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Improvement of Fibrinolytic Enzyme Production from *Bacillus* sp. ES4 by Response Surface Methodology and Exponential Fed-Batch Fermentation

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A B S T R A C T

To improve the fibrinolytic enzyme production of the mutant strain *Bacillus* sp. ES4, the response surface methodology was used to optimize the medium composition. Thirty-four flask scale experiments were carried out, and the results were fitted to quadratic models using Design-Expert software. The ANOVA analysis revealed that medium components differently affect cell density and fibrinolytic enzyme production. At optimal condition for fibrinolytic enzyme production, enzymatic activity attained 686 ± 43 FU/mL, which is increased by 1.7-fold compared to the initial medium (404 ± 4 FU/mL). One step fed-batch fermentation, in which the nutrient for optimal enzymatic activity was exponentially feed to the culture, improved fibrinolytic enzyme activity by 5.91-fold (4,057.14 ± 57.14 FU/mL) and cell density by 11.9-fold (101.2 ± 0.005). In two steps fed-batch fermentation, the nutrient for optimal cell density was first used, then followed by feeding for optimal enzyme activity. The highest cell density (OD600nm) and fibrinolytic enzyme activity reached 142.3 ± 0.0125 and 5,300 ± 100 FU/mL, respectively after 13 h fermentation. Ultimately, the medium optimization and fed-batch strategy collectively improved fibrinolytic enzyme activity by 13.12-fold.

Introduction

Fibrinolytic enzyme (nattokinase) is a serine protease enzyme capable of enhancing plasminogen activator and inactivating plasminogen activator inhibitor. It was previously isolated from natto, a Japanese traditional food (Sumi *et al.*, 1986). Today, this enzyme is commonly used in medicine to dissolve blood clots and furthermore, to prevent the clotting (Peng *et al.*, 2005; Chen *et al.*, 2018). Fibrinolytic enzyme is synthesized by various microorganisms, especially by the genus *Bacillus* (Yogesh *et al.*, 2017). With the aim of improving the biosynthesis of fibrinolytic enzyme, many methods have been applied such as mutation (Wang *et al.*, 2008; Wang *et al.*, 2016; Baig *et al.*, 2019; Mohanasrinivasan *et al.*, 2013 and Bhavani *et al.*, 2012), optimization of media composition (Deepak

et al., 2008; Liu *et al.,* 2005; Wang *et al.,* 2009; Agrebi *et al.,* 2009; Mahajan *et al.,* 2010; Eldeen *et al.,* 2015; Smitha *et al.,* 2017; Chen *et al.,* 2007; Wang *et al.,* 2009) and random mutagenesis using UV or chemical reagents was generally used to development several *Bacillus* spp. strains (Raju *et al.,* 2013; Thakur *et al.,* 2017; Gopinath *et al.,* 2020; Srivatsava *et al.,* 2018; Hui Xu *et al.,* 2011; Meraj *et al.,* 2012; Vahed *et al.,* 2013 and Effat *et al.,* 2016).

The medium composition for fibrinolytic enzyme production was optimized using the response surface methodology. Through this approach, the concentration of various medium components such as glucose, peptone, yeast extract, potassium phosphate, calcium chloride, and sodium chloride were adjusted, resulting in an increase in fibrinolytic enzyme activity from 2-fold to 6-fold (Deepak *et al.,* 2008; Agrebi *et al.,* 2009 and Wang *et al.,* 2009). However, the optimal concentration of each medium component depended on the fibrinolytic enzyme producing strains and the medium conditions.

In fermentation technology, feeding limiting nutrients to the culture media could possibily enhance the targeting products. In 2010, Cho *et al.,* improved 2.1-fold the production of fibrinolytic enzyme by feeding a mixture of glucose and peptone to bacterial culture followingpH-stat fed-batch fermentation. Optimizing the ratio glucose and peptone in feeding nutrients, Kwon *et al.,* (2011) achieved a 4.3-fold enhancement of fibrinolytic enzyme compared to that in batch culture. In addition, these authors found that fibrinolytic enzyme production was growth associated. Exponential feeding strategy (EFS) is an open-loop fed-batch strategy in which feeding nutrients increase exponentially to support the desired specific growth rate (Ramamoorthy *et al.,* 2019). This fed-batch strategy is generally used to increase microbial biomass or growth associated products.

Previously, a *Bacillus* sp. ES4 producing fibrinolytic enzyme was isolated from fermented Vietnamese soybean. And the mutant ES4capable of producing fibrinolytic enzyme at a 5-fold higher than the wild-type strain was generated from sequential mutagenesis using UV/Ethidium bromide and ethyl methyl sulfonate (Bui *et al.,* 2022). In this study, we aim to optimize the culture medium and develop the fed-batch fermentation strategy to further improve the fibrinolytic enzyme production of *Bacillus* sp. ES4 mutant.

Materials and Methods

Microorganism and media

The mutant strain *Bacillus* sp. ES4 was obtained from the collection at the School of Biotechnology and Food Technology – HUST (Bui *et al.,* 2022). It was cryopreserved at -80^{0}C. It was cultivated in petri dishes containing Luria-Bertani (LB) agar medium at 37^{0}C, for 12-24 h before use.

Cultivation

For inoculum preparation, 50 mL of LB medium were placed in a 250 mL flask and then inoculated with a single colony on the petri dish. Cultures were inoculated at 14 h, 37^{0}C, 150 rpm. Flask fermentation was carried out in a 250 mL flask with a working volume of 50 mL GYP medium (Bui *et al.,* 2022). The fresh inoculum was inoculated in flask to achieve the cell density of 0.2($OD_{600\ nm}$). Then, the flask cultures were cultivated at 37^{0}C, 150 rpm for 24 h.

Fed-batch cultures were carried out in a 2 L bioreactor (Sartorius A Plus). Temperature and pH were controlled at 37^{0}C and pH 6.5 using HCl 2N or NaOH 2N. Dissolved oxygen (DO) level was automatically controlled at ≥ 20% by changing agitation between 200 and 1200 rpm at fixed aeration 2 vvm. Feeding was started after 4 h batch culture. The feeding rate was generated from the equation 1 (Lee *et al.,* 1997).

$$F = \frac{\mu \times V_0 \times X_0 \times e^{\mu t}}{Y_{x/s} \times (S_f - S_0)} \qquad (L.h^{-1}) \quad (1)$$

Where: μ is the specific growth rate (h^{-1}); t is the feeding time (h); V_0 is the volume of culture medium at the start of the feeding (L); X_0 is the biomass concentration at the start of the feeding ($g.L^{-1}$); $Y_{X/S}$ is the biomass yield ($g.g^{-1}$); S_F is the substrate concentration in the feeding solution ($g. L^{-1}$); S_0 is the substrate concentration in the bioreactor at the start of the feeding ($g. L^{-1}$).

Optimization of the culture medium for biomass and fibrinolytic enzyme production

An optimal design was used to study the simultaneous effect of six variables (glucose, yeast extract (YE), peptone, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, and NaCl) on the fibrinolytic enzyme production.

A total of 34 experiments with three replicates at the center points were run in a random order (Table 1). The data were analyzed by Design-Expert software.

Fibrinolytic enzyme activity assay

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

Results and Discussion

Optimization for fibrinolytic enzyme production

In this study, the response surface methodology was used to optimize the concentration of six medium ingredients for cell density and fibrinolytic enzyme production. A total of 34 experiments were carried out following Table 1. The results were analyzed using ANOVA analysis following the quadratic model for both cell density and fibrinolytic enzyme activity (Table 2). The determination coefficient (R^2) of regression equations were 0.9990 and 0.9993 for cell density and fibrinolytic activity, respectively, which indicates less than 0.1% of the variability could not be explained by the model.

The ANOVA analysis showed that the regression model of both cell density and fibrinolytic enzyme activity was significant (p-value < 0.05) (Table 2). Among the independent variances analyzed, single variances such as glucose, yeast extract and peptone significantly affected cell density and fibrinolytic enzyme activity. The effect of $MgSO_4$ was nonsignificant for both biomass and fibrinolytic enzyme activity (p-value >0.05). Meanwhile, NaCl did not affect bacterial density but affected fibrinolytic enzyme production. For the quadratic interactions between six variances, there are 5/15 interactions between glucose - YE, YE - $CaCl_2$, YE - NaCl, peptone - $CaCl_2$, YE - $MgSO_4$ affected the cell density. Meanwhile, for fibrinolytic enzyme activity, 10/15 interactions were significant.

The influence of independent variances on cell density and enzyme activity is shown in Figure 1 and the coefficients of quadratic models are given in Figure 2.

Among the independent variances affected cell density, the effect of yeast extract was the highest, 8-fold higher than that of glucose and peptone, 15-fold higher than that of $CaCl_2$, while $MgSO_4$ and NaCl had a negative influence. The ratio of these variances was 1/0.12/0.12/0.07 for yeast extract/glucose/peptone/$CaCl_2$, respectively. Meanwhile for fibrinolytic enzyme activity, the effect of variances was in the order of yeast > glucose > peptone > $MgSO_4$> NaCl, with the ratios being 1/0.84/0.7/0.11/ 0.11/ 0.04, respectively. This result indicated that the influence of independent variances on cell density and enzyme activity was relatively different. From our results, the most important variance affecting cell density was the yeast extract. It is obvious that nitrogen sources are determinants in enzyme production, particularly in the case of protease. According to Chen *et al.*, (2007), the most important factor for fibrinolytic enzyme production from the recombinant strain WB700N/pUKVI-NAT2 was soybean hydrolysate. Furthermore, Deepak *et al.*, (2008) demonstrated the importance of peptone in fibrinolytic enzyme activity in the wild-type strain *B. subtilis* 1A752. For

our mutant *Bacillus* sp. ES4, interestingly, the enzyme activity was similarly affected by yeast extract, glucose, and peptone.

Optimal conditions for cell density and fibrinolytic enzyme production were generated by Design Expert software (Table 3). Under these conditions, highest enzyme activity and biomass were predicted as 704 FU/mL and OD_{600nm} = 9.44, respectively. Experiments in shake flask (triplicate) using optimum medium after 24 h showed fibrinolytic enzyme activity of 686 ± 43 FU/mL (equivalent to 97.44% desirability) and cell density of 8.505 ± 0.096 (equivalent to 90.34% desirability). Thus, the model accurately predicted both enzyme activity and cell biomass during fermentation. An increase of 1.7-fold in fibrinolytic enzyme activity was obtained using the optimum medium compared to the initial one. Our result was of same order of magnitude as others published findings. Chen *et al.,* (2007) obtained a 1.24-fold increase in fibrinolytic enzyme production by optimizing medium composition. Deepak *et al.,* (2008) reported a 2-fold increase in fibrinolytic enzyme production by *B. subtilis* 1A752 following culture medium optimization using central composite design. Depending on strain, the optimization of medium composition may increase fibrinolytic enzyme production up to 6-fold (Wang *et al.,* 2009)

One-step fed-batch fermentation

In this study, exponential fed-batch fermentation was used to improve fibrinolytic enzyme production. The feeding solution containing glucose, YE and peptone with their proportions following the optimal conditions for fibrinolytic enzyme production (Table 3) was fed in the 2 L bioreactor. The feeding rate was calculated using equation 1 with the specific growth rate of 0.57 h^{-1} and Yx/s = 2.53 $g.g^{-1}$.

In batch mode fermentation, the cell density achieved its maximum after 6 h (data not shown). In exponential fed-batch mode, the microbial density continuously increased until t = 13 h and attained an $OD_{600\ nm}$ at 101.2 (Figure 3). This value was 7.75-fold higher than microbial biomass obtained from batch fermentation. The fibrinolytic enzyme activity was increased in parallel with cell growth and reached maximum at 4057.14 ± 57.14 FU/mL. It represents an increase of more than 4.66-fold compared to batch mode.

Cho *et al.,* (2010), performed the pH-stat fed-batch fermentation using the mixture of glucose and peptone to improve fibrinolytic enzyme production by *Bacillus subtilis*. The $OD_{600\ nm}$ reached 100 after 22 h, increased 2.5-fold compared to the batch fermentation. Furthermore, using similar pH-stat fed batch fermentation, Kwon *et al.,* (2011) achieved an improvement in 4.3-fold of fibrinolytic enzyme activity in a 5 L bioreactor. An increase of 1.13-fold fibrinolytic enzyme was achieved by feeding glycerol at 3% in a 3 L bioreactor by Berenjian *et al.,* (2014).

Two-step fed-batch fermentation

Results of the optimization experiment revealed that *Bacillus* sp. ES4 preferred two different medium compositions for cell density or fibrinolytic enzyme production (Table 3). Consequently, nutrient addition to the fermentation culture was divided into two steps based on the optimal conditions for cell density and for fibrinolytic enzyme activity. In the first step, the objective of nutrient feeding was to boost cell biomass, then the second feeding was dedicated for fibrinolytic enzyme production (Figure 4).

The highest cell density in the two- step fed-batch fermentation was 142.3 ± 0.02 after 13h, which was 1.27-fold higher than in one-step fed-batch fermentation. The highest enzyme activity was 5,300 ± 100 FU/mL, 1.3-fold higher than that of one step fed-batch fermentation, 6.09-fold greater than batch mode fermentation, and 13.12-fold higher than the initial culture.

Table.1 Medium optimization for biomass and fibrinolytic enzyme production using random order design.

Run	Variables						Enzyme activity	OD 600 nm
	A: Glucose	B:YE	C: Peptone	D: $CaCl_2.2H_2O$	E: $MgSO_4.7H_2O$	F: NaCl		
	g/l	g/l	g/l	g/l	g/l	g/l	FU/mL	
1	25.00	3.15	3.36	0.35	0.22	5.00	552	6.74
2	5.00	2.88	4.64	0.26	0.30	8.23	408	5.54
3	5.00	2.88	4.64	0.26	0.30	8.23	396	5.38
4	24.20	5.00	7.16	0.60	0.12	10.00	553	9.17
5	11.20	0.00	1.00	0.10	0.26	5.00	104	2.55
6	25.00	5.00	1.00	0.10	0.30	7.38	498	7.52
7	5.00	5.00	5.16	0.10	0.14	5.00	456	7.75
8	25.00	5.00	9.00	0.18	0.10	10.00	483	8.93
9	19.40	5.00	4.92	0.60	0.10	6.03	700	9.32
10	16.00	0.00	5.88	0.42	0.10	9.75	414	3.32
11	25.00	1.40	9.00	0.60	0.19	7.85	490	4.3
12	15.70	2.40	8.00	0.10	0.24	10.00	679	5.6
13	10.40	2.73	1.00	0.34	0.10	6.50	309	5.31
14	23.90	3.60	1.00	0.48	0.27	8.53	372	7.05
15	5.00	0.00	9.00	0.19	0.16	7.63	210	4.2
16	25.00	0.00	9.00	0.10	0.30	5.00	581	3.94
17	5.00	0.38	1.40	0.60	0.19	8.65	141	4
18	5.00	5.00	1.00	0.60	0.30	5.00	219	7.46
19	5.00	0.98	7.40	0.57	0.16	5.00	252	4.27
20	12.40	5.00	1.72	0.35	0.19	9.95	492	7.55
21	5.00	0.00	1.00	0.10	0.10	10.00	24.6	2.68
22	13.00	5.00	9.00	0.38	0.27	6.15	476	8.24
23	17.40	1.18	3.96	0.60	0.30	6.53	462	4.15
24	25.00	0.48	3.40	0.10	0.15	7.50	414	4.03
25	25.00	2.90	1.00	0.60	0.10	10.00	426	6.43
26	25.00	0.00	1.00	0.60	0.10	5.00	104	3
27	25.00	5.00	6.60	0.58	0.30	10.00	497	9.39
28	25.00	5.00	1.00	0.10	0.10	8.05	468	7.68
29	19.60	2.15	9.00	0.10	0.10	5.00	595	5.27
30	25.00	0.00	1.00	0.33	0.30	10.00	103	3.04
31	10.40	2.73	1.00	0.34	0.10	6.50	294	5.07
32	25.00	3.15	3.36	0.35	0.22	5.00	540	6.94
33	5.00	0.00	9.00	0.60	0.30	10.00	210	3.45
34	5.00	4.60	9.00	0.60	0.10	10.00	329	7.29

Table.2 ANOVA analysis for quadratic models.

Source	df	Cell density (OD 600 nm)				Fibrinolytic enzyme			
		Sum of Squares	Mean Square	F-value	p-value	Sum of Squares	Mean Square	F-value	p-value
Model	27	**143.18**	**5.30**	**219.52**	**< 0.0001**	**9.851E+05**	**36484.72**	**299.59**	**< 0.0001**
A-Glucose	1	**1.71**	**1.71**	**70.89**	**0.0002**	**1.732E+05**	**1.732E+05**	**1422.39**	**< 0.0001**
B-Yeast extract	1	**91.33**	**91.33**	**3780.81**	**< 0.0001**	**1.926E+05**	**1.926E+05**	**1581.29**	**< 0.0001**
C-Peptone	1	**1.37**	**1.37**	**56.68**	**0.0003**	**1.009E+05**	**1.009E+05**	**828.68**	**< 0.0001**
D-$CaCl_2.2H_2O$	1	**0.4523**	**0.4523**	**18.72**	**0.0049**	**16552.43**	**16552.43**	**135.92**	**< 0.0001**
E-$MgSO_4.7H_2O$	1	0.0575	0.0575	2.38	0.1738	279.82	279.82	2.30	0.1804
F-NaCl	1	0.0135	0.0135	0.5576	0.4834	**2298.63**	**2298.63**	**18.87**	**0.0049**
AB	1	**2.38**	**2.38**	**98.33**	**< 0.0001**	25.11	25.11	0.2062	0.6657
AC	1	0.0628	0.0628	2.60	0.1580	**4950.29**	**4950.29**	**40.65**	**0.0007**
AD	1	0.0748	0.0748	3.10	0.1290	**1291.78**	**1291.78**	**10.61**	**0.0173**
AE	1	0.0955	0.0955	3.95	0.0939	158.58	158.58	1.30	0.2973
AF	1	0.0167	0.0167	0.6933	0.4369	**10021.10**	**10021.10**	**82.29**	**0.0001**
BC	1	0.0659	0.0659	2.73	0.1497	**31062.11**	**31062.11**	**255.06**	**< 0.0001**
BD	1	**1.14**	**1.14**	**47.13**	**0.0005**	54.09	54.09	0.4441	0.5299
BE	1	0.1301	0.1301	5.39	0.0594	**3148.49**	**3148.49**	**25.85**	**0.0023**
BF	1	**0.1537**	**0.1537**	**6.36**	**0.0451**	**6105.37**	**6105.37**	**50.13**	**0.0004**
CD	1	**2.11**	**2.11**	**87.19**	**< 0.0001**	**6438.57**	**6438.57**	**52.87**	**0.0003**
CE	1	**0.1957**	**0.1957**	**8.10**	**0.0293**	**2226.91**	**2226.91**	**18.29**	**0.0052**
CF	1	0.0314	0.0314	1.30	0.2978	**2790.80**	**2790.80**	**22.92**	**0.0030**
DE	1	0.0073	0.0073	0.3041	0.6013	**11834.46**	**11834.46**	**97.18**	**< 0.0001**
DF	1	0.0606	0.0606	2.51	0.1644	93.08	93.08	0.7643	0.4156
EF	1	0.0557	0.0557	2.31	0.1797	142.40	142.40	1.17	0.3211
A^2	1	**0.3302**	**0.3302**	**13.67**	**0.0101**	**54585.61**	**54585.61**	**448.22**	**< 0.0001**
B^2	1	**0.6359**	**0.6359**	**26.33**	**0.0022**	**25173.16**	**25173.16**	**206.71**	**< 0.0001**
C^2	1	**0.8209**	**0.8209**	**33.98**	**0.0011**	**81269.79**	**81269.79**	**667.34**	**< 0.0001**
D^2	1	0.0494	0.0494	2.04	0.2027	**16622.05**	**16622.05**	**136.49**	**< 0.0001**
E^2	1	**0.2560**	**0.2560**	**10.60**	**0.0173**	**2425.63**	**2425.63**	**19.92**	**0.0043**
F^2	1	0.0450	0.0450	1.86	0.2211	326.02	326.02	2.68	0.1529
Residual	6	0.1449	0.0242			730.69	121.78		
Lack of Fit	3	0.0833	0.0278	1.35	0.4049	474.19	158.06	1.85	0.3132
Pure Error	3	0.0616	0.0205			256.50	85.50		
Cor Total	33	143.32				9.858E+05			

Table.3 Optimal conditions for biomass and fibrinolytic enzyme activity.

Medium	Glucose (g/L)	YE (g/L)	Peptone (g/L)	$CaCl_2.2H_2O$ (g/L)	$MgSO_4.7H_2O$ (g/L)	NaCl (g/L)
Optimal cell density	24.49	4.88	1.63	0.6	0.2	7.33
Optimal Fibrinolytic enzyme	16.66	2.75	7.44	0.1	0.24	9.65

Table.4 Comparing one and two steps fed-batch fermentation.

	First step of fed-batch fermentation		Second step of fed-batch fermentation	
	1-step fed batch	2-steps fed batch	1-step fed batch	2-steps fed batch
Glucose (g)	22.63	10.51	38.99	36.89
YE (g)	5.87	10.46	17.69	17.69
Peptone (g)	15.89	3.49	47.85	47.85
μ (1/h)	0.13	0.19	0.48	0.54
Biomass (g/L)	24.42	37.36	66.30	91.92
Fibrinolytic enzyme (FU/mL)	831.43	1002.86	2942.86	4142.86
Fibrinolytic enzyme/Biomass (FU/g)	34.05	26.84	44.39	45.07
Fibrinolytic enzyme/Glucose (FU/g)	36740.17	95419.60	75477.05	112303.06
Fibrinolytic enzyme/YE (FU/g)	141567.01	95862.77	166402.66	234256.11
Fibrinolytic enzyme/Peptone (FU/g)	52326.51	287000.19	61506.36	86586.6
Biomass/Glucose (g/g)	0.05	0.19	0.30	0.63
Biomass/YE (g/g)	0.20	0.19	0.67	1.31
Biomass/Peptone (g/g)	0.08	0.58	0.25	0.48

Fig.1 Response surface for cell density and fibrinolytic enzyme production

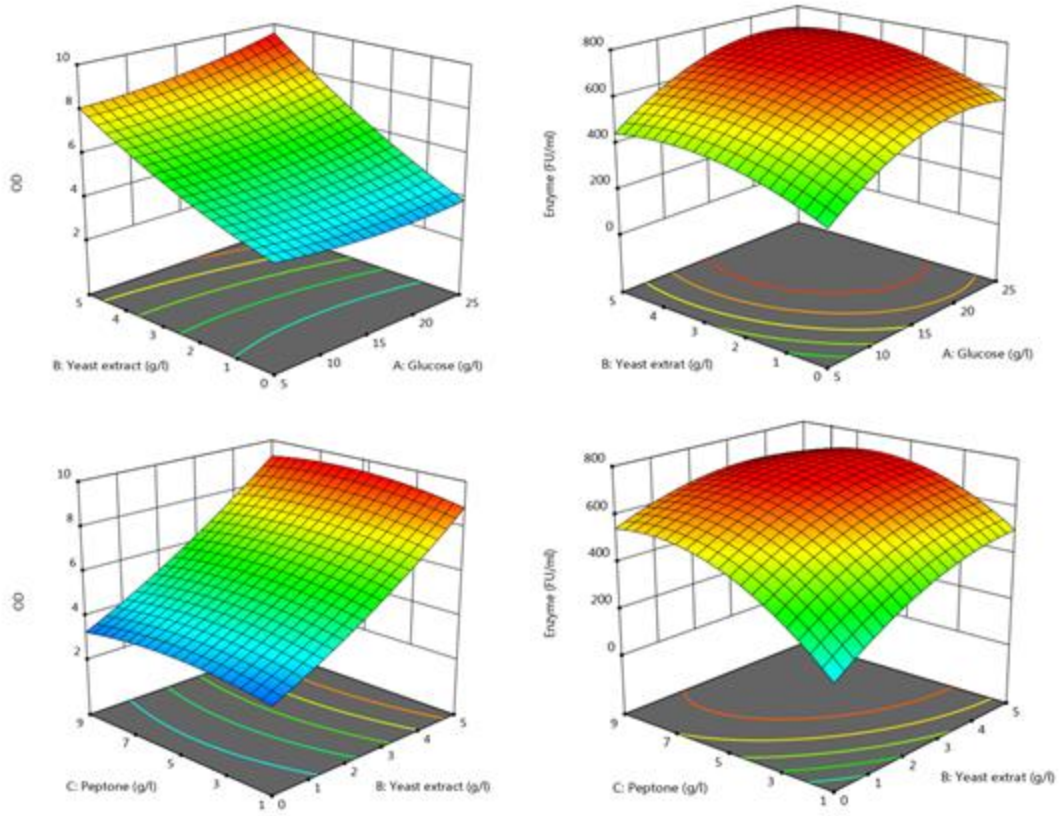


Fig.2 Coefficients of quadratic regression for cell density (dotted line) and fibrinolytic enzymeactivity (continous line) (A: glucose, B: YE, C: peptone, D: $CaCl_2$, E: $MgSO_4$, F: NaCl)

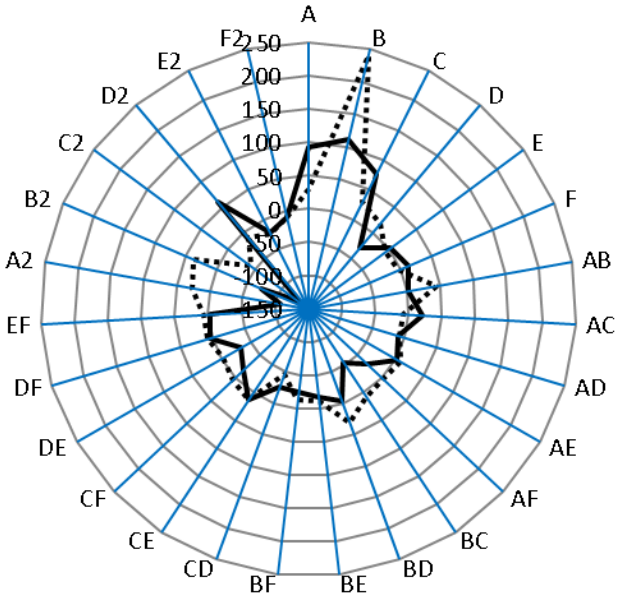


Fig.3 Kinetic of one step fed-batch fermentation (FE: Fibrinolytic Enzyme; Stirr: Stirring speed)

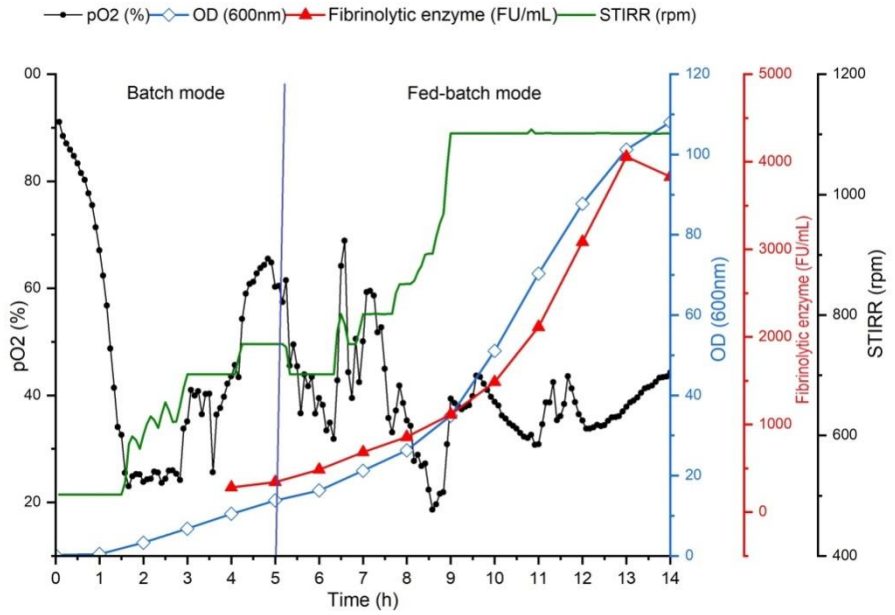
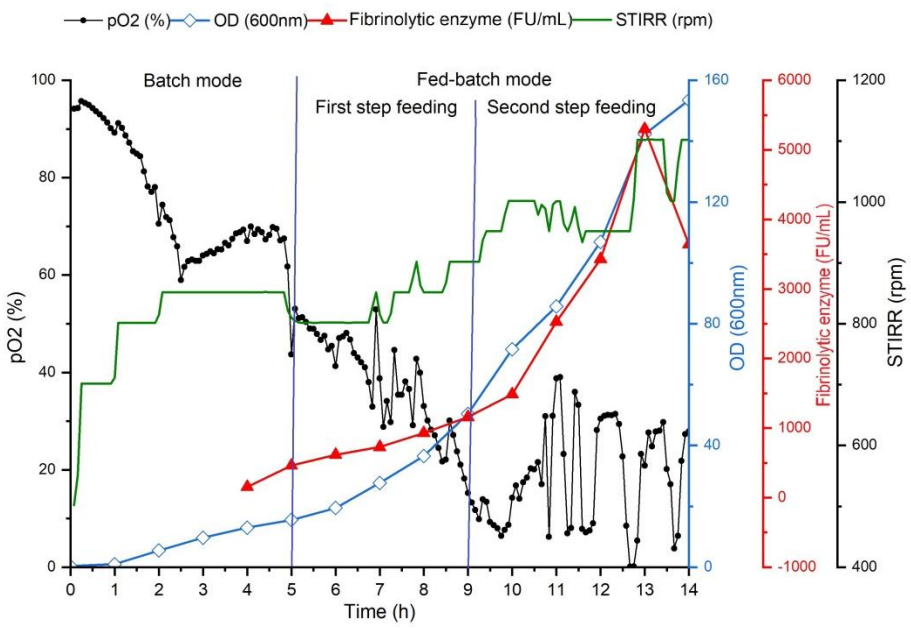


Fig.4 Kinetic of two steps fed-batch fermentation ((FE: Fibrinolytic Enzyme; Stirr: Stirring speed)



Two strategies of fed-batch fermentation were compared (Table 4). Although the total nutrient intake was similar in the second step of both experiments, the mutant *Bacillus* sp. ES4 grew more rapidly in two-step fed-batch mode, and the efficiency of using the substrate for the biosynthesis of biomass and fibrinolytic enzyme was higher than in one- step fed-batch fermentation.

The response surface methodology was conducted to

optimize the medium composition of *Bacillus* sp. ES4 for cell density and fibrinolytic enzyme production. The maximum activity of fibrinolytic enzyme reached 686 ± 43 FU/mL using medium containing glucose (16.66 g/L), YE (2.75 g/L), peptone (7.44 g/L), $CaCl_2.2H_2O$ (0.1 g/L), $MgSO_2.7H_2O$ (0.24 g/L), and NaCl (9.65 g/L) One-step exponential fed-batch fermentation using the nutrient suitable for fibrinolytic enzyme production enhanced 5.91-fold enzyme activity. Moreover, in two-step exponential fed-batch fermentation, 5,300 FU/mL fibrinolytic enzyme activity was obtained, which was 13.12-fold higher than the initial culture. Further research should be focused on scaling up fermentation for fibrinolytic enzyme production by this strain at pilot scale.

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