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EFFECT OF CARBON AND NITROGEN SOURCES ON THE PRODUCTION OF KERATINASE FROM STAPHYLOCOCCUS AUREUS

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ABSTRACT

Keratinase enzymes are mainly used in dehairing process in leather industry instead of sodium sulphides and these are also used as detergent to remove stains on cloth. The microorganisms producing keratinase were isolated from soil samples collected from a poultry farm. The isolated organism was found to be Staphylococcus aureus which was identified by various biochemical tests. The aim of this study is to use different carbon and nitrogen source to find their effect on the production of keratinase. Among the carbon sources maltose with 0.045 (µmol/min) activity showed the maximum yield and tryptone with 0.65 (µmol/min) activity showed maximum yield among the nitrogen sources used. This was compared using tyrosine as standard. The protein of interest was purified using various procedures and the molecular weight of the protein was identified using SDS PAGE.

KEYWORDS: keratinase, Staphylococcus aureus, maltose, tryptone, tyrosine, SDS PAGE.

INTRODUCTION

Keratinase is a proteolytic enzyme. They are classified as proteinases of unknown mechanism as recommended by the Nomenclature Committee of the International Union of Biochemistry (1978). Some researcher's defined keratinase as a serine protease due to its 97% sequence homologous with alkaline protease.

Keratinases are produced only in the presence of keratin-containing substrate. They are found in fungi, streptomycin and bacteria. These enzymes can degrade other fibrous protein fibrin, elastin, collagen, bovine serum albumin etc. 90% of the protein is present in feathers in which the major component is beta-keratin. Some bacteria are able to degrade keratin by attacking the native wool protein [1]. For example *Streptomyces fradiae* are capable of degrading keratin content in human hair by enzymatic decomposition[2].Keratinolytic protein from keratinophilic fungi were reported [3][4][5]. The production of keratinase by *Streptomyces sp* were reported in 1989 [6].

The objectives of this study is to develop keratinase enzyme for large-scale field application with the intent of reducing the large amount of keratin waste produced by the poultry industry each year.

[1] Isolation and identification of microbes for the production of keratinase
[2] Effect of carbon and nitrogen source on the production of keratinase
[3] Purification and characterization of the enzyme keratinase

Keratinase producing microorganisms play an important role in industrial application in fermentation technology. Submerged fermentation of poultry waste by microorganism producing keratinase helps in the conversion of non-soluble keratin (feather) into soluble protein or polypeptide [7]. These protein byproducts may be used as animal and livestock feed, and as leather filling agents [8]. It also has potential applications in dehairing process in leather industry instead of sodium sulphides [9]. It is also used as a detergent to remove stains on cloth [10]. Valorization of keratin containing wastes like feathers from poultry farms and hair from leather industries may have the potential in

development of non-polluting processes. The scope of this work is to degrade the poultry feather wastes (insoluble protein) to soluble protein.

MATERIALS AND METHODS

Materials

Sonicator, magnetic stirrer, weighing machine, SDS PAGE, ion exchange chromatography, gel filtration, calorimeter etc have been used.

Methodology

Isolation and identification of microbes for the production of keratinase

Soil sample was taken from feather dumps in a poultry farm at 5 different locations in Berigai, Tamil Nadu. Using each of the sample suspension, dilution was carried out and those samples were inoculated in an agar medium (Euro Bacter Agar) using 5 different petriplates and those plates were incubated at 37°c. The next day, the microorganisms were isolated, inoculated in a slant medium and incubated to get pure culture.

To identify the isolated organism, biochemical tests have been carried out and microorganisms have been identified.

Effect of carbon and nitrogen source on the production of keratinase

10 broths were prepared by using different carbon and nitrogen sources. These broths were inoculated with the selected organism and it was incubated for 48hrs. To check the maximum yield, enzyme activity was carried out for all the broths.

Purification and characterization of keratinase

Purification of Keratinase

The 24 hour incubated broth was centrifuged at 6000 rpm for 10 minutes and the supernatant was collected. Neutral salt ammonium sulphate was dissolved in the supernatant to carry out salt precipitation. After salt precipitation, it was kept in cold condition for an hour, and then the mixture was centrifuged at 10,000 rpm for 5 minutes. The supernatant was discarded and 10ml of TRIS-Hcl was added to the pellet and transfer this solution into a dialysis bag. The dialysis bag was maintained in cold condition overnight.

Salt precipitation

Salt precipitation was used to purify proteins by altering their solubility using ammonium sulphate. Ammonium sulphate was used since it is a neutral salt and has high solubility. In this study for purifying proteins salting out procedure was carried out. The precipitated protein obtained was centrifuged and TRIS-Hcl buffer was added for further purification process. This was later transferred to a dialysis bag.
To separate the low molecular weight compounds the dialysis bag was placed in a magnetic stirrer under cold condition and for every half an hour the water was changed. This procedure was carried on for 2 hours.

Ion Exchange chromatography

Ion exchange chromatography was used to separate low molecular weight compounds using diethylaminoethyl (DEAE) cellulose gel

Gel Filtration

Gel Filtration Chromatography (GFC) was used to separate proteins based on their molecular size using sephadex gel.

SDS PAGE

The rate of migration of SDS treated proteins is effectively determined by comparing with known proteins molecular weight.

Keratinase Assay

The keratinase activity was assayed as follows:
Casein was prepared with dipotassium phosphate buffer (pH-7.5). 5 ml of buffer was taken and equilibrated for 5 minutes then 0.5 ml of enzyme was added. The mixture was mixed gently by swirling for 10 minutes. The reaction was terminated by adding 5 ml of trichloroacetic acid (TCA) then mixed by swirling and incubated for 30 minutes. The mixture was filtered and to the 2 ml of mixture, 5 ml of sodium carbonate and 1 ml of FC (Folin Ciocalteu's

phenol) reagent (1:4) was added. The mixture was gently mixed by swirling and incubated at 37°C for 30 minutes. The mixture was filtered again and the absorbance was measured at 660 nm using spectrophotometer.

Protein Estimation

Protein level in feather was estimated by using Bovine Serum Albumin (BSA) as standard.

Enzyme kinetics

a) Activity at different pH
Various pH ranges (4-9) was used to measure the activity of the enzyme. The pH was adjusted using the following buffers - Phosphate buffer (pH 6 and 7), Acetate buffer (pH 4 and 5), and Glycine buffer (pH 8 and 9).

b) Activity at different Temperatures
Various temperatures such at 20,27,38,50 and 60°C were used to measure the activity of the enzyme.

c) Activity at different concentrations of Inhibitor
EDTA was used as an inhibitor to measure the activity by the selected organism individually at various concentrations (mg/ml) ranging from 0.01 to 0.05

d) Activity at different Times
The activity was measured at various incubation time ranging from 5 to 25minutes.

e) Activity at different concentration of Activator
$MgCl_2$ (Magnesium Chloride) was used as an activator to measure the activity of the enzyme at various concentrations (mg/ml) ranging from 0.01 to 0.05

f) Activity at different concentration of Substrate
The activity of the enzyme was measured at various substrate concentrations (mg/ml) ranging from 0.2 to 1.0.

RESULTS AND DISCUSSION

Isolation and identification of microbes for the production of keratinase

The results of the biochemical test are as follows

TABLE 1:

Tests	Results	Observation
Nitrate reduction	-	No colour change
Indole hydrolysis	-	No pink ring formation
Gelatin liquefaction	-	No liquefaction
Urease	-	Red colour formation
Lactose	AG	Acid and gas formation
Sucrose	A	Only acid formation
Dextrose	AG	Acid and gas formation
Starch hydrolysis	+	Clear zone formation
Simmon's citrate utilization	+	Turned from green colour into blue colour
Methyl Red	-	No colour change
Voges-proskauer	-	No colour change

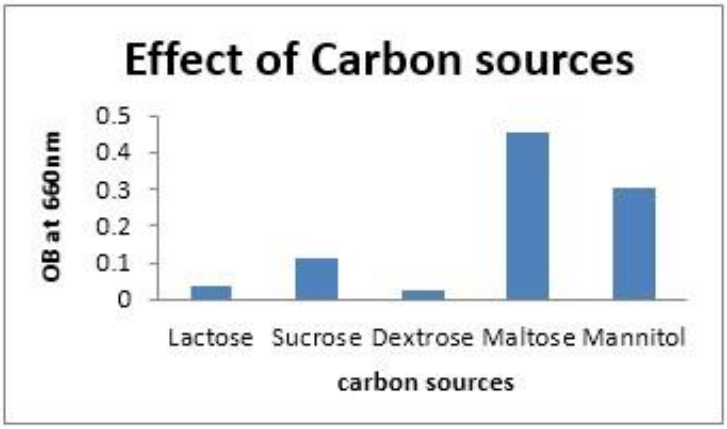
Hydrogen sulphide production	+	Formation of black precipitate
Catalase activity	-	No effervescence
Oxidase activity	+	Development of dark blue colour

+ = Positive; - = Negative; A= Acid formation; AG= Acid and Gas formation
All strains showed positive results for starch hydrolysis, simmon's citrate utilization, methyl red, voges-proskauer, H_2S, and oxidase tests. The strains had a negative reaction to nitrate, indole, gelatin, urease, and catalase tests. The results in the table reflect the ability of isolates to ferment sugars due to specific enzyme responsible for sugar fermentation and gas/ gas and acid production. By using Bergey's manual the microorganism was identified as Gram positive (cocci): ***Staphylococcus aureus***

Effect of carbon and nitrogen source on the production of keratinase
Effect of carbon source
The results for effect of carbon sources such as 1% lactose, 1% sucrose, 1% dextrose, 1% maltose and 1% mannitol are given in table 2. From the assay it was found that the maltose has the maximum effect on the production of the keratinase than other carbon source.

TABLE 2

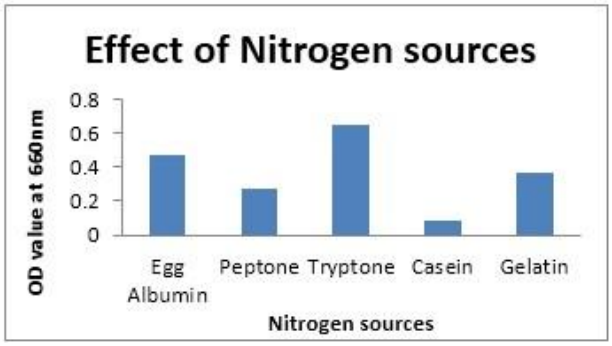
Carbon sources	OD value at 660 nm
Lactose	0.035
Sucrose	0.113
Dextrose	0.025
Maltose	0.454
Mannitol	0.305



Effect of nitrogen source
The results for effect of nitrogen sources such as 1% egg albumin, 1% peptone, 1% tryptone, 1% casein and 1% gelatin are given in table 3. From the assay it was found that the tryptone has the maximum effect on the production of the keratinase than other nitrogen sources.

TABLE 3

Nitrogen sources	OD value at 660 nm
Egg Albumin	0.472
Peptone	0.276
Tryptone	0.65
Casein	0.081
Gelatin	0.367

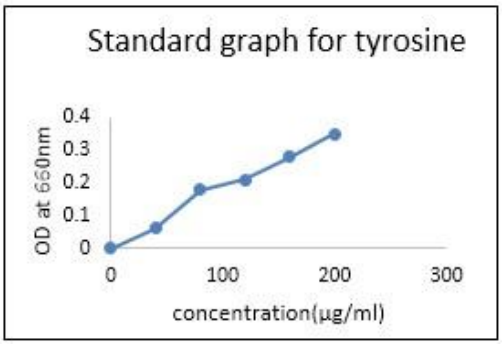


Thus, the activity of crude enzyme is accessed by incubating keratinase with different carbon and nitrogen sources.

Characterization and Purification of the enzyme keratinase
Keratinase Activity
Standard (tyrosine) graph is plotted to calculate the activity of the keratinase enzyme.

TABLE 4: Standard graph for tyrosine

Concentration(µg/ml)	OD value at 660 nm
0	0
40	0.06
80	0.18
120	0.21
160	0.28
200	0.35



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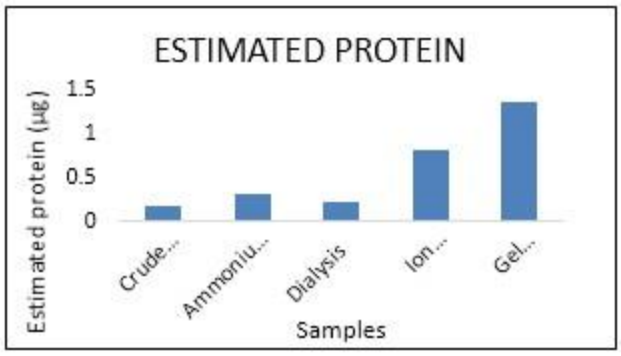
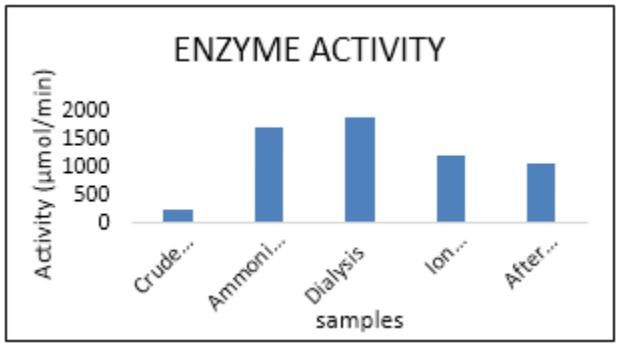
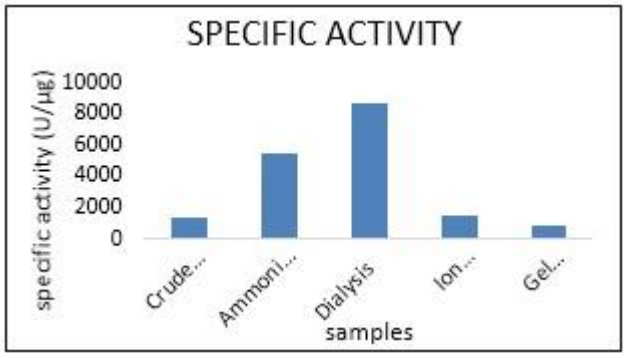
Protein Estimation
TABLE 5

Samples	Enzyme Activity (µmol/min)	Estimated protein (µg)	Specific Activity (U/µg)
Crude Enzyme	233.36	0.18	1296.44
Ammonium sulfate precipitation	1730.09	0.32	5406.53
Dialysis	1891.03	0.22	8595.59
Ion Exchange Chromatography	1212.73	0.82	1478.93
Gel Filtration Chromatography	1074.84	1.36	790.32







SDS-PAGE
SDS-PAGE was performed and the molecular weight of protein was estimated to be 30 kDa.

Enzyme Kinetics
The results of enzyme kinetics are tabulated as follows

TABLE 6: Activity at different pH

pH	OD value at 660 nm
4	0.043
5	0.069
6	0.107
7	0.085
8	0.068
9	0.055

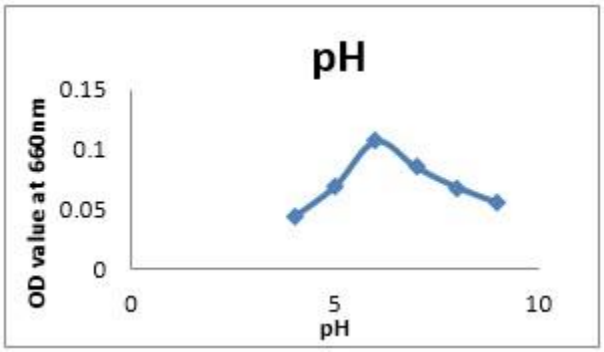


TABLE 7: Activity at different temperatures

Temperature	OD value at 660 nm
20	0.194
27	0.547
38	0.307
50	0.198
60	0.14

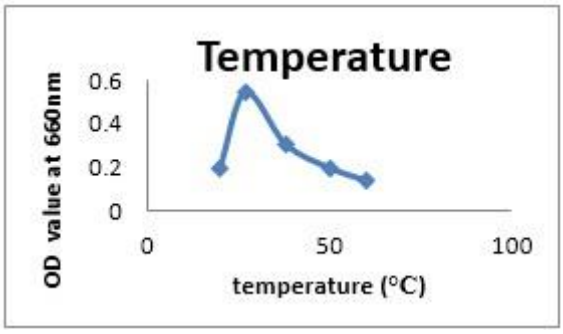


TABLE 8: Activity at different concentrations of inhibitor

Concentration (µg/ml)	OD value at 660 nm
0.01	0.112
0.02	0.101
0.03	0.086
0.04	0.075
0.05	0.055

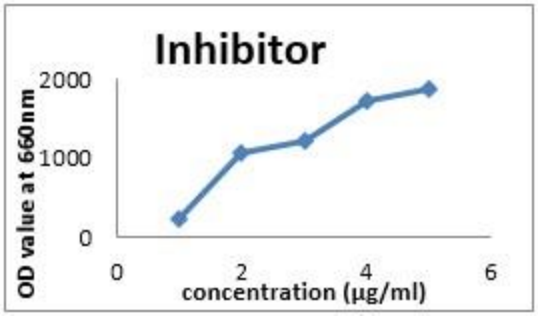


TABLE 9: Activity at different times

Time (mins)	OD value at 660 nm
5	0.032
10	0.046
15	0.07
20	0.104
25	0.115

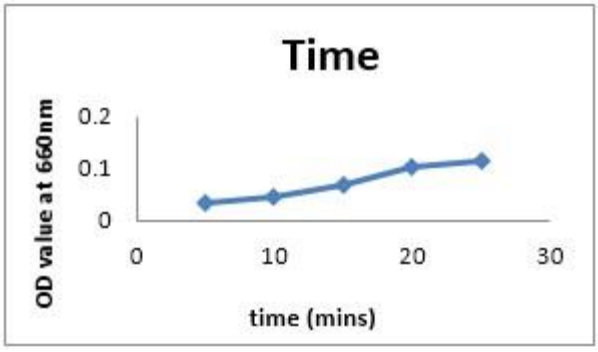


TABLE 10: Activity at different concentrations of activator

Concentration (µg/ml)	OD value at 660 nm
10	0.055
20	0.147
30	0.167
40	0.24
50	0.774

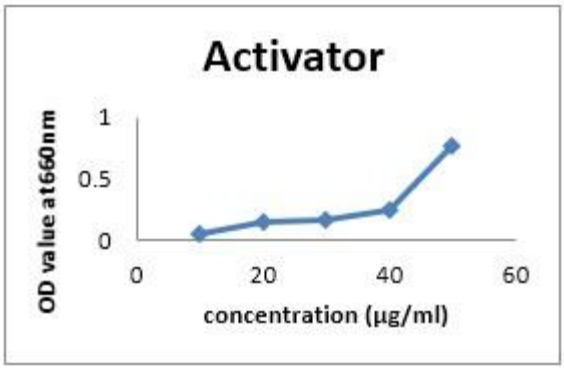
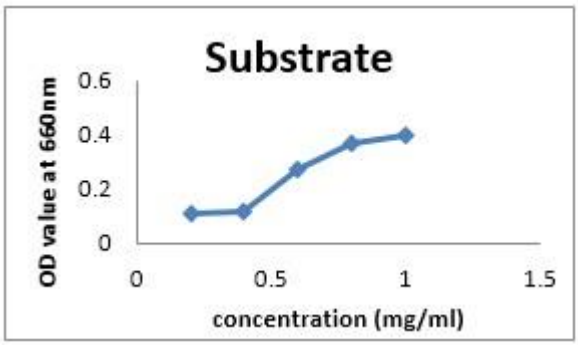


TABLE 11: Activity at different concentrations of Substrate

Concentration (mg/ml)	OD value at 660 nm
0.2	0.112
0.4	0.118
0.6	0.27
0.8	0.37
1	0.399



CONCLUSION

The keratinase producing microorganism-*Staphylococcus aureus* was isolated and identified using gram staining and biochemical techniques. Various carbon and nitrogen sources were used to identify the maximum yield of Keratinase. The enzyme showed maximum activity at pH 6, temperature 27ºC, at 0.01 (µg/ml) concentration of inhibitor, time 25 minutes and at 50 (µg/ml) concentration of substrate. The maximum amount of protein was estimated after gel filtration (1.36 µg).The specific activity of enzyme was high after dialysis (8595.59 U/µg). The maximum enzyme activity was noted after dialysis (1891.03 µmol/min). Absorbance values generated by the activity of protease are compared to the standard curve, which is generated by the known quantities of tyrosine with folin's reagent to correlate changes in absorbance with the amount of tyrosine in micromoles. From the standard curve, the activity of protease can be determined in terms of micromoles, the amount of micromoles of tyrosine equivalents released from casein per minute. The enzyme was purified using ion exchange chromatography, gel filtration. The molecular weight of the protein was estimated to be 30 kDa using SDS PAGE.

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