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**Research Article**

# INVESTIGATION OF SOME CULTURE CONDITIONS AND NUTRITIONAL SOURCES AFFECTING THE BIOSYNTHESIS OF FIBRINOLYTIC ENZYME OF *Bacillus* sp. ES4

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**ABSTRACT**

*Bacillus* sp. *can biosynthesize fibrinolytic enzymes. Culture conditions and nutrient sources are important factors affecting microbial growth and production of the Fibrinolytic enzyme. Therefore, we investigated the culture conditions of the strain Bacillus sp. ES4, such as temperature, pH, C nutrient source (glucose, sucrose, glycerol, and maltose), N source (yeast extract, meat content, peptone, and tryptone), metal ions ($Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Zn^{2+}$, $K^{+}$, $Fe^{2+}$, and $Cu^{2+}$), and time to obtain enzyme product. The results show that for Bacillus sp. ES4, the best C source was glucose, peptone and yeast extract. Ca2+ and Mg2+ are nutritional factors that greatly influence the biosynthesis of the fibrinolytic enzyme of strain ES4. At the culture conditions of 37$^0$C and pH = 6.5, the strains for the best enzyme activity at 24 hours and the enzyme activity of the strain when cultured in a 250 ml flask containing 50 ml of medium for the highest enzyme activity at 450±25. FU/ml.*

***Keywords:*** *Bacillus* sp; culture conditions; enzyme production; fibrinolytic enzyme; optimal nutrient medium

## 1. Introduction

Fibrinolytic enzymes are known to have many advantages in the prevention and treatment of blood clots and many other popular acute and chronic diseases, such as cerebrovascular accidents, myocardial infarction, stroke, and cardiovascular diseases (Zu et al., 2010). It can be eaten directly and has been proven safe during use (Weng et al., 2017). Fibrinolytic enzymes are mainly synthesized by bacterial strains isolated from traditional fermented food sources in Asian countries such as Japan (Sumi, 1987), Korea (Jeong et al., 2009; Yao et al., 2020), China (Wang et al., 2008; Chen et al., 2013; Hu et al., 2019), India (Sharma et al., 2020), Thailand (Chantawannakul et al., 2020), Indonesia (Afifal et al., 2014;

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Yanti, 2018), and Vietnam (Anh et al., 2015; Uyen et al., 2015; Huy et al., 2016; Thu et al., 2020). Isolated strains that can biosynthesize fibrinolytic enzymes include *B. subtilis*, *B. amyloliquefacien*, *B. cereus*, *B. licheniformis*, *Pseudomonas aeroginosa*. The majority are strains of *Bacillus* sp. (Raju et al., 2017; Yogesh et al., 2017; Singh et al., 2018).

The *Bacillus* sp. has high adaptability to the culture medium. However, the enzyme activity biosynthesized from *Bacillus* sp depends on many factors, such as bacterial strains, culture conditions, nutrient composition, and methods. Before that, there have also been studies on optimizing culture conditions and nutrient media of strains to increase enzyme activity, such as those by Liu et al. (2005), Wang et al. (2009), Agrebi et al. (2009), Mahajan et al. (2010), Eldeen et al. (2015), Tuan et al. (2015), Huy et al. (2016), Smitha et al. (2018), and Ju et al. (2019). Therefore, to obtain fibrinolytic enzyme products with high activity, it is necessary to investigate the factors affecting the biosynthesis of enzymes of each strain. In this study, we investigated several factors affecting the production of fibrinolytic enzymes of *Bacillus* sp. ES4 strain, including nutrient sources, culture conditions, and time obtaining enzyme during culture. *Bacillus* sp. ES4 is a mutant strain that has high biosynthesis of the fibrinolytic enzyme. This result is the basis for improving the enzyme activity of *Bacillus* sp. ES4.

## 2. Material and method

### *2.1. Microorganism and media*

*Bacillus* sp. ES4 was obtained from the collection at the School of Biotechnology and Food Technology/Hanoi University of Science and Technology (Bui et al., 2022). The strain was stored at -800C.

The medium for strain activation and propagation is Luria-Bertani (LB) medium consisting of Peptone (10g/l), yeast extract (5g/l), and NaCl (5g/l), solid medium adding agar (15g). /l). The fermentation medium GYP composed of glucose 10 g/L, yeast extract 5 g/L, peptone 5 g/L, NaCl 5 g/L, MgSO4 0.25 g/L, and CaCl20.5 g/L.

### *2.2. Method*

*2.2.1. Strain activation method*

Before use, this strain was activated on an LB agar plate and incubated at 37°C for 24 h. Colonies growing on agar plates were transferred to propagation medium in a 250 ml flask containing 50 ml of LB medium at 37°C for 12-14 h before use.

*2.2.2. Culture method to obtain enzymes*

After being activated and propagated after 12-14 hours, the strain will be transferred to a 250 ml flask containing 50 ml of GYP medium (OD = 0.2). Culture strain $37^0$C, shake 150 rpm for 24 hours, enzyme solution obtained after centrifugation to remove cell biomass at 10,000 rpm, temperature $4^0$C for 10 minutes.

*2.2.3. Fibrinolytic enzyme activity assay*

The fibrinolytic enzyme activity was determined according to the method described by Thanh et al. (2022). One unit of the fibrinolytic enzyme activity was defined as the amount of enzyme that increased absorbance at 275 nm, equivalent to 1 μg of tyrosine per minute at $37^0$C.

*2.2.4. Determine cell density*

Cell growth was monitored by measuring the optical density (OD) of the culture medium at 600 nm using a spectrophotometer (Shimadzu UV-1800, Japan).

*2.2.5. Effect of carbon sources on enzyme production*

Different strains of bacteria will have different nutritional needs for growth and enzyme production. Therefore, we investigated the effects of the carbon source on the ability to produce fibrinolytic enzymes of *Bacillus* sp. ES4, including glucose, sucrose, maltose, and glycerol. Based on the composition of the GYP medium, the carbon source in the medium was changed after 24 hours of culture shaking at 150 rpm at pH = 7 and a temperature of $37^0$C. Measure the enzyme activity to select a carbon source for the enzyme-producing strain.

*2.2.6. Effect of nitrogen sources on enzyme production*

Nitrogen is also the primary source of nutrients for bacterial growth and enzyme biosynthesis. Therefore, to determine a nitrogen source for the *Bacillus* sp. ES4 strain to biosynthesis fibrinolytic enzymes, we investigated the effects of nitrogen sources, including yeast extract, peptone, meat extract, and tryptone. Based on the composition of the GYP medium with a selected carbon source according to Section 2.2.5 and a changed nitrogen source, after 24 hours of culture shaking at 150 rpm at a temperature of $37^0$C, pH=7.0. Measure the enzyme activity to select a nitrogen source for the enzyme-producing strain.

*2.2.7. Effect of metal ions on enzyme production*

In addition to the effects of carbon and nitrogen sources, enzyme-producing strains are also affected by metal ions in the culture medium (Cui et al., 2008; Nascimento et al., 2015; Wu et al., 2019 and Salunke et al., 2019). Therefore, we investigated the effects of metal ions, such as $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $K^{+}$, $Zn^{2+}$, $Cu^{2+}$, and $Fe^{2+,}$ on the enzyme biosynthesis ability of the strain, adding metal ions in the form of salts at a concentration of 5 mM ($CaCl_2$, $MgSO_4 \cdot 7H_2O$, $MnCl_2.4H_2O$, $KH_2PO_4$, $ZnSO_4$, $CuSO_4.5H_2O$, and $FeSO_4.7H_2O$). Based on a GYP medium with C and N sources as defined in 2.2.5 and 2.2.6, with metal ions in a variable medium. The strain was cultured at $37^0$C, pH=7 for 24 hours at a shaking speed of 150 rpm, measuring enzyme activity and selecting metal ions for enzyme-producing strains.

*2.2.8. Effect of culture temperature on enzyme production*

The temperature and pH of the culture medium affect the growth and synthesis of enzyme products of the strain. Therefore, to determine the temperature of *Bacillus* sp. ES4, we investigated the temperature of the culture medium at the values of $30^0$C, $35^0$C, $37^0$C,

$40^0$C, and $45^0$C with pH 7.0. After incubation, shake at 150 rpm for 24 h. Measure enzyme activity to determine the appropriate culture temperature for the strain.

*2.2.9. Effect of environmental pH on enzyme production*

Based on determining the appropriate temperature for enzyme-producing strains in Section 2.2.8, we continue to investigate the influence of the initial pH of the medium on the enzyme's ability to produce enzymes in the culture medium at pH = 5. 5; 6.0; 6.5; 7.0; 7.5 and 8.0. Culture strains were shaken at 150 rpm for 24 hours. Enzyme activity was measured to select the appropriate pH value for the strain.

*2.2.10. Effect of the culture time to obtain enzymes of the strain*

To determine the appropriate time to collect fibrinolytic enzymes of *Bacillus* sp. ES4, we cultured the strain on 50 mL of GYP medium with a determined carbon source, nitrogen source, metal ions, pH, and temperature of the culture medium. After being cultured in flasks for 26 hours and shaken at 150 rpm, samples were taken hourly to measure OD and enzyme activity. The appropriate time to obtain enzyme is at the highest enzyme activity of the strain.

*2.2.11. Statistical analysis*

Process the data with SPSS software, which was used for all statistical analyses.

## 3. Results and discussion

### *3.1. Results on the effects of carbon sources*

This strain is grown on media with modified carbon sources: glucose, sucrose, maltose, and glycerol. The effect of the carbon source on the enzyme activity of the strain is shown in Figure 1.

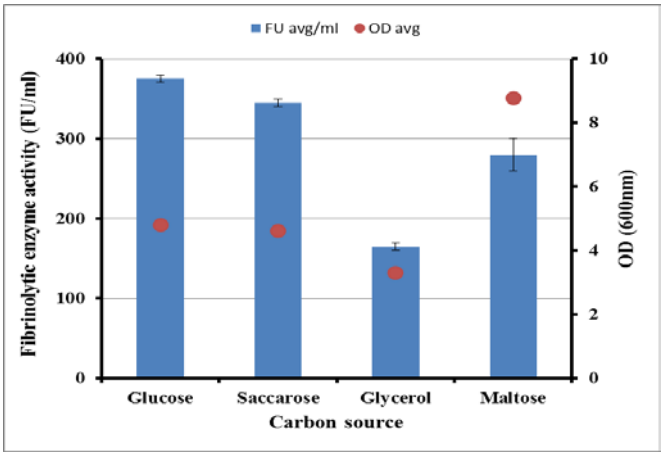


***Figure 1.*** *Effect of carbon sources on enzyme product*

Figure 1 shows that, among the four carbon sources, glucose gave the highest enzyme activity with 375±5 FU/ml, followed by sucrose (345±5 FU/ml), maltose (280±20 FU/ml), and glycerol (165±5 FU/ml). For the glycerol source, the lowest enzyme activity was not equal to half of the glucose source. Therefore, we used the carbon source as glucose to increase the enzyme product of the strain. Some previous studies used glucose as a carbon source to improve the ability to obtain fibrinolytic enzymes (Wang et al., 2009; Kwon et al.,

2011; Tuan et al., 2015). Some studies used glycerol in the culture medium to increase fibrinolytic enzyme production, such as Unrean et al. (2012) and Cui et al. (2020). Xiao et al. (2015) used maltose in the culture medium to increase enzyme production. Vijayaraghave et al. (2017) used sucrose in the culture medium to increase the enzyme product. Thus, carbon sources have different effects on bacterial strains that synthesize fibrinolytic enzymes.

### *3.2. Results on the effects of nitrogen source*

Based on the glucose selected, four nitrogen sources, including peptone, yeast extract, meat extract, and tryptone, were investigated for their effects on the enzyme product of the strain. The results of the effects of nitrogen sources are shown in Figure 2.

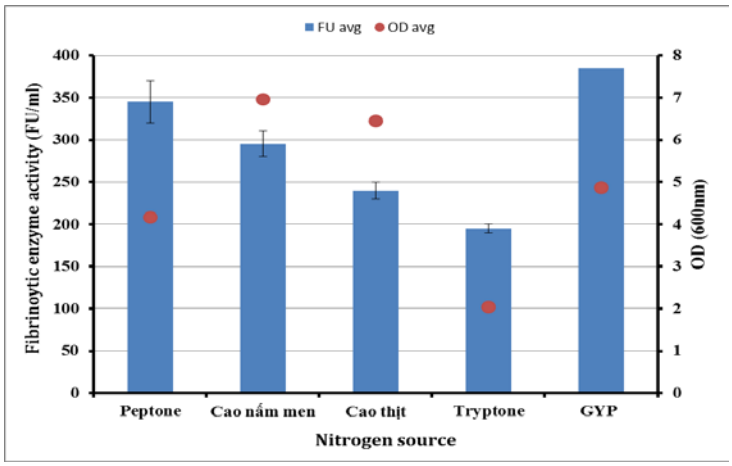


***Figure 2.*** *Effect of nitrogen sources on enzyme product*

From Figure 2, we see that the nitrogen source was peptone, and yeast extract had higher enzymatic activity than the meat extract and tryptone sources; the highest enzyme activity was 345±25 FU/ml when the strain used a peptone source, and the lowest was a tryptone source reaching 195±5 FU/ml. However, using the combination of two sources of peptone and yeast extract available in the composition of the GYP medium, the enzyme activity of the strain was higher than that of the peptone source (385±5 FU/ml). This has shown that yeast extract and peptone in the culture medium are more effective. Therefore, to obtain high enzyme activity of the strain, we used peptone and yeast extracts as nitrogen sources in the culture medium. Before, Deepack et al. (2008) and Tuan et al. (2015) used peptone in culture to increase the enzyme activity of strains. Vijayaraghave et al. (2015) used meat extract to increase the enzyme activity of the strain. From the above results, different bacterial strains have different needs for nitrogen sources. Therefore, to obtain high enzyme activity of the strain, we used peptone and yeast extracts as nitrogen sources in the culture medium.

### *3.3. Results on the effects of metal ions*

In culturing bacteria, the presence of metal ions in the medium also affects enzyme activity. Figure 3 depicts the effect of metal ions on the results.

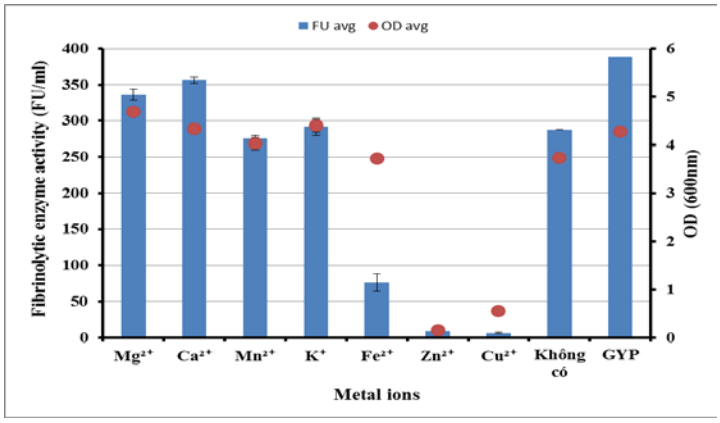


***Figure 3.*** *Effect of metal ions on enzyme product*

Enzyme activity can increase, decrease, or be inactivated in the media content metal ions. Figure 3 shows that for *Bacillus* sp. ES4, enzyme activity was higher on medium containing metal ions $Ca^{2+}$, $Mg^{2+}$, and $K^{+}$ (356±4 FU/ml, 336±8 FU/ml, and 292±12 FU/ml respectively) than on medium without metal ions (288±0 FU/ml). For the four metal ions $Mn^{2+}$, $Fe^{2+}$, $Zn^{2+}$ , and $Cu^{2+}$, when added to the culture medium, enzyme activity was lower compared to the non-added medium, indicating that the above metal ions can inhibit enzyme activity. Especially for metal ions $Zn^{2+}$ and $Cu^{2+}$, enzyme activity 9.2±1.2 FU/ml and 6.4±0.8 FU/ml, respectively, and enzyme activity has been lost (inactivated). However, when added to the two metal ions $Ca^{2+}$ and $Mg^{2+}$ in the medium, the enzyme activity of the obtained strain was higher than the medium added one ion, reaching 388±12 FU/ml. This result is similar to the study of Nascimento et al. (2015), which proves that the addition of $Ca^{2+}$ and $Mg^{2+}$ in the GYP medium is efficient. This result is also consistent with some previously published studies showing that the metal ions $Ca^{2+}$ and $Mg^{2+}$ affect the biosynthesis of enzymes of the strain (Deepak et al., 2008; Tuan et al., 2015; Xiao et al., 2015; Wu et al., 2019).

### *3.4. Results of the effect of culture temperature*

The effect of temperature on the enzyme activity of the strain during culture is shown in Figure 4.

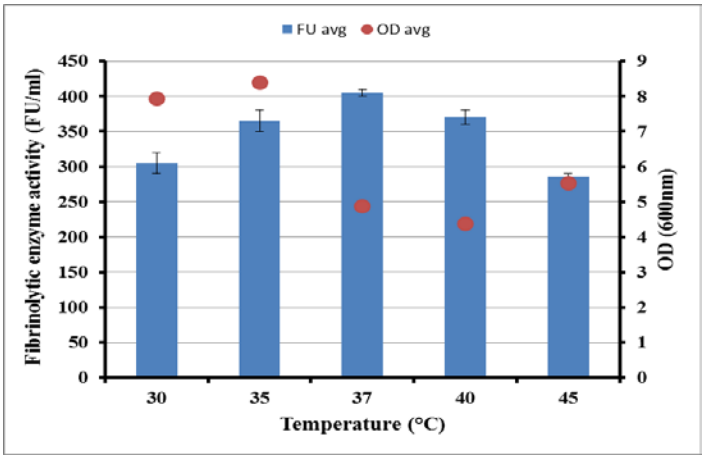


***Figure 4.*** *Effect of culture temperature on enzyme product*

Figure 4 shows that *Bacillus* sp. ES4 had the highest enzyme activity of 405±5 FU/ml when cultured at $37^0$C and the lowest enzyme activity at $45^0$C (285±5 FU/ml). This shows that, at each temperature value, bacterial strains had different enzyme activities, and when the temperature is high, the enzyme is easy to lose activity. Besides, when the temperature is high, the strain grows slowly, affecting the biosynthesis of the strain's enzyme. Thus, for *Bacillus* sp. ES4, a culture temperature of $37^0$ C was used to obtain the fibrinolytic enzyme.

### *3.5. Results of the effect of pH culture medium*

In the culture of bacteria to obtain enzymes, the pH of the initial culture medium also affects the growth and enzyme production of the strain. For *Bacillus* sp. ES4, the results of the investigation on the influence of culture medium pH are presented in Figure 5.

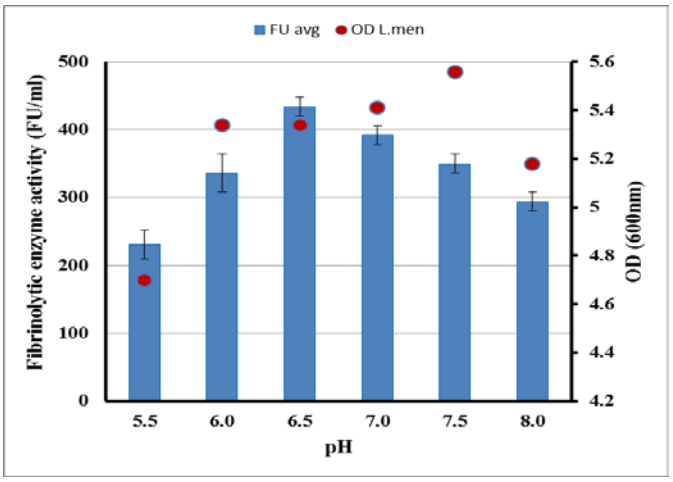


***Figure 5.*** *Effect of pH culture medium on enzyme product*

From Figure 5, it can be seen that, at pH 6.5, the enzyme activity of the strain reached the highest value of 434±14 FU/ml. At pH 7, the enzyme activity of the strain reached 392±14 FU/ml. At pH 5. 5, the enzyme activity of the strain was the lowest at 231±21 FU/ml. This shows that, at each pH value, bacterial strains give different enzyme activities. Therefore, the pH of the culture medium affects the enzyme activity of the strain. Strain Bacillus sp. ES4 at medium pH 6.5 had the highest enzyme activity, which is the suitable pH for the strain to grow and biosynthesize.

### *3.8. Result of the effect of the culture time to obtain enzymes of the strain*

Strains of bacteria grown on different nutrient mediums under different culture conditions will have a different time to obtain enzymes—Strain *Bacillus* sp. ES4 was investigated for the culture time to obtain enzymes; the results are shown in Figure 6.

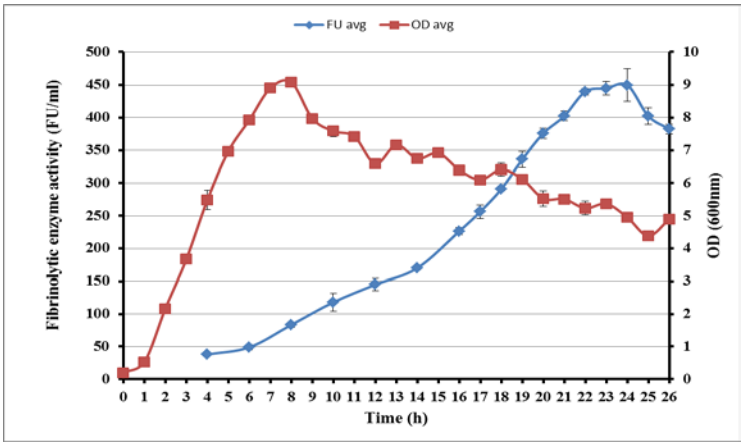


***Figure 6.*** *Effect of the culture time to obtain enzymes of the strain*

From Figure 6, it can be seen that the bacterial strain developed the strongest biomass at the time of culture from 2 hours to 7 hours, and the OD value reached the highest at 8 hours (OD = 9.085). After 8 hours of culture, strains begin to enter the equilibrium phase. Enzyme products were obtained at the end of the equilibration phase; the highest enzymatic activity of the strain was at 24 hours (450±25 FU/ml), followed by enzyme activity at 23 hours and 22 hours (445±10 FU/ml and 440 ±5 FU/ml, respectively). After 24 hours, the enzyme activity of the strain tended to decrease. At 25 hours, the enzyme activity decreased to 402.5±12.5 FU/ml. This can also be due to many reasons.It could be that during the culture process, bacteria can produce substances that are detrimental to the enzyme, that the enzyme is unstable in the culture medium, or bacterial strains can produce inhibitors that lead to a decrease in enzyme activity. The *Bacillus* sp. ES4 strain had the highest enzyme activity of 450±25FU/ml at 24 hours. Therefore, we chose to obtain enzymes of strain cultured in 250-ml flasks at 24 hours for further studies.

## 4. Conclusion

Strain *Bacillus* sp. ES4 was cultured on a medium with a carbon nutrient source of glucose, a nitrogen source of peptone, and yeast extract, with the addition of metal ions Ca2+ and Mg2+ for better biosynthesis of fibrinolytic enzymes compared to other sources.

At a temperature of 37 degrees, the initial pH of the medium was 6.5; Bacillus sp. ES4 for the highest fibrinolytic enzyme biosynthesis.

Strain *Bacillus* sp. ES4 has the highest enzyme activity at 24 hours, reaching 450±25 FU/ml in GYP medium at 37°C and pH = 6.5.

❖ ***Conflict of Interest:*** *Authors have no conflict of interest to declare.*

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# KHẢO SÁT ĐIỀU KIỆN NUÔI CẤY VÀ NGUỒN DINH DƯỠNG ẢNH HƯỞNG ĐẾN QUÁ TRÌNH SINH TỔNG HỢP ENZYME TIÊU SỢI HUYẾT CỦA CHỦNG *Bacillus* sp. ES4

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## TÓM TẮT

*Vi khuẩn Bacillus* sp. *có khả năng sinh tổng hợp enzym tiêu sợi huyết. Điều kiện nuôi cấy và nguồn dinh dưỡng là những yếu tố quan trọng ảnh hưởng đến sự phát triển và sản xuất enzyme của chủng. Vì vậy, trong nghiên cứu này, chúng tôi khảo sát điều kiện nuôi cấy của chủng Bacillus* sp. *ES4 như nhiệt độ, pH, nguồn dinh dưỡng C (glucose, sucrose, glycerol và maltose), nguồn dinh dưỡng N (cao nấm men, cao thịt, peptone và tryptone), các ion kim loại (Ca2+, Mg2+, Mn2+, Zn2+, K+, Fe2+ và Cu2+) và thời gian thu nhận sản phẩm enzyme. Kết quả cho thấy đối với Bacillus* sp. *ES4, nguồn C tốt nhất là glucose; nguồn N tốt nhất là pepton và cao nấm men; Ca2+ và Mg2+ là những yếu tố dinh dưỡng có ảnh hưởng lớn đến quá trình sinh tổng hợp enzym tiêu sợi huyết của chủng. Ở điều kiện nuôi cấy 37$^0$C và pH = 6,5, chủng cho hoạt độ enzym tốt nhất ở 24 giờ, chủng nuôi cấy trong bình tam giác 250ml chứa 50ml môi trường cho hoạt độ enzym cao nhất đạt 450±25. FU/ml.*

***Từ khóa:*** *Bacilus* sp.; điều kiện nuôi cấy; sản xuất enzyme; enzyme tiêu sợi huyết; môi trường dinh dưỡng tối ưu