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Production and Characterization of Keratinase Enzyme from Natural Isolate Bacillus Strains

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Abstract

Bacillus sp is microorganism's species which can produce keratinase enzyme. The enzyme has ability to degrade keratine substrate and it belongs to extracellular inducible enzyme group. In this study, proteolitic activities of Bacillus strains isolated from soil contaminated with poultry farm waste were determined on Skim milk Agar. 63 Bacillus strains were identified as proteolytic. Nine of these strains showed activity with the keratin-azure substrate and were determined to be keratinolytic. Among the isolated strains, strain no: 30 was selected as the best keratinase producer. The basal feather medium was used to produce keratinase. The optimum production conditions of the enzyme were determined as pH = 8.0 and 30˚C. The optimum activity of the enzyme was determined at pH: 9.0 and 50˚C. When the enzyme was pre-incubated for 30 minutes at 50˚C - 100˚C, it preserved its activity at a high rate (90%) after pre-incubation at 50˚C and showed no activity after pre-incubations above 70 degrees. After 30 minutes of pre-incubation of the enzyme with 1 - 5 mM concentrations of chemical and metal ions, it was determined that tween 20 (215%) and SDS (204%) significantly increased the activity, and EDTA (76%), Urea (40%), $ZnCl_2$ (28%), $MgCl_2$ (51%), $NiCl_2$ (24%), $CaCl_2$ (56%), $HgCl_2$ (0), $CuCl_2$ (83%) decreased the activity.

Subject Areas

Molecular Biology

Keywords

Bacillus sp., Keratin, Keratinase, Enzyme Production, Characterization

1. Introduction

Enzymes are complex organic molecules found in living cells that act as catalysts

for various and several endogenous biochemical reactions in a certain pathway [1]. Nowadays thanks to the developments in the field of biochemistry, detailed information has been obtained about the presence of enzymes in living cells and their mode of action. However, enzymes do not only show activity in living cells.

Enzymes are proteins that facilitate the cellular metabolic process by lowering their activation energies (Ea) in order to catalyze chemical reactions between biomolecules. Some enzymes lower the activation energy so much that they cause the opposite reaction of cellular reactions. However, enzymes in any case facilitate reactions without undergoing any change, such as the combustion of fuel when used. Biotechnology is one of the most developed fields of science in recent years, benefiting from the integration of biology, physics and engineering sciences in order to realize the technological applications of biological systems. Enzymes are an important resource used by the food, chemical and similar industries to produce a wide variety of biotech products and are recognized as important catalysts for various organic transformations and the production of high-quality chemicals and drugs. Most commercial enzymes are produced by microorganisms [2].

Another feature of enzymes is that they are specific molecules. That is, they only catalyze certain reactions and interact with substrates suitable for their structure. Not every enzyme interacts with every substrate, enzyme and substrate are unique to each other.

However, gene sequences with similar catalytic activity need not be similar. Therefore, conventional sequence similarity-based methods often fail to identify the enzymes involved and thus cannot be used to map an organism's metabolome [3].

$$E + S \rightarrow ES \text{ complex} \rightarrow E + P$$

2. Literature Review

Sina, A., [4], in his study, keratinolytic bacillus sp. isolated the strains and performed the production and characterization of keratinase. Bacillus sp. HSK-21 isolate was selected as the most active strain and determined the optimum production pH.

During the research, enzyme activity has been reported as pH range 5.0 - 13.0, temperature range and 20˚C - 60˚C. In the study, the optimum activity was determined at pH = 12.0 and 40˚C. When the enzyme was treated with different buffers (pH = 7.0 - 13.0) for 30 minutes, it remained active at an average of 50% - 100% reported to be preserved.

Another exploration with Nagal and Jain 2010 tested eight Bacillus strains isolated from degraded feathers for hydrolysis of feather waste in the laboratory. Among these strains, Bacillus cereus KB043 was selected as the best feather-destroying organism when grown in basal medium containing 1% chicken feather as the sole carbon and nitrogen source. It caused 78.16% ± 0.4% degradation with significant release of soluble protein (1206.15 ± 14.7 μg·mL^{-1}) and

cysteine (20.63 ± 0.4 $\mu g \cdot mL^{-1}$) in the rearing fluid [5].

In this case, Arokiyaj and Ark performed keratinase production with Bacillus cereus, which they isolated from halophilic environments in Tamil Nadu (India). Keratinase production was optimized using wheat bran substrate. 1% lactose supplement, more keratinase production (120 U/g) provided [6].

3. Method and Material

3.1. Material

As previously studied, Bacillus genus microorganisms isolated from the soil taken from Çukurova University chicken farm were used in this study. In the medium preparation, various buffers and chemicals in the Molecular Biology Laboratory of the Department for the isolation of the microorganism, the detection of the enzyme producing strain and the characterization of the enzyme; Spectrophotometer (Shimadzu); Mixer (Electro-Mag); pH meter; Oven; Autoclave; Blender; Precision Balance; Refrigerated Centrifuge; Water bath; Equipment and devices such as incubators were used.

3.1.1. Soil Samples

Samples taken from 25 different locations from the environment where Çukurova University poultry farm wastes were collected, into sterile sampling boxes, were brought to the Molecular Biology Laboratory. Samples homogenized here were stored at +4˚C for bacterial isolation.

3.1.2. Media Used

Basic Feather Medium

Bacillus sp. was used for the production of keratinase from the strains. Chicken feathers were used as substrate for enzyme production. The composition of the medium is given below.

Component	g/L
NH_4Cl	0.5
NaCl	0.5
K_2HPO_4	0.3
KH_2PO_4	0.4
$MgCl_2 \cdot 6H_2O$	0.1
Ferment	0.1
Grinded chicken feather	10
Medium pH	7.45

Nutrient Agar

Isolation of bacterial strains was used to prepare stock cultures of strains in pure culture form.

Component	g/L
Meat Peptone	5
Meat Extrait	3
Agar	12
pH	7.0

Luria-Bertani (LB) Broth

LB medium was used for resuscitation and culture renewal of stock cultured microorganisms.

Component	g/L
Casein peptone	10
Sodium chlorure	10
Ferment extrait	5
pH	7.1

Skim Milk Agar

Isolated and Bacillus sp. protease in strains found to be was used to determine its activity.

Component	g/L
Peptone	5
Ferment	2.5
Glucose	1
Skim Milk powder	28
Agar	15
pH	7.0

Keratin Azure Solution

It was used as a substrate for the determination of keratinase activity by colorimetric method. Keratin Azure-based method is considered a precision measurement method [7].

Component	Measure
Keratin azure	0.4 g
Fosfat Buffe	100 ml
pH	7.5

3.2. Method

3.2.1. *Bacillus* sp. Strains Isolations

In order to isolate bacteria belonging to the genus Bacillus, 2 g of soil samples were weighed and suspended in 50 mL of sterile saline distilled water. After be-

ing thoroughly homogenized by vortexing, it was pretreated in a water bath at 85˚C for 15 minutes in order to destroy the vegetative forms of bacteria. Following this process, serial dilution (10^{-5} to 10^{-8}) was made on Nutrient agar medium by spreading method and incubated at 37˚C for 24 hours. After incubation, strains thought to be Bacillus according to colony morphology were first inoculated on Nutrient Agar by line method and incubated at 37˚C for 24 hours. After the samples were numbered, they were stained by gram staining method and 18 hours of fresh culture was prepared from the strains that were found to be gram positive, endospore forming and Bacillus spp as a result of microscopic examination, and they were stored as stock culture on Nutrient agar in slanted solid medium at +4˚C.

3.2.2. Detection of Protease Activity

Isolated Bacillus sp. strains were inoculated on Skim Milk agar medium by line method and incubated at 37˚C for 48 hours. The hydrolysis zone was checked every 12 hours. The clear hydrolysis zone (63 strains, 53.67%) around the growth line showed the presence of protease activity.

3.2.3. Determination of Keratinolytic Activity

Upon analyses; Preliminary determination was carried out to determine the keratinolytic activity of 63 strains showing protease activity. For this purpose, basic hairy agar was prepared and protease active strains were cultivated by line sowing method. The formation of a slightly transparent zone around the colony due to the fragmentation of the feather was evaluated as a positive result.

3.2.4. Enzyme Production

Keratinase enzyme was produced in basic hairy broth. Isolate 30, which was determined to have the highest keratinolytic activity, was used as the enzyme producer. Bacteria in stock culture were grown at 30, 150 rpm for 24 hours and revived in LB Broth. 150 mL (in 500 mL flasks) of 1% of fresh culture was inoculated into the enzyme production medium. Enzyme was produced by shaking at 30˚, 150 rpm for 72 hours.

After incubation, unbroken hairs and bacteria were removed by culture. The filtrate was centrifuged at 10,000 × g for 20 minutes at +4˚C. After the upper phase was precipitated with acetone (dimethyacetone), the pellet was dissolved in pH: 7.5 phosphate buffer and stored at +4˚C. The solution was used as a crude enzyme in activity studies.

4. Exploration and Discussion

4.1. Isolation of Bacteria

In order to isolate the bacteria, (in Figure 1) spore-forming bacteria were isolated after heat pretreatment was applied from the soil samples taken from the farm area in Çukurova University. Gram staining of bacteria selected for colony morphology was performed, *Bacillus sp. 1*36 strains were found.

4.2. Determination of Keratinase Activity in Liquid Culture

The use of keratinolytic bacteria having antagonistic and plant growth promoting activities, and feather hydrolyzate can emerge as sustainable and alternative tools to promote and improve organic farming, agro-ecosystem, environment, human health, and soil biological activities [8].

We triggered this experience in liquid culture to determine in (**Figure 2**) keratinolytic activity.

4.3. Determination of Optimum Incubation Time for Keratinase Production

After 20 h of incubation at 30 ˚C, the skin was taken out and the hair was gently hand-pulled to test whether it had parted from the skin. The unhairing efficacy was assessed according to the depilated area of the skin at the end of the process and the quality of the dehaired skin was estimated according to the appearance observed by the naked eye after 24 h of treatment. The dehaired skin with high quality showed clean hair pore, clear grain structure and no collagen damage.[9] The same process was established in this case illustrated in (**Figure 3**) for keratinase incubation.

Figure 1. Nutrient agar midium Bacillus spp isolation.

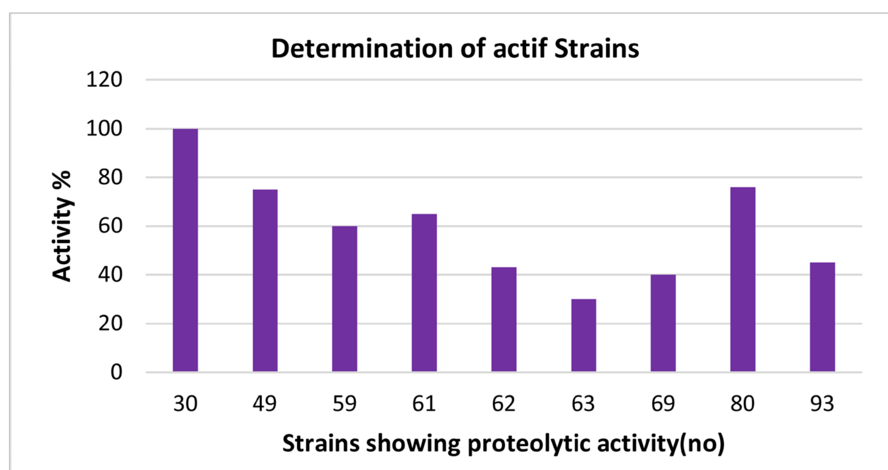

Figure 2. Comparison of strains showing keratinolytic activity.

4.4. Determination of Optimum pH and Temperature in Enzyme Production

See Figure 4 & Figure 5.

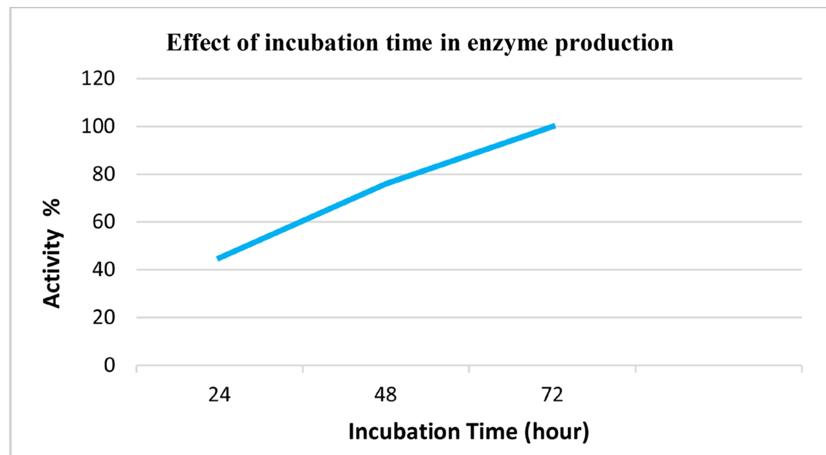

Figure 3. Effect of incubation time.

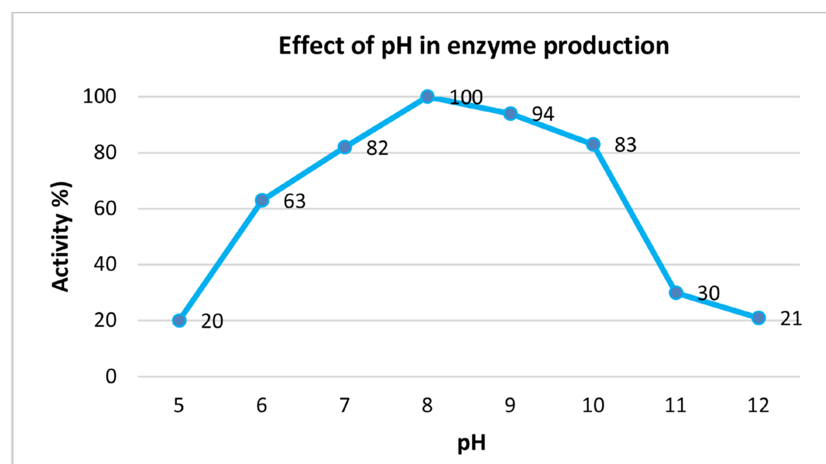

Figure 4. Optimum pH effect on enzyme activity.

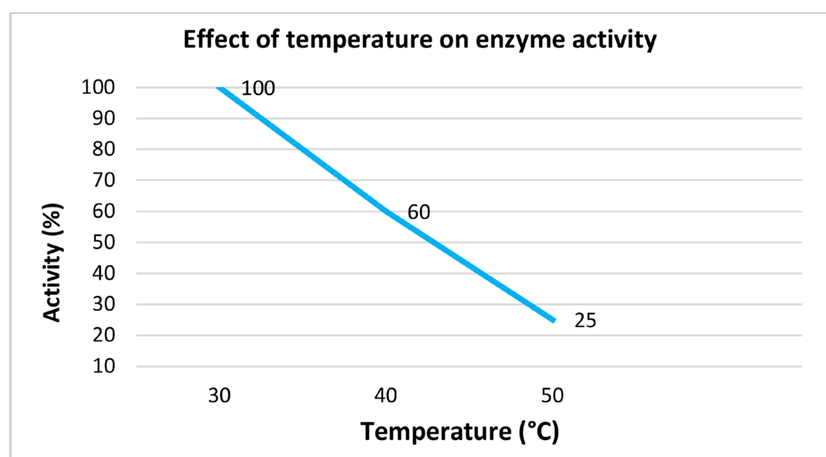

Figure 5. Incubation temperature effect in production.

4.5. Determination of the pH at Which the Enzyme Shows Optimum Activity

The protein content was determined using the standard method described by [10]. Keratinase was precipitated using Acetone precipitation. Keratinase activity and protein content of dissolved pellet and supernatant was assessed. The pellet was dissolved in Tris-HCl buffer (10 mM, pH 9.0) and dialyzed extensively for 24 h using dialysis membrane showing in the graphic in (Figure 4 and Figure 6).

4.6. Determining the Temperature at which the Enzyme Shows Optimum Activity

See Figure 7.

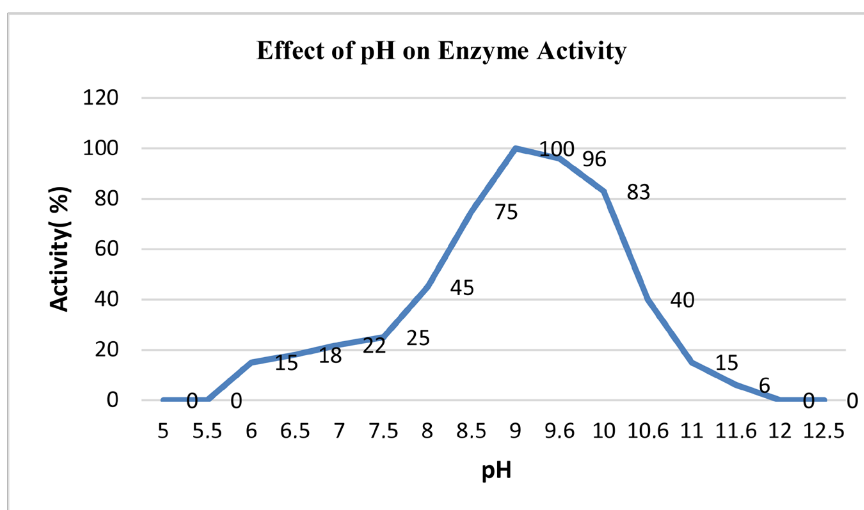

Figure 6. Effect of pH on enzyme activity.

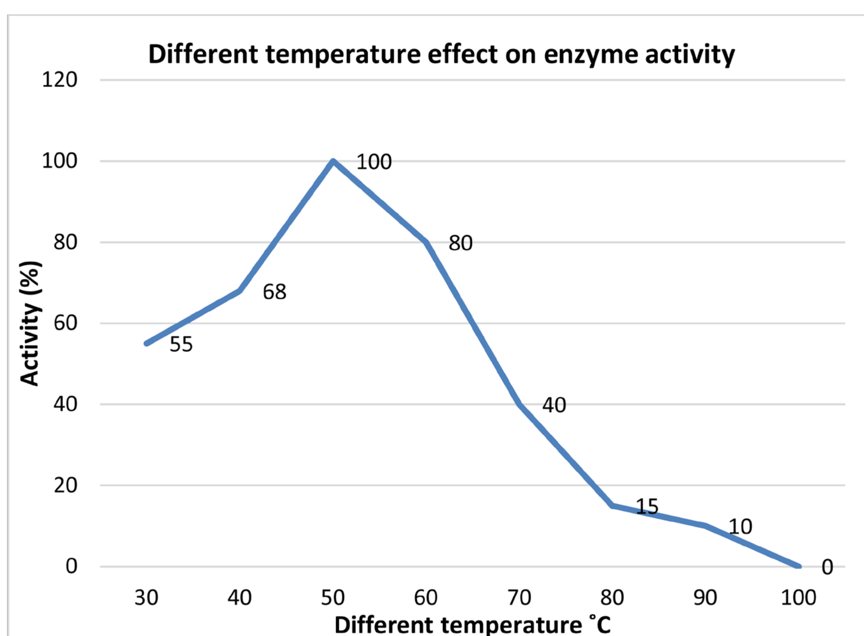

Figure 7. Effect of temperature on enzyme activity.

4.7. Determination of Thermal Stability of Enzyme

The enzyme was not able to regain activity upon inactivation and storage at 4˚C for 24 h, which indicates that the inactivation observed was irreversible [11]. Melting temperature for keratinase production is listed in Table 1.

4.8. Effect of Chemicals and Metal Ions on Enzyme Activity

Technically many researches showed immobilised cells of Bacillus sp. khayat were able to produce keratinase protease and efficiently degrade chicken feather in the presence of elevated concentration of different heavy metals ions in a submerged fermentation. Beads of the immobilised cells can be used for many cycles in the process of continual degradation of heavy metal laden feather and keratinase enzyme production. The stability of the cells in gellan gum and the ability of the beads to be used for degrading different types of heavy metal laden feather make it a potential to be utilized in the bioremediation of feather wastes and industrial production of keratinase protease [12]. The effect of chemical and Metal ions illustrated in [1] (Table 2).

Table 1. Thermal Stabilité.

Temperature (˚C)	Remaining activity (%)
50	90
60	40
70	10
80	0
90	0
100	0

Table 2. Effect of chemicals and metal ions.

Chemical	Relative activity (%) 1 mM concentration	Relative activity (%) 5 mM concentration
Control	100	100
EDTA	84	76
SDS	150	204
Urea	85	40
$ZnCl_2$	28	44
$MgCl_2$	51	72
$NiCl_2$	47	24
$CaCl_2$	56	80
$HgCl_2$	20	0
$CuCl_2$	96	83
Tween20	127	215

5. Conclusions

The gradual increase and diversity of the usage areas of enzymes and the search for economical enzymes suitable for the industry by biotechnologists and the studies in this field have gained importance. Many countries are insufficient in the production of their own enzymes and are dependent on foreign sources in this regard. The development of enzyme technology is becoming increasingly important.

According to these results, it can be said that the enzyme is mesophilic and alkaline. Considering its features, it can be said that it is an enzyme that can be used for biotechnological applications by increasing the activity with the chemicals in question.

Enzyme production is of great importance by using chicken feathers as a substrate. The sulphurous amino acids that are released can be used in many areas that require amino acid support, especially in feed and food production. In addition, while chicken feather wastes, which are seen as pollutants, are made useful, an important economy will be provided by using them in the above areas.

Conflicts of Interest

The author declares no conflicts of interest.

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