

Production, Characterization and Application Of Keratinase Produced By Soil Isolates Using Chicken Feather As Substrate

Submitted in partial fulfillment of the requirements for the award of Bachelor of
Technology degree in Biotechnology

by

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DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING

SATHYABAMA
INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)
Accredited with Grade "A" by NAAC

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of S. SWETHA (37230089) who carried out the project entitled "Production, Characterization and Application Of Keratinase Produced By Soil Isolates Using Chicken Feather As Substrate" under our supervision from November 2020 to March 2021.

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ABSTRACT

There are a lot of feathers that are waste on a daily basis worldwide. These feathers constituent about 90 per cent of keratin. These can be used as a substrate to produce keratinase enzyme from soil isolates instead of wasting and dumping this waste feather and causing environmental pollution. So this study is done based not only to efficiently remove feathers from environmental waste, but also it can be used as different by-products. For production of keratinase, soil isolate was isolated, identified and used for enzyme production. This produced enzyme is isolated and then screening procedures will be done to confirm the enzyme. Later optimization of keratinase enzyme will be carried out with different concentration of feather as substrate. For optimized keratinase growth curve, protein estimation and enzyme assay is carried out. Later characterization of keratinase enzyme is done using HPLC and its application in stain removal in fabrics is observed.

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LIST OF ABBREVIATIONS

VP	Voges Proskauer
M	Molar
HCl	Hydrochloric Acid
NaOH	Sodium Hydroxide
TCA	Trichloroacetic Acid
KH ₂ PO ₄	Potassium Dihydrogen Phosphate
K ₂ HPO ₄	Dipotassium Hydrogen Phosphate
KOH	Potassium Hydroxide
hrs , h	Hours
°C	Degree Celcius
NaCl	Sodium Chloride
rpm	Revolutions Per Minute
mins	Minutes
UV	Ultra-Violet
ml	Millilitre
µl	Microlitre
cm	Centimetre

CHAPTER 1 INTRODUCTION

1.1 MICROBES AND ENZYMES:

Microbes in the soil are one of the most important components of the ecosystem, as they improve soil productivity and thus plant growth. In the soil, there are a variety of microbes that have beneficial, neutral, or pathogenic effects. (Miransari, M., 2013). Microbes have long been and continue to be one of the most important and valuable sources of enzymes. Many industrial processes, such as chemical synthesis for the production of chemicals and pharmaceuticals, have a number of drawbacks, including low catalytic performance, lack of enantiomeric specificity for chiral synthesis, and the need for high temperature, low pH, and high pressure. Furthermore, the use of organic solvents results in the processing of organic waste and contaminants. Enzymes are more useful for these applications because they operate under mild reaction conditions (e.g., temperature, pH, and atmospheric conditions), do not need substrate functional group safety, have a long half-life, and have a high selectivity of yielding (Adrio, J. L., & Demain, A. L., 2014). The majority of industrial enzymes currently in use are hydrolytic in nature and are used to degrade a variety of natural substances. Proteases are still the most common enzymes, they are in widespread use in the detergent and dairy industries. The second most common form of carbohydrase is amylases and cellulases, which are used in industries such as starch, textiles, detergents, and baking. (Gurung, N., et al., 2013). High temperatures and intense pH denature enzymes. They are dependent on pH, ionic strength, temperature and the presence or absence of inhibitors or activators, as well as the effect they have on chemical reactions. (Tabatabai, M. A. 1994).

1.2 WASTE INTO ENZYME PRODUCTION:

Different types of enzymes are produced from different waste products and used wisely such as production of protease from rice mill waste (Paranthaman, R., et al

2009). Amylase production using banana waste as substrate (Krishna, C., & Chandrasekaran, M 1996). Cellulose production using agricultural waste (Milala, M. A., et al 2005). Cellulose not only using agricultural waste but also using fruit waste production is carried out. (Rathnan, R. K et al., 2011). Also, vegetable processing industry produce lot of waste on daily basis these wastes are stored as renewable feed stock for enzyme production. (Sharma, R et al., 2016). Fish waste from the food industries are used for isolation and activation of collagenase. (Daboor, S. M et al., 2012) Similarly keratinase production was also done using different substrate such as wool (Eslahi, N., et al 2013) and chicken feather as substrate since chicken feathers consist of 90% of keratin. (Cheng, S. W.,et al1995).

1.3 KERATIN:

Keratin is one of the most abundant biopolymers in the world, it is a tough, fibrous, insoluble material that functions as an outer coat of human and animal organs, to prevent the loss of body fluids. Keratin is predominantly found in tissues of reptiles, birds, amphibians, and mammals. (Gopinath, S. C., et al 2015). The extraction and use of soluble keratin from feather waste in the creation of new, useful bio-products would be very beneficial because environmental concerns will be minimized. Keratin-based materials are acceptable for medicinal, cosmetic and farming use. (Sinkiewicz, I., et al2017). Keratins are structural proteins that humans, birds, and reptiles make. Keratins serve as a defensive shield or a mechanical support in most cases. Every year, the poultry, meat processing, leather, and wool industries produce millions of tonnes of keratin wastes and low-value co-products. The presence of pathogenic microbes on keratin waste has necessitated effective and immediate keratin waste treatment. In many countries, the vast amount of keratin waste causes a severe solid waste problem. Keratin waste has been related to the spread of odors and pathogens in soil and water. It's difficult to get rid of keratin waste. Keratinases are proteases that can break down keratin and provide a rare hydrolysis opportunity (June Pinheiro De Oliveira Martinez et al, 2020). Poultry feathers are often made up of the protein keratin, which is rich in α -pleated sheets and therefore proteolysis resistant. The

creation of a high-throughput screening method for proteases hydrolyzing disease-related protein aggregates, as well as mechanically resilient keratin-based polymers, is clearly therapeutic and industrial potential for the soluble keratin-based assay. (Hyeon-Su Jin et al, 2007). Feather wastes are pure keratin proteins, which result in large amounts as a byproduct of industrial poultry manufacture. In the absence of common proteolytic enzymes such as trypsin, pepsin and papain, keratin is not degradable. Feather waste is currently used for animal feed on a limited basis as a dietary protein supplement. (Kim, J. M et al 2001). Chicken consumption is growing every day because of technical advancements in food production; chicken feather is meanwhile the by-product of commercial poultry processing plants and chicken slaughtering shops and is piling up. These poultry feathers are normally discarded using land filling or burning ground, causing large soil, water and air pollution. Sahoo, D. K., et al 2012). Because of their crude protein content of more than 85%, chicken feathers, a byproduct of the poultry processing industry, are considered a possible high-quality protein substitute. Despite this, due to a lack of appropriate recycling practices, chicken feathers have been listed as waste (Zheng Peng, et al, 2019). Feathers are composed of over 90% protein and produced in large amounts as a waste by poultry processing worldwide. Accumulation of feathers will lead to environmental pollution and feather protein wastage (Cai, C. G et al., 2008). Chicken feathers have unique structure and properties not found in any natural or synthetic fibers. Poultry feathers is a Cheap and organic protein fiber source. (Reddy, N., & Yang, Y. 2007).

1.4 KERATINASE:

The conversion of these wastes is very important, the waste is converted Effectively and economically for valuable industrial and commercial products and also to reduce the harmful impact of such waste in the environment. (Bharathiraja, S., et al 2017). So, these feathers as used for production of keratinase a type of protease enzyme. A diverse microbial group uses Keratin as a substrate for Production of a specific protease class, i.e. keratinase (EC)3.4.21/24/99.11), has been reported which targets

amino acid-rich hydrophobics, Proteins that are insoluble. (Jana, A., et al 2020). Keratinases (E.C. 3.4.99.11) belong to the group of serine hydrolases that are capable of degrading keratin, The structural protein is fibrous and insoluble, crosslinked extensively to disulfide and hydrophobic linkages. (Anbu, P et al., 2007). These enzymes are also used or could be applied in the food industry, textiles, medicine, cosmetics and leather and poultry processing industry (Farag, A. M et al., 2004). Keratinaceous materials in nature are widely available (e.g., Feather, hair, wool, horn) but only have limited uses. They are insoluble and commonly are not degradable because of a high degree of disulfide linkage, Hydrogen bonding and interactions with hydrophobes by proteolytic enzymes like triypsin, pepsin and papain. (Ignatova, Z., et al 1999). Keratinase constituted of *Streptomyces* Sp. A11 decomposed human hair, hen feather, wool, silk and natural keratin extracted from human epidermis. The enzyme become inducible, extracellular, homogeneous with a molecular weight of 49,000. The enzyme pastime changed into inhibited through decreased glutathione, phenylmethyl sulphonyl fluoride and 2-mercaptoethanol. (RP Mukhopadhyay et al, 1990). Keratinase is a broad-spectrum protease that has the unusual ability to degrade keratin, allowing for the environmentally friendly conversion of keratin wastes to useful amino acids. The ability of a feather-degrading thermophilic bacterium, *Meiothermus taiwanensis* WR-220, to apparently complete feather decay at 65 C in two days was investigated (Wan-Ling Wu, et al, 2017). The performance and cost effectiveness of a wide range of industrial systems and processes were speeded up using keratinase. The keratinolytic proteases, have predominantly been purified from a variety of microorganisms often bacterial species such as *bacillus subtilis*. (Paul, T., et al 2014). In the presence of dimethyl sulfoxide, ethanol, isopropanol, and acetonitrile, keratinase I40 exhibited good stability. Chicken feathers, dove feathers, duck feathers, human hair, fur, and hog bristle all had the highest activity of the enzyme. Seven common proteases observed no change in chicken feathers treated with keratinase I40 after one day (Jen- Min Kuo et al, 2020). *Arthrobacter* sp. KFS-1, previously isolated from a dump site, was used to produce keratinase in the basal medium. The physico-chemical conditions were modified to improve keratinase performance, and the enzyme's biochemical properties were

evaluated (Nonso E Nnolim et al, 2020). A keratinolytic protease interest become secreted by *Kocuria rosea* whilst cultured in bioreactors while the usage of feathers as particular carbon and nitrogen source turned into purified and characterized. (C Bernal et al, 2006). The use of keratinous hydrolysate by keratinase enzymes to produce vitamin B-complex is described. *Bacillus thuringiensis* strain MT1, which was recently isolated from a cattle-yard using donkey hairs, was used to manufacture the keratinase enzyme (Mohamed A Hassan, et al, 2020). The isolated keratinase from the culture supernatant is a monomer with a molecular mass of 28.7 kDa. The enzyme is relatively thermostable, meaning it can work at a variety of temperatures and pH levels. Keratinase can function on a wide range of soluble and insoluble protein substrates, according to specific enzymatic assays (E Lingaa et al, 2008). The enzyme had a molecular weight of 28.5 kDa and was homogeneous, as shown by a single band of protein. The purified keratinase's amino acid profile showed that it was made up of 14 different amino acids (Eman F Shared, Naveen M Khalil, 2011) Keratinases are proteolytic enzymes predominantly energetic while keratin substrates are to be had that assault disulfide bridges within side the keratin to transform them from complicated to simplified forms. Keratinase activity may well be detected by unleash of amino acids from solubilized Keratin and by creation of zones of lysis in gel diffusion assay. (Wawrzkievicz, K., et al 1987)

1.5 APPLICATION:

Enzymes are life-threatening protein molecules. Proteases are the only form of enzymes that play a major role in almost all organisms' metabolism. The key interest from biotechnological perspectives are extracellular proteases produced by bacillus and cocci species. They are used in detergents, food, tannery, drug and leather industries. Keratinase (EC 3.4.25) belongs to hydrolase which is more effective than other protease in the hydrolysis of insoluble keratins. (Raju, E. V. N., & Divakar, G. 2013). Keratinases are crucial in guidance of animal nutrients, protein supplements, leather-based manufacture, fabric processing, detergent formulation, feather meal processing for feed and fertilizer, the pharmaceutical and biomedical industries, and waste management. (Subhash CB Gopinath et al, 2015.). Keratinase has various

application it can be used for converting feather into feed in feed industry, it is used in de-hairing goat hair in leather industry and also it is used in clearing obstruction in sewage system during waste water treatment (Vigneshwaran, C., et al 2010). Recently keratinase is also used in different stain removal like blood, chocolate etc in addition to detergent (Reddy, M. R., et al 2017). Agricultural use of animal wastes, including poultry waste, has been investigated. Several million tonnes of turkey and chicken feather are produced by-products for the poultry processing industry. This makes it of interest to animal nutritionists to recycling poultry waste. Feather food is currently produced by high temperature and pressure treatment of feather. This method is energy-intensive and contributes to the loss of essential amino acids that are heat sensitive. In the treatment of keratin waste such as feather and hair keratinase from these micro-organisms may be used, which increases feather food's nutritional consistency. (Ramnani, P., & Gupta, R. 2004). The scope of this work is to produce keratinase which can be used to remove stain from the cloth.

CHAPTER 2

LITERATURE SURVEY

Feathers are the most complex and ill waste by-product of poultry slaughter. Poultry farms produce millions of tons of feather biomass per year, causing solid waste management issues as well as human diseases like chlorosis and fowl cholera. Keratin accounts for about 90% of the overall nutrient content of the feather. The extraction of keratin with sodium sulfite can be a simple and cost-effective method that yields a good yield while maintaining the protein's secondary structure. (Alahyaribeik, S., & Ullah, A. 2020).

Several categories of microbial organisms have been noted, to produce keratinase, from keratin (e.g., fungal species (*Doratomyces microsporos*, *Alternaria radicina*, *Aspergillus* sp., *Rhizomucor* sp. etc.) and several bacteria (*Pseudomonas aeruginosa*, *Microbacterio* sp., *Bacillus licheniformis* and *B. pumilus*). Abdel-Fattah, A. M., El-Gamal, M. S., Ismail, S. A., (Emran, M. A., & Hashem, A. M. 2018).

The use of keratinases to degrade poultry waste is an environmentally friendly biotechnological process that transforms this abundant waste into low-cost, nutrient-rich animal feed. Keratinolytic enzymes are used in two enzyme systems. Investigate the detergent, medical, cosmetic, and leather industries; they can also be used to degrade prion proteins and as pesticides. The development of keratinases and peptidases by three *Bacillus* species isolated from poultry waste was investigated in the current report. Since keratinase production is typically induced by the existence of keratinous substrates, the study's main objective was to compare the effects of feather and feather meal on keratin production. (Mazotto, A. M., et al., 2011)

In addition to destructing several amino acids, the traditional methods (chemical and Steam Pressure) used in feather quenching require a great deal of energy. In former

times, feather's economic degradation is great for many people who search for it. Microbial feather degradation was investigated using *Bacillus licheniformis*. (Emran, M. A et al., 2020)

Cultivation conditions are critical for good enzyme development, and optimizing parameters like pH, temperature, and media composition is critical for creating the best fermentation conditions. Various nitrogen sources and concentrations, such as raw feather, feather meal, powdered chicken nails, bovine fur, and wool, affect keratinase development. (Sivakumar, T et al., 2012)

Microorganisms that produce keratinase have a significant industrial application in fermentation technology. The conversion of non-soluble keratin (feather) into soluble protein or polypeptide is aided by submerged fermentation of poultry waste by microorganisms producing keratinase. These protein byproducts may be used as animal and livestock feed, as well as fillers in leather. Keratinase is also being used as a detergent to eliminate stains on cloth, rather than sodium sulphides, in the dehairing process in the leather industry. Deivasigamani, B., & Alagappan, K. M. (2008).

It has also been stated that *Actinobacterium actinoalloteichus* MA-32 produces keratinase, which is used in detergent formulation. It's worth remembering that keratinase-containing detergents are effective at removing blood, paint, and dye stains from fabrics. (Sharma, S., & Gupta, A. 2016)

CHAPTER 3

AIM AND OBJECTIVES

3.1 AIM:

The aim of the study is to produce keratinase for stain removal in industries using waste chicken feathers as substrate.

3.2 OBJECTIVE:

1. Collection soil sample .
2. Isolation of feather degrading microbe in soil.
3. Production of keratin using chicken feathers
4. Partial Purification of keratin
5. Keratinase enzyme production
6. Partial Purification of keratinase
7. Keratinase characterisation and optimization
8. Application in stain removal.

CHAPTER 4

MATERIALS AND METHODS

4.1 MATERIALS:

- Soil sample
- Chicken feathers
- Sodium- bi sulphite
- Sodium hydroxide
- Beaker
- Pipette
- Conical flask
- Test tubes
- Ammonium sulphate
- Dialysis bag
- Phosphate buffer
- Distilled water
- Alkaline copper sulphate solution
- Folin's phenol
- Feather medium
- Nutrient broth
- Crystal violet
- Gram's iodine
- 95% ethyl alcohol
- safranin
- Skim milk
- Surf excel
- Gauss cloth with stain
- Glucose phosphate broth
- Tryptone broth
- Kovac's reagent

- Methyl red
- VP reagent A (5% solution of alpha naphthol in absolute alcohol)
- VP reagent B (40% KOH containing 0.3% creatine) VP solution B
- Simmon citrate agar
- Sulfanilic acid (Nitrate reagent A)
- alpha-naphthylamine (Nitrate reagent B)
- 5% iodine solution
- Carbohydrate fermentation medium
- Durham's tube
- Starch agar
- Gelatine broth
- Urease agar
- Nitrate medium
- 3% hydrogen peroxide
- 0.1M tris-HCl buffer
- Phosphate buffer
- Hcl
- NaOH
- TCA
- oxidase disc
- Alkaline copper reagent:

50ml of solution A is mixed with 1ml of solution B to make alkaline copper reagent.

 - Solution A: 1g of sodium carbonate is + 0.4g of sodium hydroxide and made to 50ml.
 - Solution B: 0.125g sodium potassium tartarate + 0.06g of CuSO_4 and made to 25ml.
- Folin's phenol: 1:2 ratio (phenol reagent: distilled water)

Table 4.1: Tryptone broth

Medium	Concentration (g /ml)
tryptone	10g
Distilled water	1000ml

Table 4.2: Glucose phosphate broth

Medium	Concentration (g /ml)
Peptone	5g
Glucose	5g
K ₂ HPO ₄	5g
Distilled water	1000ml

Table 4.3: Simmon citrate agar

Medium	Concentration (g /ml)
Magnesium sulphate	0.2g
KH ₂ PO ₄	1g
K ₂ HPO ₄	1g
Sodium Citrate	2g
Bromothymol blue	0.08g
Agar	15g
Distilled water	1000ml

Table 4.4: Nitrate medium

Medium	Concentration (g /ml)
Peptone	5g
Meat extract	3g

Potassium nitrate	1g
Distilled water	1000ml

Table 4.5: Nutrient broth

Medium	Concentration (g /ml)
Peptone	5g
Yeast extract	3g
NaCl	5g
Distilled water	1000ml

Table 4.6: Carbohydrate fermentation

Medium	Concentration (g /ml)
Peptone	10g
Beef extract	1g
Phenol red	0.018 g
NaCl	5g
Carbon source	10g
Distilled water	1000ml

Table 4.7: Starch agar

Medium	Concentration (g /ml)
Peptone	5g
Yeast extract	1.5g
Beef extract	1.5g
NaCl	5g
Starch	2g
Agar	28g
Distilled water	1000ml

Table 4.8: Gelatin broth

Medium	Concentration (g /ml)
Peptone	5g
Beef extract	1g
Yeast extract	2g
NaCl	5g
Gelatin	8g
Distilled water	1000ml

Table 4.9: Skim milk

Medium	Concentration (g /ml)
Tryptone	5g
Yeast extract	2.5g
Skim milk powder	28g
Glucose	1g
Agar	20g
Distilled water	1000ml

Table 4.10: Feather medium:

Medium	Concentration (g /ml)
Chicken feathers	5g
NaCl	0.5g
MgSO ₄ .7H ₂ O	0.1g
KH ₂ PO ₄	0.7g
K ₂ HPO ₄	1.4g
Distilled water	1000ml

4.2 METHODS:

4.2.1 BACTERIAL ISOLATION AND SCREENING:

4.2.1.1 Collection of soil sample:

Soil sample was collected from roadside of poultry farm in Madhavaram, Chennai.

4.2.1.2 Serial dilution of soil sample:

1g of soil sample was weighed and then mixed in 9ml of sterile distilled water. Here the dilution is 10^{-1} and this has to mixed properly. Using a sterile pipette 1ml from 10^{-1} dilution was transferred to next test tube which contains 9ml sterile distilled water now this is 10^{-2} dilution. Subsequent dilutions made up to 10^{-8} . Before this nutrient agar was prepared as in Table 4.5 and poured in to petri plate for solidifying. Once this both is done, now 0.1ml of last four dilutions was transferred over the solidified agar and spreader using "L" rod. This is repeated for the other dilutions. The plate was incubated for 24hrs at 37°C in inverted position tapped with masking tape.

4.2.1.3 Biochemical test:

The colony was selected from the plate and then screening was done to find the isolated colony.

4.2.1.3.1 Gram's staining:

With help of inoculating loop bacteria colony was taken and placed in center of clean slide in which a drop of water is kept for proper smear. Now show the slide in flame to fix the smear. Now the fixed smear was covered with crystal violet for 1 minute. Then the stain was rapidly washed off with distilled water. Again, the smear was covered with gram's iodine solution for 1 minute. Using the distilled water, the iodine was washed off. Now the smear was decolorized rapidly with 95% ethyl alcohol and washed immediately with distilled water. Then the smear was again covered with safranin for 1 minute. This stain was washed off with distilled water and the slide was allowed to air dry and was viewed under microscope for identification.

4.2.1.3.2 Indole production test:

Initially tryptone broth was prepared as mentioned in table 4.1 and it was transferred in to two test tubes one serving as control. Using sterile technique, a loop full of

organism was inoculated into the test tube. The test tube was incubated for 48hrs at 37°C. then Kovac's reagent was added and gently shaken and result was observed.

4.2.1.3.3 *Methy red:*

Glucose phosphate broth was prepared as mentioned in table 4.2 and it was transferred in to two test tubes one serving as control. Using sterile technique, a loop full of organism was inoculated into the test tube. The test tube was incubated for 48hrs at 37°C. Then 0.5ml of Methyl Red solution was added and gently shaken and results was observed immediately.

4.2.1.3.4 *Voges-proskauer:*

Glucose phosphate broth was prepared as mentioned in table 4.2 and it was transferred in to two test tubes one serving as control. Using sterile technique, a loop full of organism was inoculated into the test tube. The test tube was incubated for 48hrs at 37°C. Then 0.5ml of reagent A (5% solution of alpha naphthol in absolute alcohol) and then 0.5ml of reagent B(40% KOH containing 0.3% creatine) was added and gently shaken and results was observed.

4.2.1.3.5 *Citrate utilization test:*

Simmon citrate agar was prepared using the table 4.3 mentioned above and transferred in to two test tubes one serving as control. Using sterile technique, a loop full of organism was inoculated into the test tube. The test tube was incubated for 48hrs at 37°C. then the growth and color change was observed.

4.2.1.3.6 *Catalase test:*

The organism was transferred into a clean glass slide using sterile technique, then three to four drops of 3% hydrogen peroxide was allowed to overflow the entire surface of the culture and formation of bubbling was observed.

4.2.1.3.7 *Oxidase test:*

The oxidase disc was placed in the clean slide and then using sterile technique, the

organism was transferred on to the disc and then color change was observed.

4.2.1.3.8 Nitrate reduction test:

Nitrate broth was prepared using the table 4.4 mentioned above and transferred in to two test tubes one serving as control. Using sterile technique, a loop full of organism was inoculated into the test tube. The test tube was incubated for 48hrs at 37°C. Then the nitrate reagent A and nitrate reagent B was added and color change was observed.

4.2.1.3.9 Gelatin hydrolysis test:

Gelatin broth was prepared using the table 4.8 mentioned above and transferred in to two test tubes one serving as control. Using sterile technique, a loop full of organism was inoculated into the test tube. The test tube was incubated for 24hrs in refrigerator. Then the solidified medium was observed.

4.2.1.3.10 Carbohydrate fermentation test:

Carbohydrate broth was prepared using the table 4.6 mentioned above with different carbohydrate source like (Lactose, maltose, mannitol, glucose) and transferred in to two test tubes one serving as control. And Durham's tube was transferred to the test tube to analyze the gas formation. Using sterile technique, a loop full of organism was inoculated into the test tube. The test tube was incubated for 48hrs at 37°C. then the color change and was observed and checked if gas formation is present.

4.2.1.3.11 Starch utilization test:

Starch agar was prepared as mentioned in table 4.7 and it was transferred in to petri plate and allowed to solidify. Using sterile technique, a loop full of organism was streaked into the petri plate. The petri plate was incubated for 48hrs at 37°C. Then 20ml of 5% iodine solution was added and starch utilization was observed.

4.2.1.3.12 Growth in 7% NaCl:

7% NaCl agar was prepared and it was transferred in to petri plate and allowed to

solidify. Using sterile technique, a loop full of organism was streaked into the petri plate. The petri plate was incubated for 48hrs at 37°C. Then the growth of organism was observed.

4.2.2 PRODUCTION OF KERATIN:

4.2.2.1 Collection and processing of substrate:

Collection of chicken feather from ayanavarm, Chennai chicken shop. These feathers were first washed with tap water and removed from blood and dirt. Once the feathers are free from everything it was washed again twice with distilled water. Once the feathers are washed it was dried under the sun and in hot air oven at 60°C for 48hrs. To remove the fatty material from feather, the feathers were immersed in chloroform and kept in rotatory shaker overnight. Next day these feathers were removed from chloroform and kept in hot air oven at 60°C for 48hrs to dry the feather completely. Now these feathers are grinding and stored in a plastic bag free from dust. (Sinkiewicz, I., et al 2017).

4.2.2.2 Extraction of keratin:

In the extraction process defatted feathers were weighed and 1g was put in 50 mL of aqueous solutions containing 0.5 M sodium bisulfite, 8 M urea, 0.5 M SDS and the mixture was shaken at 50 C for 2 h with a magnetic stir bar to prevent aggregation of the feathers during the reaction. After the processes, the resulting mixture was centrifuged at 9000 rpm for 15 min to separate the insoluble material, and the supernatant was filtered through a folded filter. (Sinkiewicz, I., et al 2017).

4.2.2.3 Partial purification of keratin:

The filtrate obtained with various reducing agents was purified by adding ammonium sulphate at 30% and then it was centrifuged at 6000 rpm for 15mins. And then the pellet is dissolved in phosphate buffer (pH-6.8) and dialyzed in phosphate buffer (pH-6.8) using dialysis membranes for 72 h changing the outer buffer every day. The keratin is then taken out from the bag and stored in refrigerator.

4.2.2.5 Protein estimation of keratin:

Protein estimation of the keratin was carried out by Lowry's method (Lowry, O. H.; et al 1951). The sample was taken in different volume such as 100 µl, 150 µl and 200 µl. And distilled water 400µl, 350µl, 300µl and 500µl(serving as blank) and then 2ml of alkaline copper reagent was added and then 0.2ml of folin's phenol was added after 10mins. Now this mixture is kept undisturbed for 30mins and then the optical density value is taken in UV spectrophotometer at 540nm, and then the concentration was found from standard.

4.2.3 KERATINASE PRODUCTION:

4.2.3.1 Extraction of keratinase:

Feather medium was prepared as mentioned in table 4.10 and pH was 7.0. (Fakhfakh, N., et al 2009). Now the bacteria which was isolated from soil was inoculated into the feather medium. Now the bacteria start utilizing the minerals present and breaks down the chicken feather and production of keratinase takes place. This enzyme is taken and centrifuged at 9000rpm and the supernatant is collected.

4.2.3.2 Partial purification of keratinase:

The filtrate obtained was purified by adding ammonium sulphate at 30% and then it was centrifuged at 5000 rpm for 15mins. And then the pellet is dissolved in 0.1M tris-HCl buffer (pH- 8) and dialyzed in 0.1M tris-HCl buffer (pH-8) using dialysis membranes for 72 h changing the outer buffer every day. The keratinase is then taken out from the bag and stored in refrigerator. (Syed, D. G., et al 2009).

4.2.3.3 Skim milk plate assay:

To check the presence of keratinase skim milk plate assay was done. Skim milk was prepared as mentioned in table 4.9. Now the skim milk was transferred into sterilized petri plate and allowed it to solidify. Once the plate is solidified a well is cut in center

of the plate and with help of pipette 50µl of keratin was transferred into the well and diffusion was observed but a clear zone around the well.

4.2.3.4 Protein estimation of keratinase:

Protein estimation of the keratin was carried out by Lowry's method (Lowry, O. H.; *et al* 1951). The sample was taken in different volume such as 250µl and 500µl. And distilled water 750µl, 500µl and 1000µl (serving as blank) and then 2ml of alkaline copper reagent was added and then 0.2ml of folin's phenol was added after 10mins. Now this mixture is kept undisturbed for 30mins and then the optical density value is taken in UV spectrophotometer at 540nm, and then the concentration was found from standard.

4.2.3.5 Optimization of keratinase with different concentration of substrate:

To optimize the keratinase production different concentration of chicken feather as substrate is given in feather medium and production is done. For this 0.5% and 1% feather as concentration is prepared and inoculated with the isolated bacterial and production is done as already mentioned. Once the production is over, different parameters were checked for the keratinase in different interval of time to note the increase and stability of the enzyme. The different parameters that were checked was the growth rate, protein estimation using Lowry's method and keratinase enzyme assay.

4.2.3.5.1 Growth rate of keratinase:

The growth rate of the enzyme for two different concentration 0.5% and 1% was checked on regular basis say 1st, 2nd, 3rd, 4th, 5th, 6th, 7th and 8th day. This was checked by taking absorbance in UV- spectrophotometer at 560nm. To compare basal salt medium without feather was prepared and similar growth rate was determined.

4.2.3.5.2 Protein estimation of keratinase:

As mentioned already protein estimation of keratinase was done using Lowry's

method. (Lowry, O. H.; et al 1951). This is done for both the concentration of feather medium and compared. Here blank is 1500 μ l of water and the keratinase sample was taken in two different volume (100 μ l, 200 μ l) and this is made up to 500 μ l using distilled water in volume 400 μ l and 300 μ l and taking 500 μ l of distilled water has blank. Now 2ml of alkaline copper reagent is added and allowed to rest for 10mins and then 0.2ml folin's phenol is added and the mixture is shaken well and 30mins of incubation time is given and left undisturbed then, the absorbance is measured using UV-spectrophotometer at 660nm and then the values are converted to 1ml and using BSA standard graph the concentration of protein is calculated and graph is plotted. To compare basal salt medium without feather was prepared and similar protein estimation was done and concentration was found and graph was plotted for all the three concentration.

4.2.3.5.3 Keratinase Enzyme assay:

A constant volume of substrate is taken and then different volume of enzyme is added to the substrate and allowed for incubation. Here the substrate is keratin so 300 μ l of keratin is taken in two different test tube and then 200 μ l and 400 μ l of keratinase is added to the test tubes. This is done for 0.5% and 1% feather medium and the results were compared with basal salt medium without feather which serves as control. This substrate and enzyme is allowed to incubate for 30mins and then the reaction is stopped by adding TCA to the mixture. This mixture is centrifuged and then lowry's estimation is done to the supernatant by taking 200 μ l and 400 μ l of sample and then making up to 4500 μ l by adding 4300 μ l and 4100 μ l distilled water and then for blank 4500 μ l of distilled water is taken. Now to these 2ml of alkaline cooper reagent and then after 10mins 0.2ml of folin's phenol is added and allowed for 30mins of incubation and absorption was measured at 660nm. (Cheng, S. W., et al 1995) then using tyrosine as standard the concentration of the enzyme was found and graph was plotted and then enzyme activity is calculated using the formula:

Enzyme (units/ml) = μ mol of sample released * total volume of assay in milliliters / (Volume (in milliliters) used in Colorimetric Determination * volume of enzyme used for assay* time (in minutes) of assay. (Assay, U. P. A. Casein as a Substrate).

Here,

μmol of sample released = concentration of the keratinase (found using tyrosine standard);

Total volume of assay in milliliters = 4.5ml ;

Volume (in milliliters) used in Colorimetric Determination = 2ml ;

Time (in minutes) of assay = 30min.

1 unit of enzyme is defined as liberation of 1 mg of tyrosine equivalent of substrate per ml of enzyme in specific time, so here from the calculation the value will be in $\mu\text{g/ml}$ as per standard so to convert them to units that is mg/ml, it should be divided by molecular weight of tyrosine (181.19 g/mol). The the enzyme activity in units can be calculatd.

4.2.3.6 HPLC:

After optimization of keratinase and checking for different parameters, to check the purity of keratinase HPLC is carried out using C18 column and the mobile phase as acetonitrile: water in the ratio 90:10, this is carried out in the flow rate 0.5ml/min and the protein was detected at 280nm using UV detector. (Rajput, R., et al 2010)

4.2.4 APPLICATION:

Keratinase has various types of application, keratinase is used in many industries, here keratinase is used for effective removal of stain from cloth. When the cloth is stained with chocolate and blood, it is very hard to remove the stain from the cloth but using keratinase it can be removed. Here gauss cloth of square shape of 4*4 cm is cut and stained with blood and chocolate in four pieces. Now the stain is tried to remove by tap water, only detergent (surf excel), only enzyme (keratinase), both enzyme + keratinase. The solid detergent of 0.7% is weighed and dissolved in tap water. The stained cloth is placed in petri plate and the mentioned 10ml of solutions are added to the piece of cloth, for the enzyme+ detergent it is added is ratio 1:1. This is kept in heat at 50°C for 30mis and the results were observed. (Paul, T., et al 2014)

CHAPTER 5

RESULTS AND DISCUSSION

5.1 BACTERIAL ISOLATION AND SCREENING:

5.1.1 SERIAL DILUTION OF SOIL SAMPLE:

The soil sample after serial dilution a colony was selected from the plate.

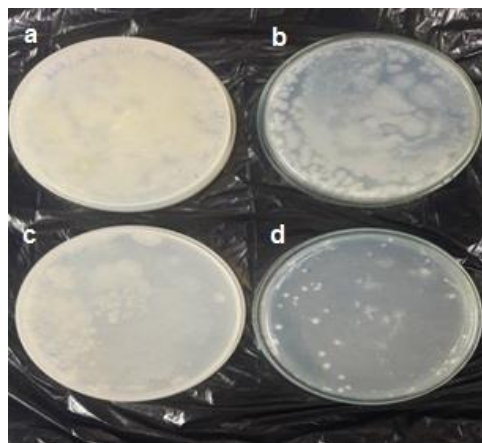


Fig 5.1.1 serial dilution of soil sample

a) 10^{-5} dilution b) 10^{-6} dilution c) 10^{-7} dilution d) 10^{-8} dilution

5.1.2 BIOCHEMICAL TEST FOR CHARACTERIZATION:

For the isolated bacteria different biochemical test was carried out to find out the bacteria.

5.1.2.1 GRAM'S STAINING:

Gram staining was done and it was observed as gram positive and rod shaped, so from this this is found that it is bacillus species due to aerobic and spore forming of bacteria. Later different test was done to find the species.

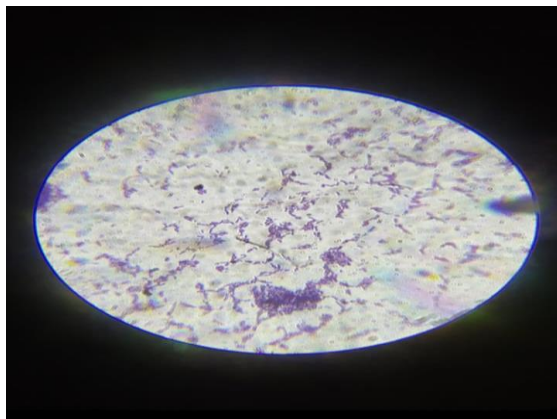


Fig 5.1.2.1 gram staining

5.1.2.2 INDOLE PRODUCTION TEST:

Indole production test is observed to be negative here since there is no formation of red ring after addition of Kovac's reagent to the inoculated organism.

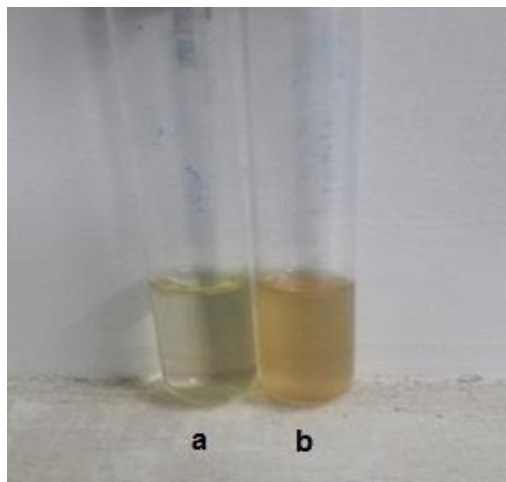


Fig 5.1.2.2 Indole production test

a) Control b) Test sample

5.1.2.3 Methyl red:

Methyl red test is observed to be positive here since there is formation of red color after addition of methyl red reagent to the inoculated organism.

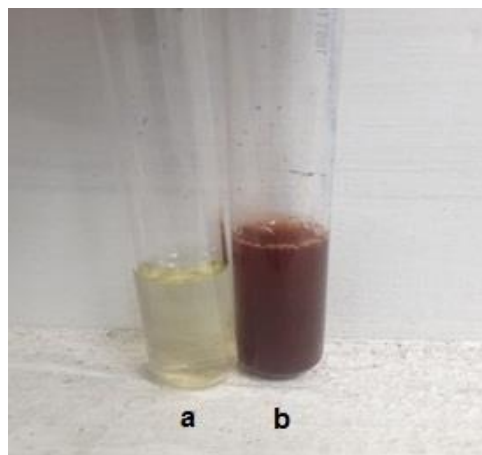


Fig 5.1.2.4 Methyl red test
a) Control b) Test sample

5.1.2.4 Voges-phosphate test:

VP test is observed to be positive here since there is formation of red color after addition of VP reagent “A” and VP reagent “B” to the inoculated organism.

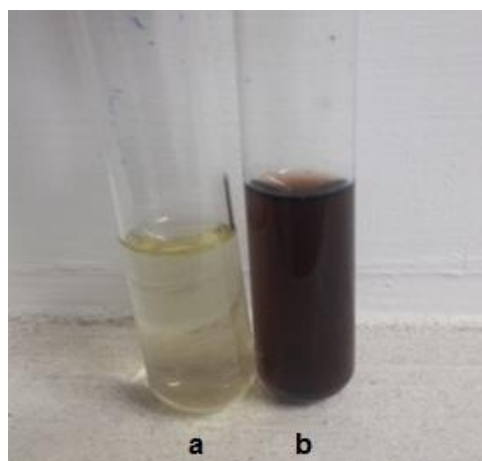


Fig 5.1.2.5 Voges-phosphate test
a) Control b) Test sample

5.1.2.5 CITRATE UTILIZATION TEST:

Citrate utilization test is observed to be positive here since there is change of color

from green color to blue color after the growth of bacteria in the medium.

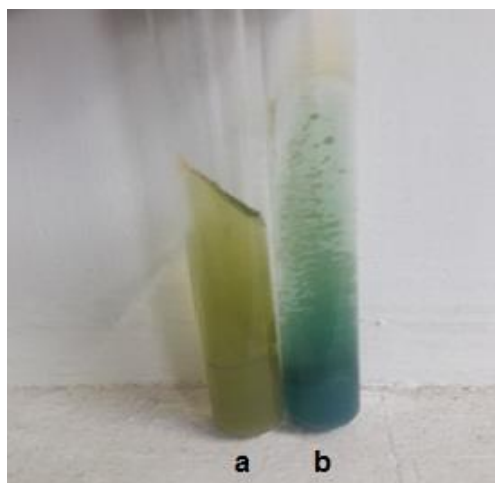


Fig 5.1.2.5 Citrate utilization test
a) Control b) Test sample

5.1.2.6 NITRATE REDUCTION TEST:

Nitrate reduction test is observed to be positive here since there is formation of red color after addition nitrate reagent “A” and nitrate reagent “B” to the inoculated organism.

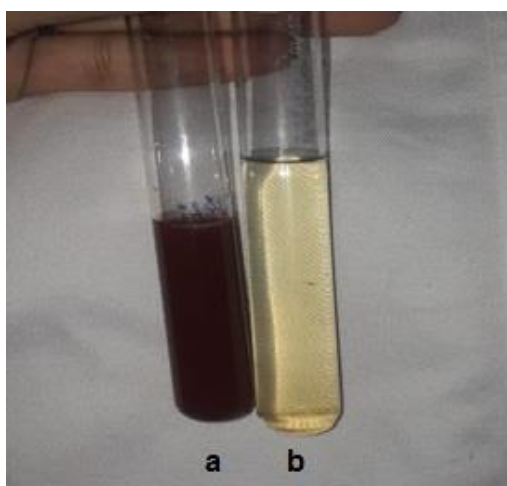


Fig 5.1.2.6 Nitrate reduction test
a) Control b) Test sample

5.1.2.7 OXIDASE TEST:

When the inoculum is kept on the oxidase disc there is formation of blue color, this indicates it is positive.



Fig 5.1.2.7 Oxidase test

5.1.2.8 CATALASE TEST

Here when the 3% hydrogen peroxide is added to the inoculum there is formation of bubbles which indicates it is positive reaction.

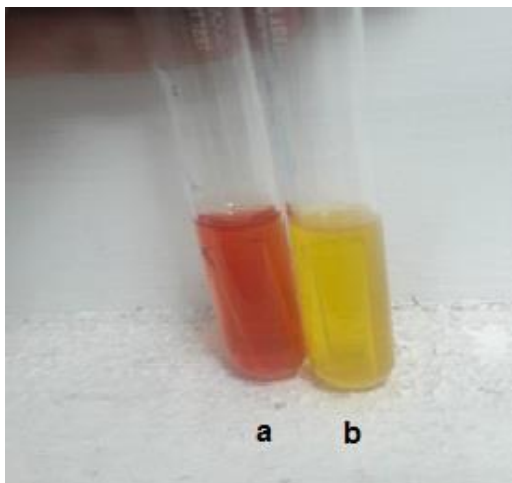


Fig 5.1.2.8 Catalase test

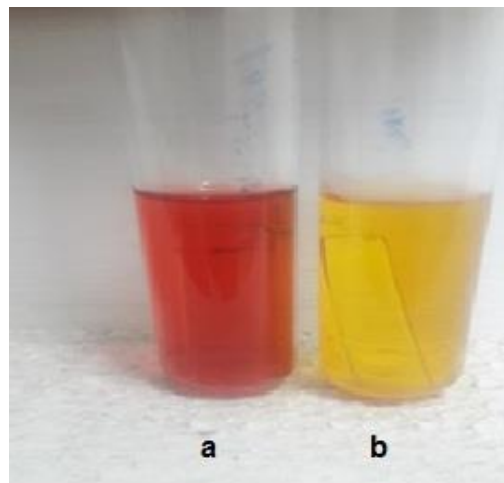
5.1.2.9 CARBOHYDRATE FERMENTATION TEST:

Here the bacteria is tested for four different carbohydrate source such as glucose, lactose, mannitol and maltose. All the four sugar had shown positive result by change

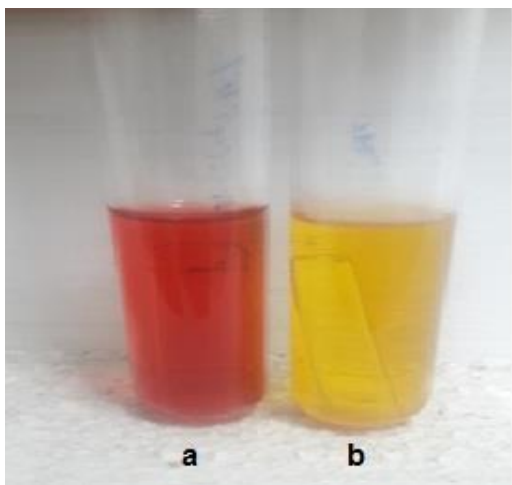
of color from red color to yellow color, also there is no formation of gas in Durham's tube in all the four sugar.



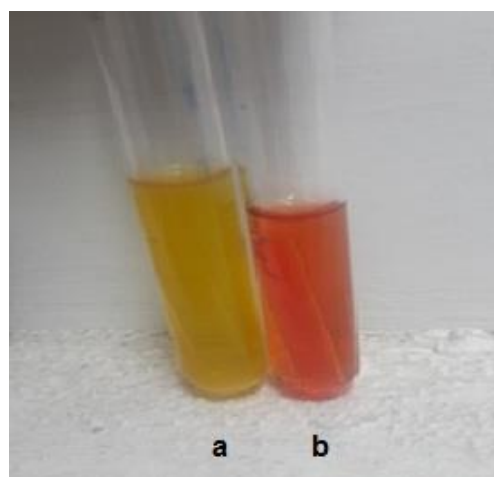
***Fig 5.1.2.9.1 Glucose test
a) Control b) Test sample***



***Fig 5.1.2.9.2 Lactose test
a) Control b) Test sample***



***Fig 5.1.2.9.3 Mannitol test
a) Control b) Test sample***



***Fig 5.1.2.9.4 Maltose test
a) Test sample b) Control***

5.1.2.10 GELATIN HYDROLYSIS TEST:

Here when the inoculated tube was taken from the fridge and the result was observed the tube inoculated with gelatin did not get solidified but the control tube was solidified this showed the bacteria had hydrolyzed the gelatin and it is positive reaction.

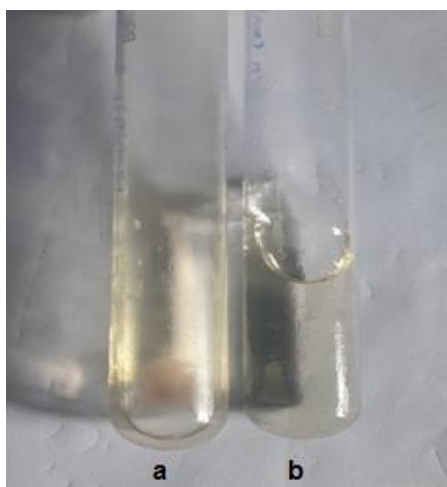


Fig 5.1.2.10 Gelatin hydrolysis test
a) Test sample b) Control

5.1.2.11 STARCH UTILIZATION TEST:

Here after the growth of bacteria iodine was poured and left for 10mins and then it was rinsed off with water, the clear zone was seen around the bacteria which shows the bacteria has hydrolyses the starch and it is a positive result.

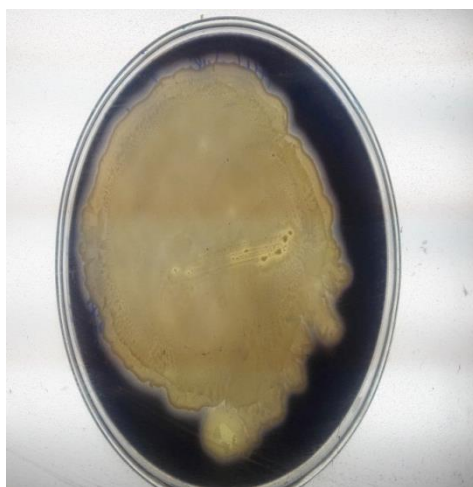


Fig 5.1.2.11 Starch utilization test

5.1.2.12 GROWTH IN 7% NaCl:

In this media the organism has grown and shows positive result.

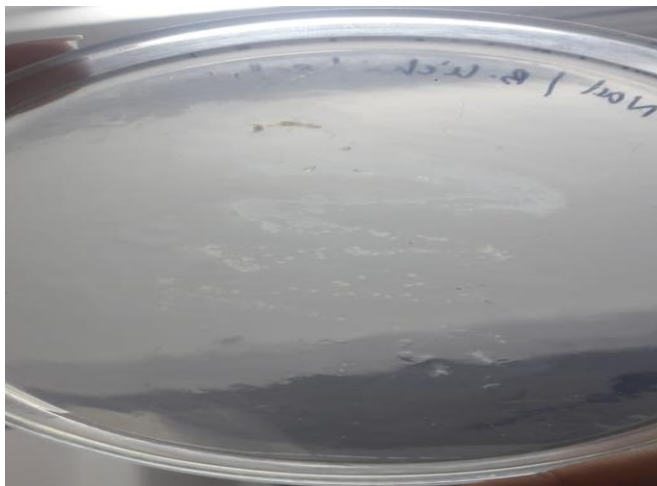


Fig 5.1.2.12 Growth in 7% NaCl

Table 5.1 : Result of characterization of bacterial isolate

Test for characterization	Result
Gram's staining	+ (rod-shaped)
Indole production	-
Methyl red	+
Voges- Proskauer	+
Citrate utilization test	+
Nitrate reduction test	+
oxidase	+
catalase	+
carbohydrate fermentation test	

▪ Glucose ▪ Lactose ▪ Mannitol ▪ maltose	+ + + +
Starch utilization test	+
Gelatin hydrolysis	+
Growth in 7% NaCl	+

From all the results of biochemical characterization the isolated bacteria was found to *Bacillus licheniformis*. (Berkeley, R. C. W., et al 1984) (Santini, F., et al 1995).

5.2 PROTEIN ESTIMATION OF KERATIN:

Protein estimation of keratin using Lowry's method was done and found that there was increase in concentration when increase in volume of keratin, this verified the presence of protein (keratin)

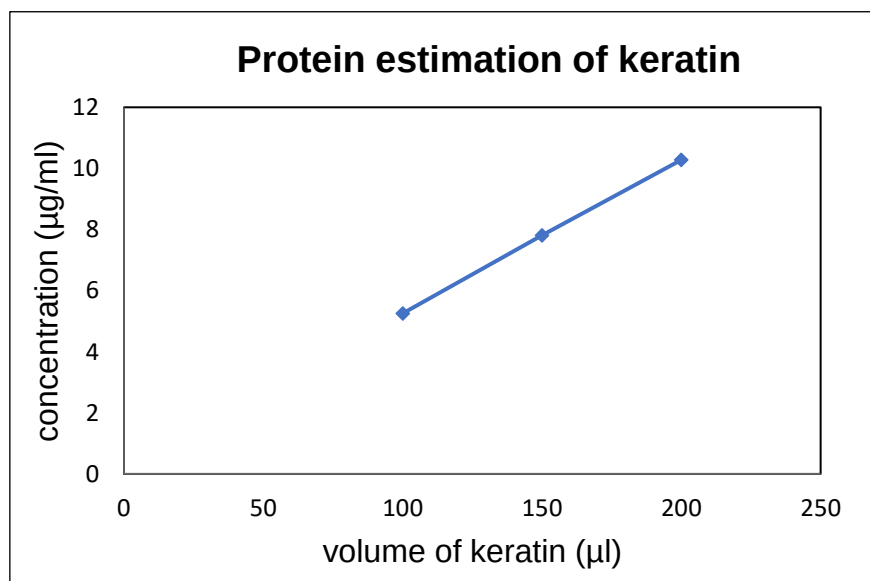


Fig 5.2 Protein estimation of keratin in graph

5.3 KERATINASE SCREENING:

5.3.1 SKIM MILK PLATE ASSAY FOR KERATINASE:

Skim milk plate assay was carried to check the zone and to check if protease is present in the sample and is capable of hydrolysis casein. This was carried out since keratinase is type of protease enzyme. There was zone formation of 1cm in diameter around the well.



Fig 5.3.1 Skim milk assay

5.3.2 PROTEIN ESTIMATION OF KERATINASE:

Protein estimation of keratinase using Lowry's method was done and found that there was increase in concentration when increase in volume of keratinase, this verified the presence of enzyme (keratinase).

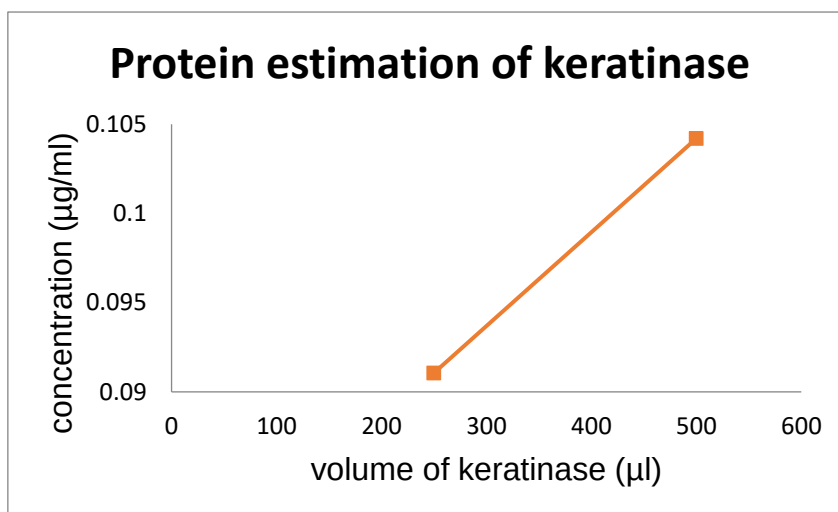


Fig 5.3.2 Protein estimation of keratinase in graph

5.4 KERATINASE SCREENING AFTER OPTIMIZATION:

5.4.1 GROWTH RATE OF KERATINASE:

The growth rate of keratinase was plotted in graph for 0.5% and 1% feather as substrate and also for basal medium without substrate, from the graph it is understood that without substrate the growth rate decreases after the 2nd day itself but with substrate the growth rate decreases only after 6th day also increase in percentage of substrate from 0.5% to 1% there is increase in the growth of the organism.

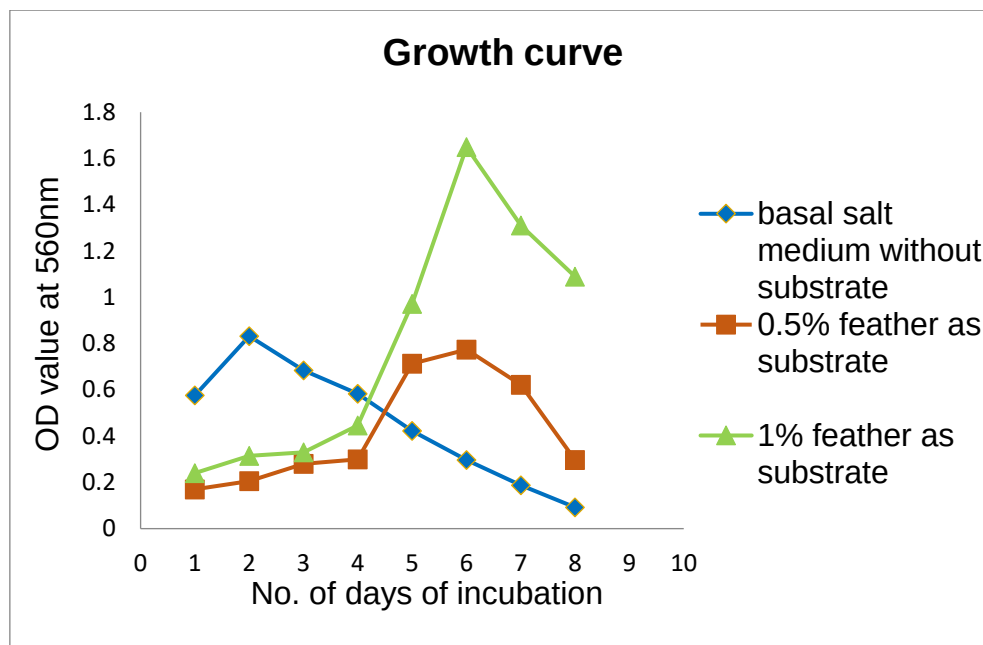


Fig 5.4.1 Growth curve of keratinase

5.4.2 PROTEIN ESTIMATION OF KERATINASE OF (0.5% AND 1% SUBSTRATE):

The protein concentration of keratinase was calculated for 1ml and concentration was found from the standard BSA graph using $y = m \times x + c$ ((OD value- intercept)/slope) and concentration was plotted in graph for 0.5% and 1% feather as substrate and also for basal medium without substrate, from the graph it is understood that without substrate the protein concentration decreases after the 2nd day itself but with substrate the protein concentration decreases only after 6th day also increase in percentage of substrate from 0.5% to 1% there is increase in the concentration of protein.

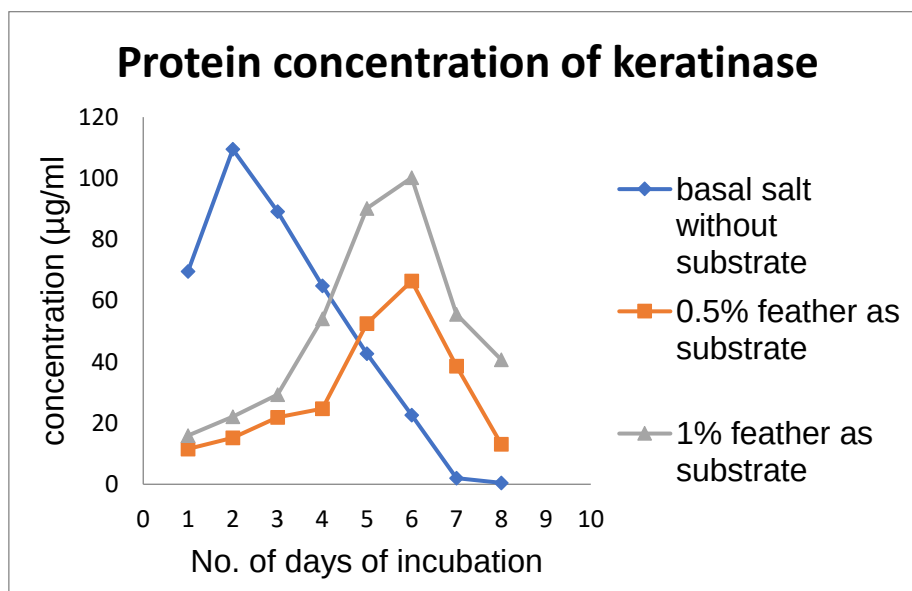


Fig 5.4.2 Protein concentration of keratinase

5.4.3 KERATINASE ENZYME ASSAY:

The enzyme assay of keratinase concentration was found from the standard tyrosine graph using $y = m \times c + c$ ((OD value- intercept)/slope) and concentration was plotted in graph for 0.5% and 1% feather as substrate and also for basal medium without substrate, from the graph it is understood that without substrate the enzyme assay decreases after the 2nd day itself but with substrate the protein concentration decreases only after 6th day also increase in percentage of substrate from 0.5% to 1% there is increase in the enzyme activity.

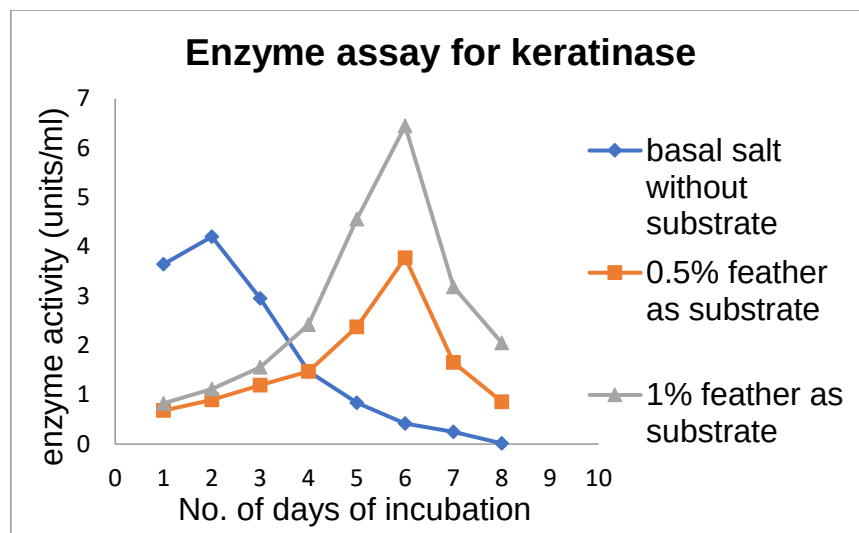


Fig 5.4.3 Enzyme assay of keratinase

5.4.4 HPLC RESULT:

The keratinase after partial purification was mixed in TCA buffer and was analyzed and the retention time was observed to be 40.0283 minutes .

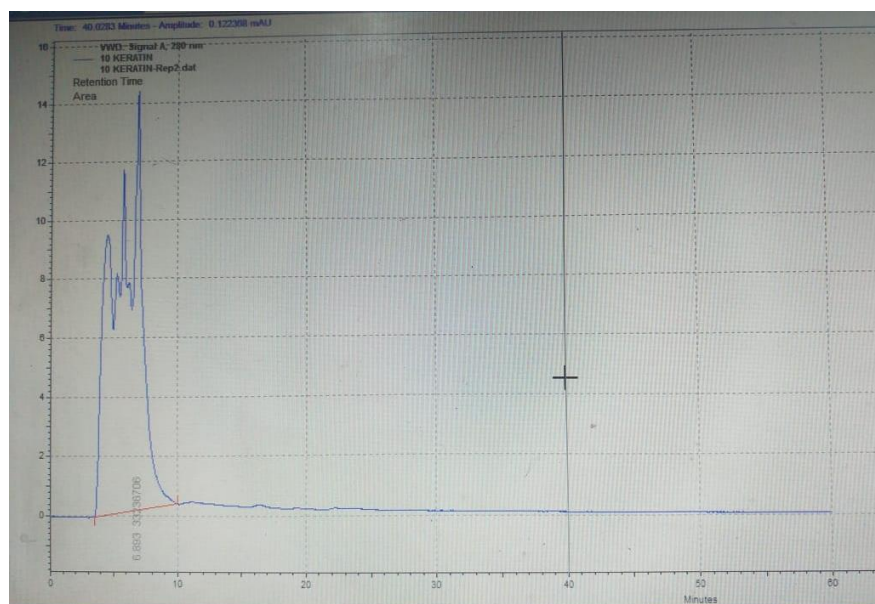


Fig 5.4.4 HPLC result for keratinase

5.5 APPLICATION:

Keratinase is an enzyme which is broadly used in many industries such as cosmetics, textile etc, here keratinase is used in effective removal of stain such as chocolate and blood. After washing the cloth with tap water, detergent, only enzyme and enzyme + detergent. The best result was observed when enzyme + detergent is combined and used rather than using them separately. So this study proves that keratinase can be combined with detergent and can be used in effective removal of stain in textile industry for large scale, even detergent manufacturing industries can incorporate enzyme to their powder and manufacture them which will have best stain removal property.

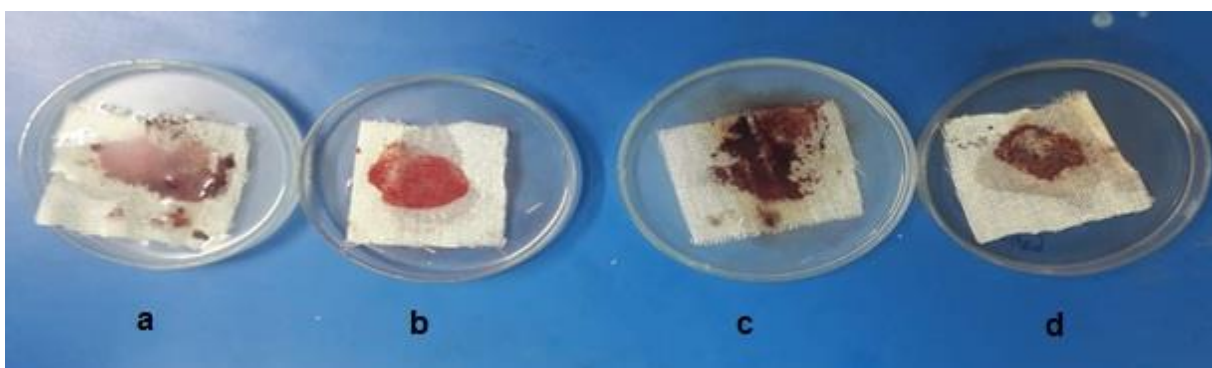


Fig 5.5.1 blood stain before wash a) only tap water b) only enzyme c) only detergent d) detergent+ enzyme.

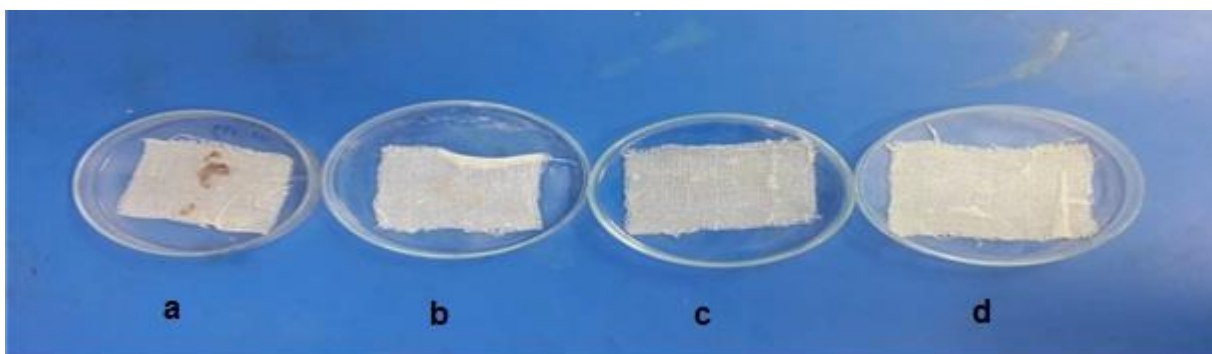


Fig 5.5.2 blood stain after wash a) only tap water b) only enzyme c) only detergent d) detergent+ enzyme.

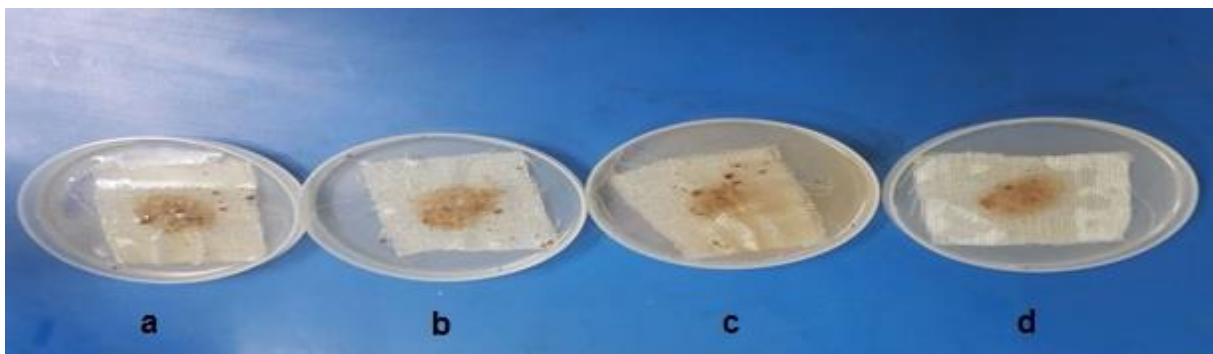


Fig 5.5.3 chocolate stain before wash a) only tap water b) only enzyme c) only detergent d) detergent+ enzyme.

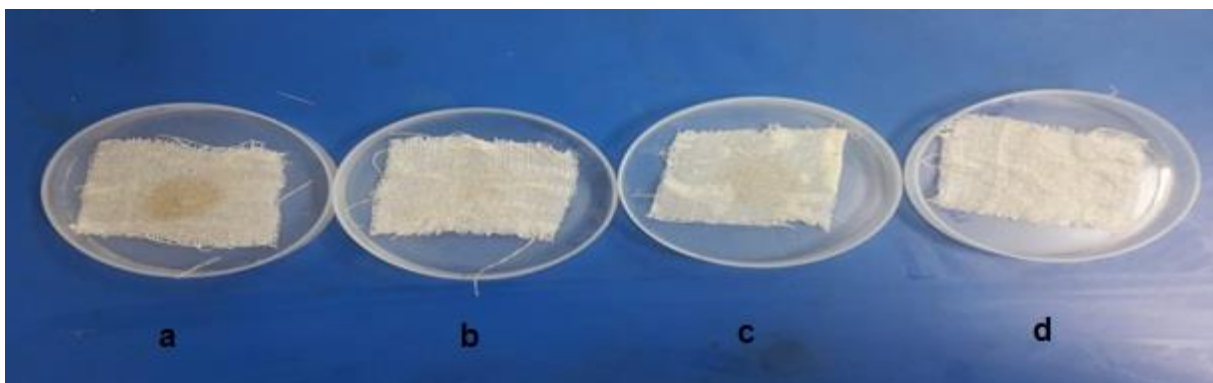


Fig 5.5.4 chocolate stain after wash a) only tap water b) only enzyme c) only detergent d) detergent+ enzyme.

DISCUSSION

This study showed that chicken feathers have a lot of source of keratin and this can be used as a substrate for production of keratinase and these can be used in variety of applications.

Optimization of substrate concentration as shown that chicken feather with 1% feather as substrate has more concentration of keratinase than the substrate with 0.5% feather, the growth curve, protein concentration and enzyme assay for keratinase was checked on 1st, 2nd, 3rd, 4th, 5th, 6th, 7th and 8th day and it is understood that there is more concentration increases daily and on the 6th day the concentration was in peak and then there is decreases in enzyme activity slowly on the upcoming days.

The keratinase was given for HPLC analysis and 40.0283 minutes was observed in the graph.

This study has also showed that keratinase when combined with normal detergent and used for stain removal there is best results, and stain has completely removed from the cloth.

CHAPTER 6

SUMMARY AND CONCLUSION

Keratinase enzyme was extracted from chicken feather, chicken feathers are a lot of waste on daily basis which pollute the environment, so effective removal of chicken feathers and using them as substrate and used for the production of keratinase. The extracted keratinase was analyzed for its growth rate, protein concentration and enzyme assay for which keratin was also extracted from chicken feathers, and it was observed that 6th day has more concentration of keratinase. The keratinase was given for HPLC analysis and the retention time was at 40.0283 minutes. Later this keratinase was used for the effective removal of stain from cloth where detergent cannot perform the task but detergent when combined with keratinase has completely removed the stain from the cloth. Thus, keratinase can be produced from waste chicken feathers and it can be used in textile industries as bio-detergent in laundry industries.

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