ISOLATION AND SCREENING FOR *STREPTOMYCES* STRAIN CAPABLE OF EFFICIENT FEATHER DEGRADATION

PHÂN LẬP VÀ TUYỂN CHỌN CHỦNG *STREPTOMYCES* CÓ KHẢ NĂNG PHÂN HỦY LÔNG GIA CẦM HIỆU QUẢ

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Abstract - Feathers cause environmental problems worldwide when poultry processing industries produce it as a waste of billions of tons yearly. This waste is not easily decomposed by natural agents in a short time. Currently, *Streptomyces* spp. are known as a highly enzymatic activity species which can efficiently degrade feathers to products. Therefore, the aim of this study was to screen for *Streptomyces* strains with high feather-degrading activity. A total of 46 strains of *Streptomyces* spp. were isolated from different soils of Danang city and other vicinities, in which 13 isolates showed the ability to degrade casein and gelatin. The protease activity of H3, N5, and N7 isolates were higher than others. In particular, N7 strain had the highest protease activity with 70.33 U/mL and degraded 97.79% feather after 5 days of cultivation. Based on the sequence analysis of 16S rRNA, N7 was identified as homologous to *Streptomyces anandii*.

Key words - Feather; feather degradation; *Streptomyces*; protease activity; keratine.

Tóm tắt - Hằng năm các ngành công nghiệp chế biến gia cầm thải ra hàng tỷ tấn lông gia cầm và gây ra các vấn đề về môi trường trên toàn thế giới. Loại rác thải này khó bị phân hủy bởi các tác nhân tự nhiện trong thời gian ngắn. Hiện nay, *Streptomyces* spp. được biết đến như một loài có hoạt tính enzyme cao, có thể phân hủy lông gia cầm một cách hiệu quả. Vì vậy, mục đích của nghiên cứu này là sàng lọc các chủng *Streptomyces* có hoạt tính phân hủy lông gia cầm cao. Tổng cộng có 46 chủng *Streptomyces* được phân lập từ các loại đất khác nhau của thành phố Đà Nẵng và các vùng lân cận, trong đó có 13 chủng phân lập có khả năng phân hủy casein và gelatin. Hoạt tính protease của các chủng H3, N5, N7 cao hơn các chủng khác. Đặc biệt, chủng N7 có hoạt tính protease cao nhất với 70,33 U/mL và phân hủy 97,79% lông sau 5 ngày nuôi cấy. Dựa trên phân tích trình tự của 16S rRNA, N7 được xác định là tương đồng với loài *Streptomyces anandii*.

Từ khóa - Lông gia cầm; phân hủy lông gia cầm; *Streptomyces;* hoạt tính protease; keratine

1. Introduction

The application of science and technology has created a wide variety of food products today. Along with the rapid development, by-products are also increasing and becoming an environmental issue in the world. No exception, the poultry meat processing industry produces thousands of tons of feather by-products every year. This by-product consists of approximately 90% keratin, which is difficult to decompose in nature [1]. Their accumulation contributes to environmental pollution if no appropriate treatment is taken. Besides, due to their protein nature, poultry feathers can be considered a source of nitrogen organic fertilizer for plants if they are broken down efficiently [2], [3]. These wastes were usually collected and subjected to high temperatures and pressure, then further milled to form a powder. After they're dried, they're then packaged up for use in fertilizer mixes. This process requires high energy, high cost, and emits a large amount of carbon dioxide. Limitations of conventional methods for producing readily digestible feather meals demand the use of biotechnological treatment of feather wastes as an ecologically safe and low-cost method [3]. In this respect, microorganisms that produce specific proteases (keratinase) may have an important role in biotechnological processes involving keratin-containing wastes by developing non-polluting processes [4]. When keratinases are mostly extracellular enzymes, feathers are degraded during the fermentation process where a consortium of thermophilic/mesophilic bacterial cultures such as *Bacillus, Streptomyces, Vibrio, Chryseobacterium* strains are used [5]. Despite several keratinases, there is still a necessity to identify keratinase-producing organisms for commercial applications.

Streptomycetes are a group of gram-positive filamentous bacteria, belonging to actinobacteria, that can be isolated in soil and found worldwide. Most Streptomycetes can degrade complex and persistent organic materials, such as pectin, cellulose, keratin, elastin, lignocellulose, and aromatic compounds [6]. Current research reflects on the isolation of actinobacteria from extracted soil samples, and the identification of keratinolytic Streptomycetes isolates through primary and secondary screening tests [7], [8], [9].

The aim of this study was to isolate feather-degrading *Streptomyces* strain from soils of Danang city and other vicinities, and investigate their feather-decomposing capacity.

2. Methods

2.1. Materials treatment

Chicken feathers were collected from a local poultry market (Hoa Khanh, Danang) and washed with tap water. The washed feathers were dried at 60°C for 24 h and were separated into Ziploc bags. These bags were then kept at 4°C for subsequent analysis [10].

2.2. Isolation of Streptomyces

Soil samples were taken from different places in Danang city and other vicinities in Vietnam. To isolate

Streptomyces spp., soil samples were pretreated with $CaCO_3$ (10:1 w/w) at 37°C for 4 days. Each sample was then diluted with sterile Ringer's solution at 10^{-2} and placed in a water bath at 45°C for 16 h. After that, 0.1 mL of the dilutions were inoculated on the surface of the Actinomycete Isolation Agar (Difco 0957) plates and the plates were incubated at 28°C for 2 days. *Streptomyces* colonies that appeared on the incubated plates were transferred to the Inorganic Salts-Starch agar (ISP4) slants (Himedia M359) in 7 days [11]. Isolated *Streptomyces* strains were stored in 10% glycerol (v/v) at -20°C for further screening studies.

2.3. Primary screening of Streptomyces with protein degradation ability

Isolated Streptomyces strains were screened for protease production on both gelatin agar (gelatin 10.0 g/l; peptone 5.0 g/l; beef extract 5.0 g/l; agar 15.0 g/l) and casein agar (casein 3.0 g/l; KNO_3 2.0 g/l; NaCl 2.0 g/l; K_2HPO_4 2.0 g/l; $MgSO_4$ 0.05 g/l; $CaCl_2$ 0.02 g/l; agar 15.0 g/l), and incubated at 28 °C for 5 days. After incubation, clear zones that developed around the colony were considered positive for protease activity. The selected strains consisted of both casein degradation ability and gelatin degradation ability. All samples were analyzed in duplicate.

The zone of proteolysis was calculated with the following formula:

Zone of proteolysis (cm) = D (clear zone diameter)

- d (colony zone diameter)

2.4. Secondary screening of Streptomyces with feather degradation ability

Isolates obtained from the primary screening were subjected to secondary screening with the intention to isolate the feather-degrading *Streptomyces*. Liquid whole-feather medium (whole chicken feather 10 g; beef extract 5g; peptone 5g; NaCl 5g; K_2HPO_4 1g; $MgSO_4.7H_2O$ 0.5g; $CaCl_2$ 5g; Na_2CO_3 5g, dH_2O 1000 mL, and pH 8.0) to check feather degradation ability was prepared in 250 mL Erlenmeyer flasks and sterilized at 115°C for 15 minutes. Each flask (100 mL) was inoculated with 1 mL of selected strains ($8x10^9$ CFU/mL), which was prepared in ISP4 liquid medium and incubated for 2 days [10]. The control flask was carried out the same way without *Streptomyces* strain and was not autoclaved. After that, feather meal was filtered through the filter membrane (pore size = 0.2 mm), and residual feathers were dried at 50°C till constant weight to calculate the rate of feather degradation. The degradation rate was determined as follows: chicken feather degradation (%) = [(dry weight of feathers before degradation - dry weight of feathers after degradation)/ dry weight of feathers before degradation]x100. The residual solution was centrifuged at 10000xg for 10 min and the supernatant was used as a crude enzyme solution for the following studies.

2.5. Protease activity

Protease activity was conducted with the Folin–Ciocalteu method [12]. Briefly, 1 ml of casein 1% (w/v) was preincubated for 15 min at 37°C. The reaction was initiated by the addition of 1 ml of crude enzyme solution and kept at 37°C for 1 h reaction. Then, 2 ml of trichloroacetic acid 0.4 M was added to terminate the reaction and the mixture was allowed to stand at room temperature for 25 min. The reaction mixture was centrifuged at 8000 rpm at room temperature for 10 min to collect the supernatant. 150 µl of supernatant was added with 750 µl of sodium carbonate 0.4 M and 200 µl of Folin Ciocalteau's reagent. The mixture was incubated for 20 min at 40°C in the dark and the absorbance of the mixture then was measured at 660 nm. The tyrosine standard curve was established to convert the corresponding enzymatic activity. One unit of protease activity was defined as the amount of enzyme required to release 1µg of tyrosine per minute under experimental conditions. All samples were analyzed in triplicate.

2.6. Identification of Streptomyces strains

All isolates were morphologically observed to confirm *Streptomyces* characterized [6]. The selected strain was identified by rRNA 16S sequence and compared to the sequences presented in the GENBANK network at the National Center for Biotechnology Information (NCBI) using BLAST software. This work was done by VNU Institute of Microbiology and Technology

2.7. Statistical analysis

Measurements described with mean± S.D were from at least three replicates. Data were calculated by Microsoft Excel.

3. Results and Discussion

3.1. Isolation of Streptomyces

A total of 46 strains were isolated from 8 different soil areas, of which, 8 strains were from Hue city, 19 strains were from Danang city, 12 strains were from Quang Binh province and 10 strains were from Quang Ngai province. All strains grew at 28°C on ISP4 medium for 7 days. The aerial mycelium color of the isolates ranged from light white, ivory white, pink, light pink, deep pink, grey, black grey, yellowish green, and grayish olive (Figure 1). *Streptomyces* strains can produce a wide variety of pigments represented by the color of aerial mycelia [6]. Otherwise, some pigments can diffuse into the agar medium. In this study, the H7, M1, M'2, and H8 isolates produced colored soluble pigments (Figure 1).

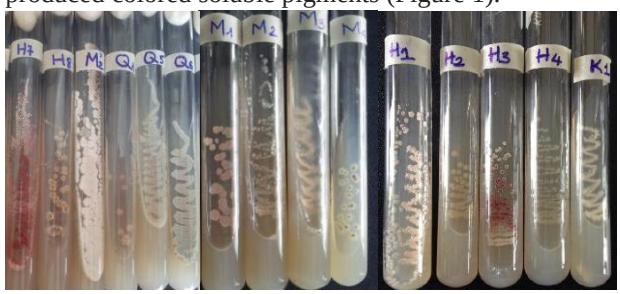

Figure 1. *Morphological characterization of some Streptomyces strains on ISP4 agar*

3.2. Screening of Streptomyces with protein degradation ability

All isolated strains were first screened for protease production on casein agar medium and gelatin agar medium. The zone diameters of the plates were clearly mentioned in Table 1. Among 46 strains, 16 strains were formed as the clear zone, which supported the degradation and utilization

of casein. These strains accounted for 34.78% of the total isolates. Besides, 13 strains degraded both casein and gelatin (H3, H7, K2, M1, M3, Q5, M'2, N2, N3, N4, N5, N6, N7), made up 28.26% of total 46 isolates. Furthermore, these trains showed higher casein degradation levels than gelatin degradation levels. The results indicated that casein agar medium was the best for the primary screening of feather degradation ability. In fact, casein contributes to about 78% of the total milk proteins in milk [13]. Some authors used milk agar medium or casein agar medium for the primary screening of keratinolytic actinobacteria [14], [15]. Another author also mentioned the screening of keratinolytic bacteria isolated from waste disposal sites using skim milk and casein agar medium [16].

Table 1. *Primary screening of proteolytic Streptomyces*

Isolate	Zone of hydrolysis (cm)	
	Casein agar medium	Gelatin agar medium
H3	3.05 ± 0.07	0.6 ± 0.14
H7	1.6 ± 0.14	0.55 ± 0.07
K2	3.3 ± 0.14	0.9 ± 0.14
M1	1.8 ± 0.14	0.6 ± 0.00
M3	0.85 ± 0.35	0.55 ± 0.21
Q5	**3.05 ± 0.07**	**1.05 ± 0.07**
M'2	1.8 ± 0.00	0.6 ± 0.28
N1	0.65 ± 0.07	-
N2	2.9 ± 0.14	0.75 ± 0.07
N3	2.45 ± 0.07	0.55 ± 0.07
N4	3.15 ± 0.07	0.45 ± 0.07
N5	1.45 ± 0.35	0.55 ± 0.21
N6	0.9 ± 0.14	0.2 ± 0.00
N7	**3.55 ± 0.21**	**1.55 ± 0.07**
N8	0.7 ± 0.28	-
N10	2.25 ± 0.21	-

Data are expressed as mean ± S.D of two replications

3.3. Screening of Streptomyces with feather degradation ability

The secondary screening had been done to find out the feather-degrading *Streptomyces* among the 13 positive isolates which were found in the first screening. Results showed that N3, N5, N7, and H3 isolates had higher proportion of feather degradation than others, approximately 98% after 7 days of incubation. Meanwhile, the control sample had the lowest proportion of feather degradation, at 9.78%. N2 and M3 isolates had the second lowest proportion of feather degradation, at 18.72% and 31.43%, respectively. The remaining strains were in the range of 60% to 91% of feather degradation (Figure 2).

We continued to choose N3, N5, N7, and H3 strains to check feather degradation levels in 3 days, 5 days, and 7 days. The results were shown in Figure 3.

In the first 3 days, N3 showed feather degradation ability higher than N5 and N7, however after 5 days, N7 had a dramatic increase in the proportion of feather degradation (at 97.79%) and it remained the same level after 7 days. The feather degradation proportion of N5 strains had steadily risen during 7 days. On the 7th day, N3, N5, and N7 had the same level of feather degradation ability, at just over 97%.

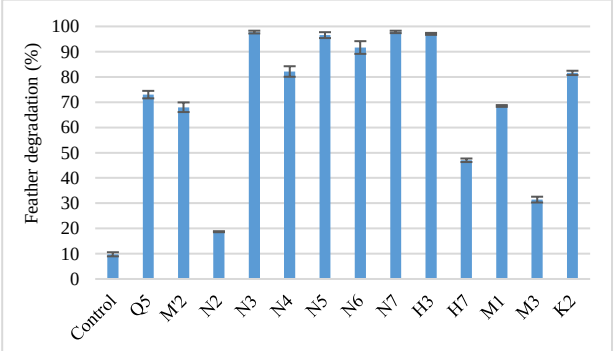

Figure 2. *Feather degradation proportion of selected strains after 7 days*

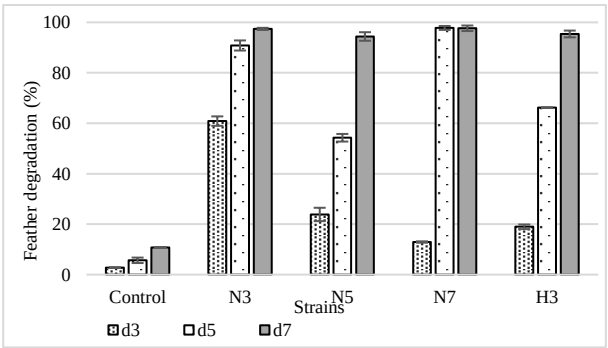

Figure 3. *Feather degradation of N3, N5, N7, and H3 isolates after 3 days, 5 days, and 7 days of incubation*

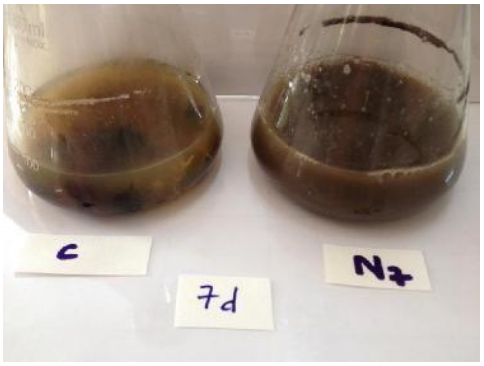

Figure 4. *Incubated solution of the control sample (C) and N7 strains (N7) after 7 days*

In feather-degrading bacteria isolated from soil, for instance, the percentage of remaining feathers was within 20–90% after 3 weeks of incubation with some isolates [17]. Other isolates completely degraded chicken feathers within 48 h [14], meanwhile another isolate completed this work with 168 h [18]. Therefore, the level of degrading whole feather seems to be highly variable among feather-degrading microorganisms.

3.4. Protease activity of selected Streptomyces strains

Due to high feather degradation ability, crude enzyme solutions produced by 4 strains (N3, N5, N7, and H3) after 7 days were collected to determine protease activity. The results were shown in Figure 5.

After 3 days, 3 isolates (N5, N7, and H3) secreted protease enzyme with high activity value. In particular, sample N7 showed the highest value of protease activity after 5 days of cultivation, which was also consistent with the result of secondary screening.

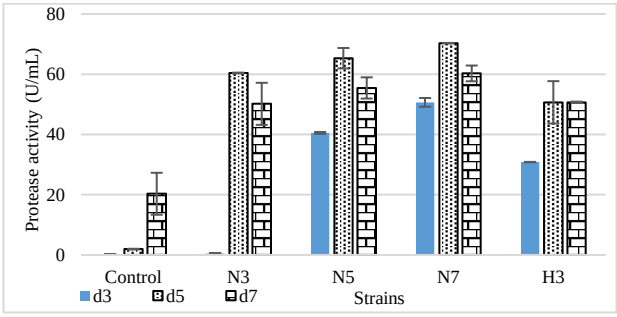

Figure 5. *Protease activity of isolated strains and control during 7 days of incubation*

The production of extracellular proteases during the stationary phase of growth is characteristic of many bacterial species [19]. Keratinases released by microorganisms are mainly extracellular enzymes [20]. For Streptomycetes, their growth transitioned to the stationary phase by day 4 or day 5 of cultivation [21]. Therefore, enzyme activity at that time usually reaches the highest value.

Due to the highest ability in degrading feather, the N7 isolate was then identified through rRNA 16S sequence.

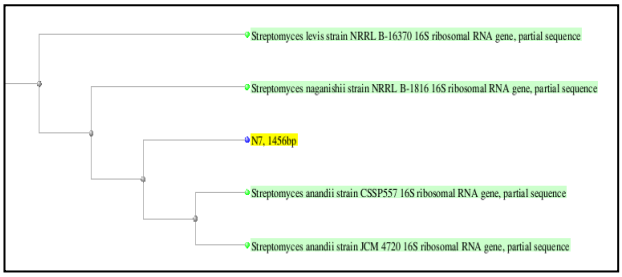

Figure 6. *Partial 16S rDNA gene sequences based on the phylogenetic tree of N7 strain and closely associated strains*

The phylogenetic tree indicated that N7 isolate belonged to the genus *Streptomyces*. The levels of similarity between the 16S rRNA gene of the *Streptomyces* species are shown in Figure 6. Accordingly, N7 isolate was identified as homologous to *Streptomyces anandii.* This strain was first identified and isolated from soil in the region of Saudi Arabia [22]. *S. anandii* was known to produce some antitumor agents such as pentaene G8, gilvocarcin V, gilvocarcin M and gilvocarcin E [23]. Therefore, the finding of this study demonstrates the other functions of this strain including feather degradation.

4. Conclusion

In the current study, 46 strains of *Streptomyces* capable of decomposing chicken feathers were isolated from the soil of Danang city and surrounding areas. Based on the results presented here, the protease activity of H3, N5, and N7 isolates were higher than others. Especially, N7 strain showed the highest proportion of feather degradation, approximately 97.79% with highest protease activity at 70.33 U/mL after 5 days of incubation with chicken feather medium. N7 was identified as homologous to *Streptomyces anandii* by 16S rRNA gene sequencing. This result suggests the use of *Streptomyces anandii* to treat feather meal as fertilizer for plants.

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