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Research Article

PRODUCTION OF KERATINASE BY USING *PSEUDOMONAS AERUGINOSA* ISOLATED FROM POULTRY WASTE

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ABSTRACT

Pseudomonas aeruginosa strain isolated from poultry waste was tested for its abilities to hydrolyze the feather. The effect of different production parameters such as pH, temperature, carbon source, nitrogen source (Organic and inorganic) incubation time inoculum sizes and surfactants on keratinase production by the isolated bacterial strain was studied. The enzyme production was assayed in submerged fermentation (SmF) condition. The maximum keratinase production was observed with maltose (120±2.6 U/ml), yeast extract (90±3.6 U/ml), ammonium sulphate (61±3.0 U/ml), pH 7.0 (114±4.1), temperature 40°C (110±1.9), Tween-80 (104±2.8U/ml), inoculum size level 5% (112±2.0U/ml) and incubation time 48 hours (101.01± 0.56U/ml) in the production medium.

Keywords: Poultry waste, *Pseudomonas aeruginosa*, submerged fermentation, Keratinase.

INTRODUCTION

Enzymes are delicate protein molecules necessary for life. Proteases are the single class of enzymes which play an important part in the metabolism of almost all organisms (Plants, Animals, Fingi, Bacteria and Viruses).Investigation of proteases is a central issue in enzymology due to their wide applications in Landry detergents, Pharmaceutical. Leather products, Photography, Food, Agricultural products and Bioremediation process. Among the various proteases, bacterial extracellular proteases are the most significant, compared with animal, Plants, viruses and fungal extracellular proteases. Extracellular proteases produced by Bacillus and cocci species are of main interest from a biotechnological perspective, and are not only in scientific fields of protein chemistry and protein engineering but also in applied fields such as detergents, foods, tannery, pharmaceutical and leather industries. These proteases account for 60% of the total worldwide production of enzymes. The genus Bacillus and cocci contains a number of industrially important species and approximately half of the present commercial production of bulk enzymes derives from the srtains of Bacillus and cocci. Keratinase (EC 3.4.4.25) belongs to the class hydrolase which are able to hydrolyse insoluble keratins more efficient than other proteases and their action are very specific, i.e., they acts only on keratin substrates, Keratin are insoluble fibrous proteins found in hair, wool, feather, nail, horns and other epithelial covering which is rich in beta helical coil linked through cysteine bridges. Keratinases are hydrolyze both native and denatured keratin, and the enzymes are widely used not only in chemical and medical

industries but also in food and basic biological science .In this study an attempt was made for the screening , isolation of of Keratinase producing bacteria from poultry waste and production of Keratinase by using different substrates.

The Keratinase producing bacteria (such as *Bacillus subtilis, B. cereus, B. amyloloquefaciens* and *B. megaterium*) and fungi (such as *A. niger, Penicillum, Cephalosporium, Neurospora* and *Rhizopus*) are major keratinase producing microorganisms. Microorganisms such as fungi, bacteria, actinomycetes and algae are effectively producing keratinase.

The industrial use of enzymes often requires enzymatic reaction to be conducted at higher temperatures. Generally under those conditions productivity improved with less microbial contamination. Therefore, thermostable enzymes have been the heart of numerous studies involving in the elucidation of thermal denaturation mechanism and development of rational strategies for the enhancement of enzyme thermostability.

The present study was mainly focused on the production of keratinase form *Pseudomonas aeruginosa* by optimizing various parameters such as carbon sources, inorganic nitrogen sources, organic nitrogen sources, pH, temperature, substrate concentration, inoculums concentration, incubation time and surfactants.

MATERIALS AND METHODS

Collection and isolation of sample

Samples were collected from dump yards of poultry wastes at Solddevanahalli, Chikkabanavara, Devasandra, K.R.puram, Tannary road and Yashwanthpur in and around Bangalore, Karnataka, India. The samples were labeled after collected. These were spread onto isolation media Feather powder agar and incubated at 37^{o}C for 48 hours after serial dilution of 10^{-1} to 10^{-6}.

Screening of Keratinase production by plate assay

The isolates were screened for Keratinase activity. This was done by inoculating the organisms on the feather powder agar plates containing0.4 % feather powder (Washed feathers were dried at 50^{o}C in a forced draught oven (Gallenkamp, Ltd UK). The dried feathers were ground into fine fractions (<90, 90, 150, 300, 425 and 850μM) with test sieves of appropriate diameters) incubated at 37^{o}C for 48 hours. A clear zone around the growth of the bacteria was indicated to Keratinase activity

Morphological and Biochemical Characteristics

Gram staining, motility, indole production, methyl red, Voges Proskauer's, citrate utilization, triple sugar iron, nitrate reduction, catalase, oxidase, gelatin liquefaction, urease, hydrolysis of casein, hydrolysis of starch were carried out[8].

Keratinase Enzyme Assay

Cell Separation: The cells from the culture were separated by centrifugation. Around 2ml of culture was taken in a sterile eppendorf tubes and were centrifuged at 6,600 rpm for 2 min in a cold room. The supernatant was transferred carefully into another test tube.

Keratinase Assay

Keratinase activity was determined by measuring the hydrolysis ability on azokeratin. 200 μL of enzyme extract was added to 800 μL of azokeratin (5 mg/mL) in 50 mM phosphate buffer (pH 7.5). After 60 min reaction at 50°C, equal volume of 15% trichloroacetic acid (TCA) solution was added to stop the reaction. Absorbance at 450 nm was then measured after 5 min centrifugation at 10,000 × g. One unit of activity was defined as the amount of keratinase that caused an increase in absorbance of 0.01 at 450 nm within 60 min reaction at 50°C[17].

Protein Estimation of Crude Enzyme

The protein content of the crude enzyme was measured according to Bradford (1976) method[4].

MEDIUM OPTIMIZATION FOR KERATINASE PRODUCTION

Carbon Source

To identify the suitable carbon sources for Keratinase production by the *Pseudomonas aeruginosa*. The following different carbon sources were tested such as glucose, sucrose, maltose, lactose, galactose, fructose and dextrose with sample concentration of 0.5% in the optimized carbon sources in production medium at 37°C.

Organic and Inorganic Nitrogen Sources

The Keratinase production by the selected bacterium was also optimized by supplementing different organic and inorganic nitrogen sources individually at the concentration of 0.5% such as potassium nitrate, ammonium sulphate, sodium nitrate, ammonium nitrate, ammonium chloride, casein, malt extract, peptone, urea, gelatin and yeast extract .

Effect of pH

The effect of pH for Keratinase production was determined by culturing the bacterium in the production media with different pH. The experiment was carried out individually at various pH 5, 6, 7, 8, 9 and 10. The enzyme assay was carried out after 72 hours of incubation at 37°C.

Effect of Temperature

Temperature is an important role for the production of Keratinase. The effect of temperature on Keratinase production was studied by the incubating the culture media at various temperatures 10, 20, 30, 40, 50, 60,70 and 80°C along with arbitrary control at 37°C.

Effect of Surfactants

To identify the surfactants facilitating Keratinase production, four different surfactants were used for experimentation. They were Tween-20, Tween-80, SDS (Sodium dodecyl sulphate) and PEG (Poly Ethylene Glycol). The surfactants were tested individually at the concentration of 0.2% in the optimized production medium.

Effect of Various Incubation Times on Keratinase Production

The Keratinase production by the selected experimental microorganisms was determined by optimizing the media by adding different bacteria in the production media. The experiment was carried out individually at various incubation times such as 24, 48, 72, 96 and 120 hours. The enzyme assay was carried out individually after 72 hours of incubation.

Effect of Various Inoculum Concentrations on Keratinase Production

The Keratinase production by the selected experimental microorganisms was determined by adding bacterium at different inoculum's concentrations such as 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5.0 % to test its ability to induce Keratinase production in the production medium.

RESULTS

Screening of Keratinase Producing Bacteria from Poultry waste

The bacteria isolated from Poultry waste were screened for Keratinase production on feather powder agar medium. From the soil samples 9 bacterial strains were isolated. But later during screening it was found that only 3 strains showed Keratinase activity. Later only one potential isolate was identified by standard morphological and biochemical characterization. After careful biochemical tests, it was confirmed that the isolate was *Pseudomonas aeruginosa.*

Effect of Carbon Sources on Keratinase Production:

Table 1 shows the effect of carbon sources on Keratinase production after 48 hours of incubation period at 37°C. The maximum Keratinase production was recorded in maltose (120±2.6U/ml) supplemented medium and minimum Keratinase production was recorded in glucose (40±1.0U/ml).

Effect of Inorganic Nitrogen Sources on Keratinase Production

Table 2 shows the effect of different kinds of inorganic nitrogen sources on Keratinase production after 48 hours of incubation period at 37°C. The maximum amount of enzyme production was observed in ammonium sulphate (61±3.0U/ml) supplemented medium and minimum amount of Keratinase production was observed in sodium nitrate (36±1.2U/ml) supplemented medium.

Effect of Organic Nitrogen Sources on Keratinase Production

Table 3 shows the effect of different kinds of organic nitrogen sources on Keratinase production after 48 hours of incubation period at 37°C. The maximum amount of Keratinase production was observed in yeast extract (90±3.6U/ml) with supplemented medium and minimum enzyme activity was observed in gelatin (61±1.7U/ml).

Effect of pH on Keratinase Production

Table 4 shows the effect of various pH on Keratinase production after 48 hours of incubation period at 37°C. The maximum Keratinase production was observed at pH 7.0 (114±4.1U/ml) and minimum amount of Keratinase production was recorded at pH 10 (72±2.5U/ml).

Effect of Temperature on Keratinase Production

Table 5 shows the effect of various temperatures on Keratinase production. The maximum Keratinase production was obtained at 40°C (110±1.9U/ml). Followed by this, 50°C temperature (107±1.2U/ml) was the second best temperature on Keratinase production. On the other hand, the minimum amount of Keratinase production was observed at temperature 80°C (27±0.7U/ml).

Effect of Surfactants on Keratinase Production

Table 6 shows the effect of various surfactants on Keratinase production after 48 hours of incubation at 50°C. The maximum amount of enzyme was recorded in Tween-80 (104±2.8U/ml) and minimum amount of Keratinase was observed in PEG (60±1.0U/ml).

Effect of Incubation Time on Keratinase Production

Table 7 illustrates the effect of different incubation times on Keratinase production. The maximum amount of Keratinase production was observed with 48 hours incubation time (101.01± 0.56U/ml). The minimum amount of Keratinase production was obtained with 120 hours incubation (65.15±1.24U/ml).

Effect of Various Inoculum Sizes on Keratinase Production

In the present study, the initial inoculum level has played an important role in Keratinase production by *Pseudomonas aeruginosa*. The maximum Keratinase specific activity was registered at the 5% (112±2U/ml) of inoculum level. On the other hand, the minimum amount of Keratinase production was observed at 0.5% of (46±2.80 U/ml) inoculums level (Table 8).

DISCUSSION

The addition of carbon source in the form of either monosaccharide or polysaccharides may influence the production of Keratinase enzyme. In our present study, the influence of maltose was more (120±2.6U/ml) than the other carbon sources tested. Galactose was the second best supplementary carbon source (106±1.5U/ml). Glucose gave the lowest Keratinase enzyme activity (40±1.0U/ml).

In the present study, ammonium sulphate was found to be the most suitable inorganic nitrogen source for *Pseudomonas aeruginosa* and the enzyme activity observed was 61±3.0U/ml. The lowest Keratinase production was observed in sodium nitrate (36±1.2U/ml) supplied medium. Ramachandran *et al.* [13] reported that ammonium salts enhanced the enzyme activity. Sodium nitrate showed a negative influence, showing a steep decrease in keratinase activity.

The nitrogen sources are of secondary energy sources for the organisms, which play an important role in the growth of the organism and the production. The nature of the compound and the concentration that we used might stimulate or down modulate the production of enzymes. In the present study experiment on the effect of supplementary nitrogen sources on Keratinase production under SSF, showed that yeast extract was found to be a better nitrogen source for this isolate (90±3.6U/ml).

Yeast extract is the best nitrogen source for Keratinase production, probably due to its high content in minerals, vitamins, coenzymes and nitrogen components. Ramachandran *et al.* [14] reported that peptone gave an increase in enzyme yield in SSF using coconut oil cake as substrate.

The effect of initial pH on SSF of Keratinase showed that the pH range of 5-7 produced more amount of Keratinase and it was relatively high in pH 7.0 (114±4.1U/ml) and pH 8 (104±2.3U/ml).Above this level, the Keratinase production decreased, because the metabolic activities of microbes are very much responding to pH change.

Ellaiah *et al.* [11] stated that at high pH, the metabolic action of bacterium may be suppressed and thus it inhibits the enzyme production. Physical factors are important in any fermentation for optimization of biochemical production. The important physical factors that determine the bioprocess are pH, temperature, aeration and agitation. In the present study, the effect of temperature on Keratinase enzyme activity by SSF revealed that 40°C was optimum (110±1.9U/ml) and at the tested higher temperatures, the enzyme production decreased which might be due to growth reduction and enzyme inactivation or suppression of cell viability.

Surfactants in the fermentation medium are known to increase secretion of proteins by increasing the cell membrane permeability. In the present study, the addition of Tween-80 increases the Keratinase production for *Pseudomonas aeruginosa* (104±2.8U/ml). The effect of incubation time on Keratinase production showed that 48 hours was the optimum duration for maximum Keratinase enzyme activity (101.01± 0.56U/ml). Above this period the Keratinase enzyme activity started to decrease. This is because, the cells may reach the decline phase and displayed low Keratinase synthesis.

Since the carbon source represents the energetic source that is available for the growth of the microorganism, it could be that the enzyme production is associated and the presence of feathermeal in the medium stimulated the increased production of the enzyme. An inoculums concentration higher than the optimum value may produce a high amount of biomass which rapidly depletes the nutrients necessary for growth and product synthesis. On the other hand, lower inoculums levels may give insufficient biomass and allow the growth of undesirable organisms in the production medium. This increases the necessary time to grow to an optimum number to consume the substrate and synthesize the desired product.

In the present study, the highest enzyme activity (112±2U/ml) was obtained at an inoculums level of 5% by *Pseudomonas aeruginosa* under SSF.

Table 1: Effect of various Carbon sources on Keratinase production

Carbon Source (0.5%)	Specific Activity (U/ml)
Glucose	40±1.0
Galactose	106±1.5
Maltose	**120±2.6**
Sucrose	100±2.5
Lactose	97±2.8
Fructose	90±1.
Dextrose	58±1.0

Table 2: Effect of various Inorganic nitrogen sources on Keratinase production

Nitrogen Source (0.5%)	Specific Activity (U/ml)
Potassium nitrate	43±10
Ammonium Sulphate	**61±3.0**
Sodium nitrate	36±1.2
Ammonium nitrate	58±1.2
Ammonium chloride	50±2.9

Table 3: Effect of various Organic nitrogen sources on Keratinase production

Organic nitrogen source (0.5%)	Specific Activity (U/ml)
Casein	82±2.2
Malt extract	80±2.0
Peptone	75±2.7
Urea	86±2.0
Gelatin	61±1.7
Yeast extract	**90±3.6**

Table 4: Effect of various pHs on Keratinase production

pH	Specific Activity (U/ml)
5	90±2.9
6	95±2.4
7	**114±4.1**
8	104±2.3
9	86±1.7
10	72±2.5

Table 5: Effect of various temperatures on Keratinase production

Temperature (ºC)	Specific Activity (U/ml)
10	82±0.6
20	95±1.3
30	102±1.8
40	**110±1.9**
50	107±1.2
60	90±1.5
70	71±0.9
80	27±0.7

Table 6: Effect of various surfactants on Keratinase production

Surfactants	Specific Activity (U/ml)
Tween-20	92±2.0
Tween-80	**104±2.8**
SDS	84±1.0
PEG	60±1.0

Table 7: Effect of various Incubation times on Keratinase production

Incubation Time(hrs)	Specific Activity (U/ml)
24	96±1.20
48	**101.01± 0.56**
72	81.36±0.11
96	78.11±0.98
120	65.15±1.24

Table 8: Effect of various Inoculum sizes on Keratinase production

Inoculum sizes (%)	Specific Activity (U/ml)
0.5	46±2.80
1.0	50±1.80
1.5	62±1.00
2.0	72±2.80
3.0	84±0.36
4.0	102±0.58
5.0	**112±2**

CONCLUSION

The above report stated the evidence for the production of Keratinase with substrate interactions of bacterial strains with simple and effective manner. More over this study gives us values as well as the microbial wealth of Keratinase producing bacteria which can be boon for the development of biotechnological processes.

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