, raised, dull, opaque, grayish white, frosted glass appearance
3.	Capsule	Present
4.	Size	23 dm
5.	Indole production	Negative
6.	Methyl red	Negative
7.	Vogesprousker	Positive
8.	Citrate	Positive
9.	Oxidase	Negative
10.	catalase	Positive
11.	Litmus milk reaction	Alkaline
12.	Hydrogen sulphide	Negative
13.	Urease test	Negative
14.	Starch hydrolysis	Positive
15.	Casein hydrolysis	Positive
16.	Fructose & Glucose fermentation test	Acid production ;no gas

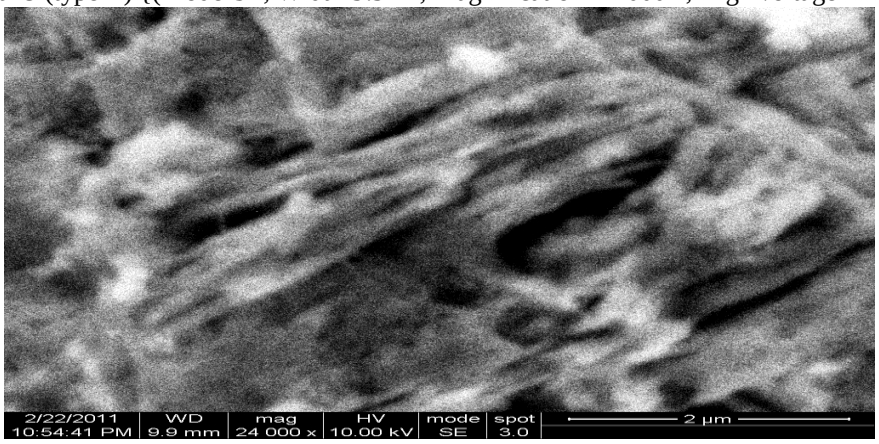
Then, the organism is undergone with 16sr RNA molecular sequence. Thus, the organism is identified as ***Bacillus subtilis.*** The sample sequence was submitted to NCBI-FASTA (Accession Number: N- 10273.1).



(iv) *Bacillus subtilis – DNA* with marker DNA

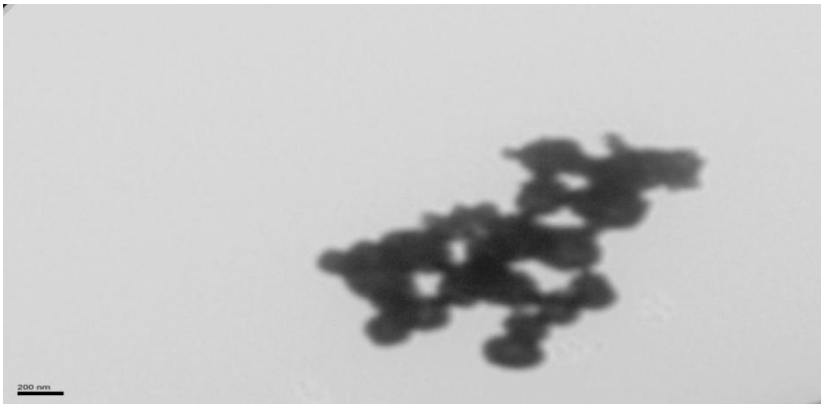
(iv) differential media

(iii) **1.3. Scanning Electron Microscope: -** The surface of the feather degraded, up to 2µm (size) in *Bacillus subtilis* (type II) {(Mode-SE, Width-9.9mn, Magnification- 24000X, High voltage – 10.00KV)}.



(v) SEM- Observation.

1.4. Transmission Electron Microscope: - the thickness of the feather degraded were found to be 200nm (size) in) in *Bacillus subtilis* (type II),a pellet of stationary phase cell from casein broth was fixed in 3% glutaraldehyde in 1XPhosphate buffer. Post fixed in 1% OsO4, dehydrated through a graded series of Ethanol, embedded in an epoxy resin



(vi) TEM - Observation

1.5. Keratinase assay:-5mg of keratin azure was suspended in 1ml of 50m mol/L Tris – HCL buffer (pH 8) was prepared. Reaction mixture contained 1ml keratin azure suspension and 1ml of aspirated diluted enzyme. It's carried out at 50^0C with constant agitation of 200r/min for 1 hr. After that, reaction gets stopped by adding 2mLof 0.4mol/L

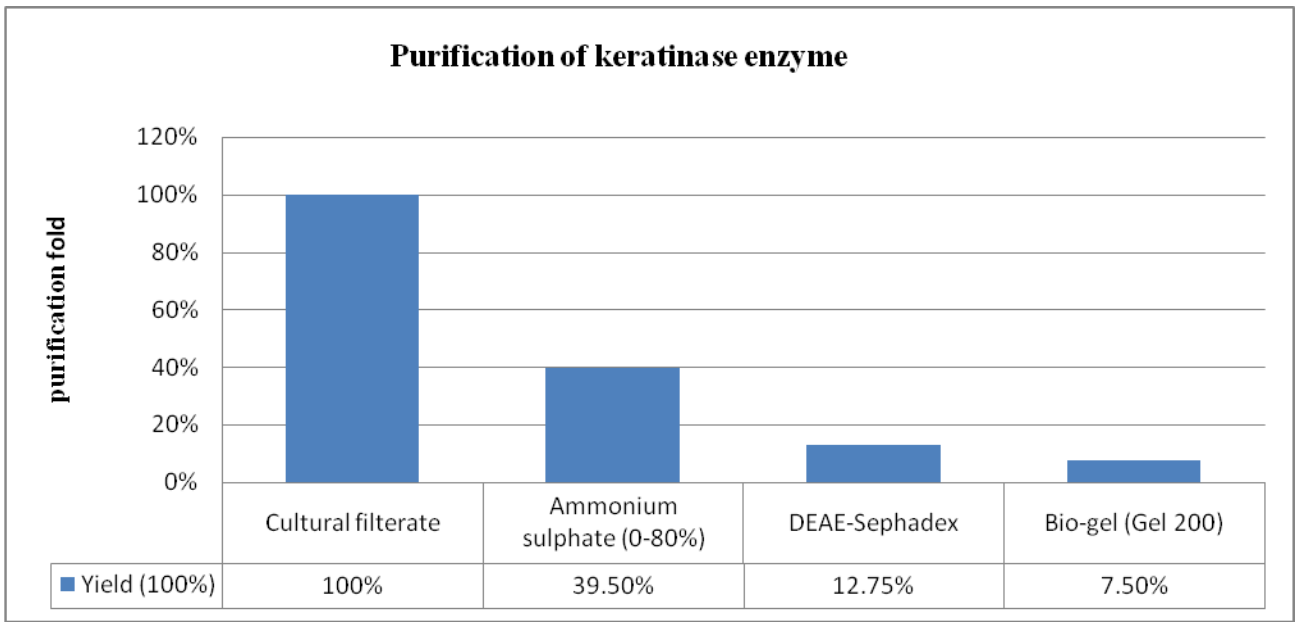
TCA and it was followed by filtration technique in order to remove the substrate. Then, the filterate is observed under spectrophotometer at 595 nm. The *Bacillus subtilis* produce 1500 KU/ml keratinase production, (Table: 2) Keratinase production by bacterial isolates from soil sample in different places.

S.no	Isolates	Keratinase KU/ml
1	CPE-1	0.40
2	CPE-2	0.57
3	CPE-3	1.37
4	CPE-4	3.75
5	CPE-5	2.01
6	CPE-6	6.02
7	CPE-7	10.20
8	CPE-8	2.38
9	CPE-9	3.35
10	CPE-10	15.27
11	CPE-11	1.56
12	CPE-12	2.00

4. Purification of keratinase enzyme:-

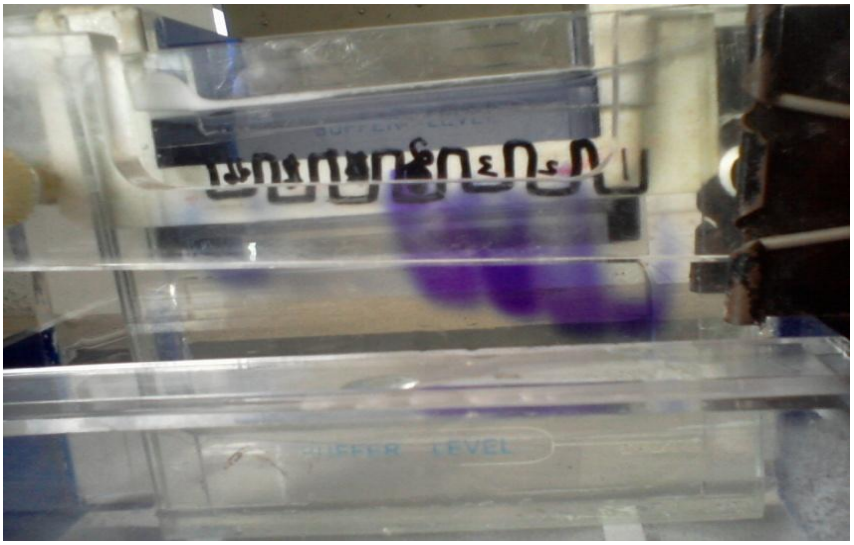
Enzyme was purified by using ammonium sulphate, gel permeation; ion exchange technique. Polyacrlamide gel electrophoresis of native PAGE according by Laemmli (1978), (Table 6) Keratinase purification-using culture supernatant of ***B.subtilis*** by Anionic chromatography and gel permeation method.

S.no	**Purification**	**Total enzyme activity**	**Total protein (mg)**	**Specific activity (U/mg)**	**Purification fold**	**Yield (100%)**
1.	Cultural filterate	4200	60	83.33	1	100%
2.	Ammonium sulphate (0-80%)	1585	23	110	1.30	39.5%
3.	DEAE-Sephadex (K-50)	510	2.4	347	2.80	12.75%
4.	Bio-gel (Gel 200)	306.74	.175	2860	34.30	7.5%



4.1. SDS-PAGE:-

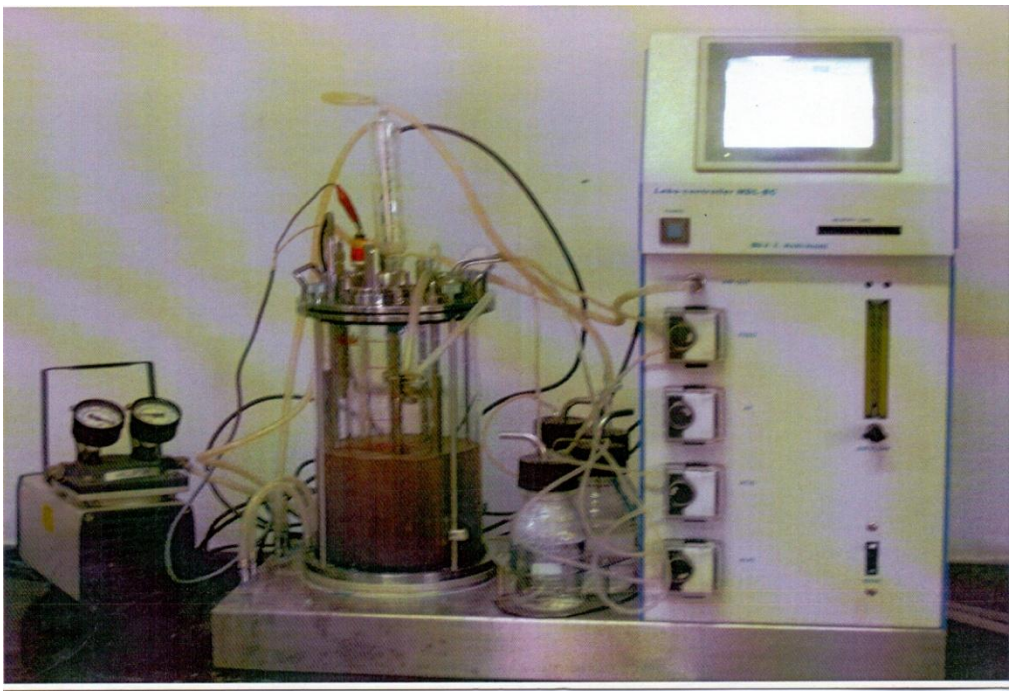
In this, the molecular mass of the protein are compared with marker protein. Specific protein obtained from the ***Bacillus subtilis*** for about 40KDa.



(vii) SDS-PAGE[40 KDa]

5. Large scale production in 5L bioreactor:-

For the production of keratin, both the type of (solid and submerged) fermentation process was carried out in the research work.



(viii) 5 L Bio-reactor

5.1. Solid state fermentation:-

Feather degrading microbes (*Bacillus subtilis)* was grown aerobically to a concentration of approximately 10^7 cell/ml in the chicken feather broth containing 1% raw chicken feather (C.M. Williams *et al*., 1990), (Table :7) shows an solid state fermentation technique.

s.no	Dry weight (gm)	*Bacillus subtilis* (KU/ml)
1	2	650.52
2	4	722.10
3	6	821.35
4	8	1456.10

(viii) Before Inoculation [left]., After Inoculation(Right)

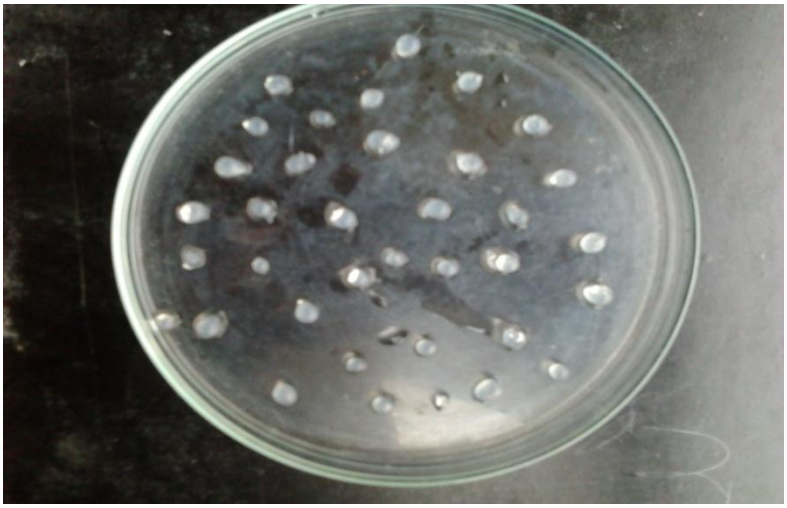
5.2. Submerged fermentation:-

In this, the keratin powder (sterile chicken feather were ground) was taken directly to the fermentation medium (J.Friedrich *et al*., 1990). The powder was sifted through a 0.5mm size. The fermentation was prepared according to Nickerson *et al.,* (1963).The flask was autoclaved for 1 hour 15 minutes at 121^0C. The 1ml of sample (10^6-10^7) *Bacillus subtilis* was taken inoculated into the flask. The flask was shaken at 120 rev/min and 50^0C for up to 1-7 days. The *Bacillus subtilis* shows a highest keratinase assay for about 18000 KU/ml at 48 hours with the specific pH 8 and temperature 50^0C, (Table: 8) shows a submerged fermentation technique.

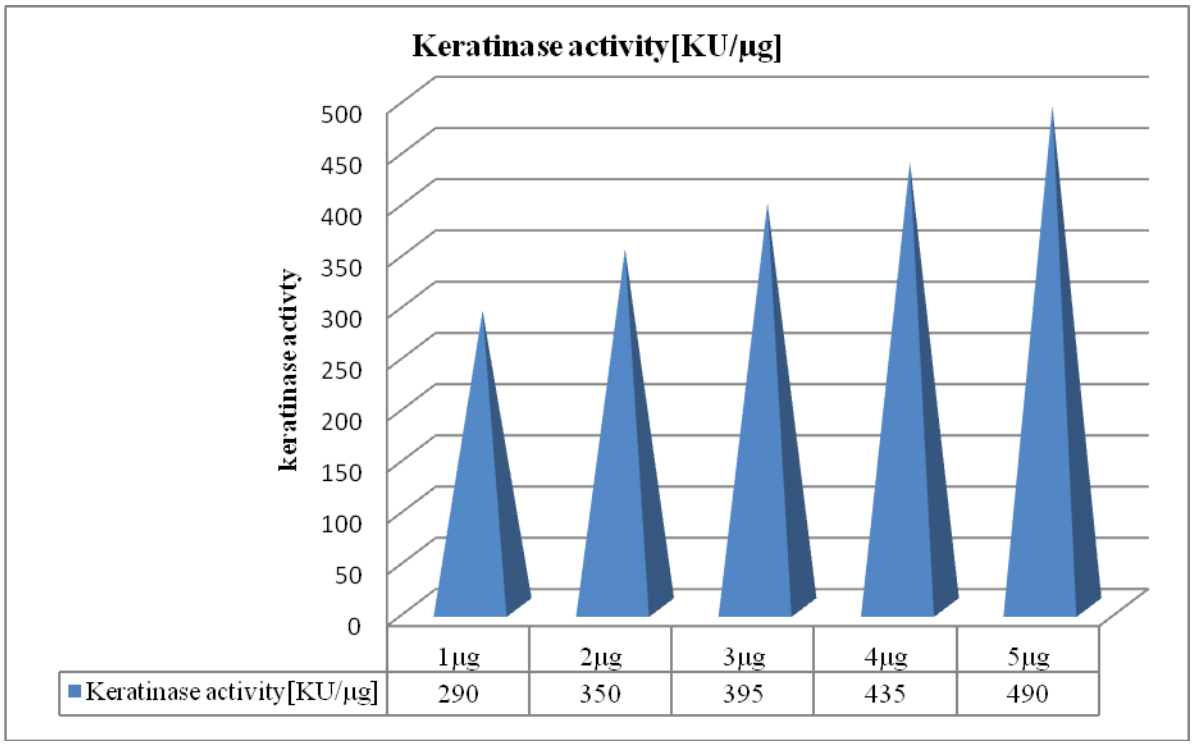
S.no	Sterile Chicken feather Powder (gm)	Keratinase activity (U/ml)
1.	2gm	730
2.	4gm	810
3.	6gm	1410
4.	8gm	1490
5.	10gm	1800

6. Immobilization of keratinase enzyme:-

Immobilization of keratinase in calcium alginate gel was the most favorable, since the percent entrapped activity was maximum in calcium alginate beads 45.77% (Susmita *et al*., 2012). 1 beads having an 1µg of the keratin. These beads undergone with the reaction time for immobilized beads increase from 2 hours to 5 hours. The activity of the calcium alginate entrapped keratinase beads was assayed for three cycle using the substrate. Immobilized beads displayed high level of heat stability and increased tolerance towards acidic pH compared with the free keratinase. These beads stored in 1XPO_4 buffer. These beads retained its 50% of the original enzyme activity after 7 days. These beads can be easily stored, easily filtered from the fermentation taken, maintain activity, in-contaminated for long life.



(ix) Immobilization – keratinase enzyme.



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