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Optimization of fibrinolytic enzyme production by newly isolated *Bacillus subtilis* Egy using central composite design

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ABSTRACT

In the present study a fibrinolytic enzyme producer was isolated and identified as *Bacillus subtilis* using 16S rDNA sequencing. Central Composite Design was used for optimization of enzyme production using fodder yeast as a cost effective growth medium. The obtained results revealed that fodder yeast concentration, incubation temperature, aeration level followed by yeast extract concentration and incubation period are significant factors affect the enzyme production yield by the tested organism. Optimum levels of the selected variables were 3.05% fodder yeast, 0.71% yeast extract, initial pH 7,20% aeration level, 3.2% inoculum size (16 × 10^6CFU), 36.7 °C incubation temperature and 4 days incubation period. At these conditions the predicted enzyme activity was 18.9 U/ml and the practical enzyme activity was 16.6 U/ml which revealed that the model was valid by 87.83%. The results were discussed in the light of possible application as a thrombolytic agent.

1. Introduction

Formation of a blood clot in a blood vessel, intravascular thrombosis, is the main cause of cardiovascular diseases. Heart attacks and strokes are usually acute events and are mainly caused by a blockage that prevents blood from flowing to the heart or brain. About 17.9 million people died from cardiovascular diseases in 2016, representing 31% of all global deaths (http://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)) and Zipes et al. (2018).

Microbial fibrinolytic enzymes are agents that dissolve the fibrin clots.

Fibrinolytic agents are highly effective therapy for cardiovascular diseases. These fibrinolytic agents were classified into two groups namely plasminogen activators and plasmin like protein type fibrinolytic enzyme as reported by Yong et al. (2005). Thrombolytic drugs such as streptokinase, urokinase, and tissue plasminogen activator (t-PA) were found to cause unrequired side effects such as gastrointestinal bleeding and sometimes allergic reactions (Wang et al., 2006a,b). Therefore, searching for new, effective and safe sources of fibrinolytic enzyme is a challenge (Xin et al., 2018). Gram-positive *Bacillus* species are among the bacterial champions in enzymes production. In biotechnological processes, *Bacillus subtilis* has become the most popular due to their excellent fermentation properties, high product yields and the complete absence of undesirable by-products. *Bacillus subtilis* has been intensively studied over many years and it is presently the best-characterized safe bacterium (Earl et al., 2008). Submerged fermentation, a process in which microorganisms grow in liquid medium, with high content of free water. Bioprocesses carried out in submerged fermentation have notable advantages regarding instrumentation and control (monitoring of temperature, pH, dissolved oxygen, concentration of water soluble nutrients), separation of biomass after the fermentation, mixing and scaling up (Farinas, 2015). Submerged fermentation in general, has been used in scaling up the production of pharmaceutically important fibrinolytic enzymes and other industrially important enzymes. (Shanjing, 2003; Mukherjee and Rai, 2011). Optimization of bioprocess parameters is highly significant in reducing the final yield cost (Kumar et al., 2018). Usually used one-factor at a time strategy has limitations such as, it is time consuming, labor intensive and lack of detecting interactions between the variables influencing enzyme productivity (Box et al., 1978; Rao et al., 2000). In addition, market price of thrombolytic agents depends on the costs of the composition of the production medium (Haddar et al., 2010; Zaman et al., 2016). Thus, our present study is principally focused on introducing a new and cost-effective source of fibrinolytic enzyme

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produced by newly isolated *Bacillus subtilis* Egy. Optimization of enzyme production conditions using fodder yeast as a cheap growth medium by applying Central Composite Design was studied.

2. Materials and methods

2.1. Materials

Fibrin was purchased from MP Biochemicals, Germany. L-tyrosine was purchased from BDH, England. All other used chemicals were of analytical grade.

2.2. Microorganism and inoculum preparation

Bacillus subtilis Egy was isolated from the Egyptian soil. The pure culture was maintained on Nutrient agar (NA) (0.5% peptone, 0.3% beef extract and 1.5% agar) slants.

Inoculum was prepared by inoculating Luria-Bertani medium (LB) with tested bacterial culture and incubated at 30 °C overnight under shaking conditions. LB composed of 1% tryptone, 1% sodium chloride and 0.5% yeast extract.

2.3. Isolation and screening of fibrinolytic bacteria

Soil samples were collected from slaughter-houses, mixed with 10 ml sterile water and heated at 80 °C for ten minutes. Serial dilutions were plated on NA plates and incubated at 30 °C for 48 h. Bacterial colonies were purified by transferring on NA plates and stored on NA slants. In the primary screening, each isolate was examined for proteolytic activity on skimmed milk agar plates which composed of NA and 1% skimmed milk powder. Bacterial isolates which showed clear zone around them exhibited as proteolytic active bacteria and were subjected to secondary screening on fibrin agar plates to ascertain their fibrinolytic activity. Fibrin agar plates composed of NA and 1% fibrin. Plates were incubated at 37 °C for 24 h. Appearance of the clear zone around the bacterial growth on fibrin plate was indicative of fibrinolytic activity of isolated bacteria.

2.4. Fibrinolytic enzyme production

Five standard bacterial media namely Nutrient broth (0.5% peptone, 0.3%beef extract),LB, Nutrient yeast salt medium (Yousten and Davidson, 1982), *Bacillus* minimal medium (Spizizien and Anagnostopoulos, 1961), and M9 Minimal medium (Maniatis et al., 1982) in addition to nine media based on dried ground industrial by-products (final concentration 3%) were tested under submerged fermentation using the tested bacterial isolate for fibrinolytic enzyme production. These substrates include sesame meal, fodder yeast, soybean meal, wheat bran, feather meal, cotton seed meal, offals meal, Lenin meal, and black seed meal. Fermentation was carried out at 30 °C under shaking at 125 rpm. Samples were centrifuged at 4000 rpm for 10 min at 4 °C and the supernatant was used as enzyme source for assaying the enzyme activity.

2.5. Assay of enzyme activity and protein content

Fibrinolytic activity was determined by using fibrin as the substrate according to the method of Raafat et al. (2012) with minor modifications. Assay mixture containing 10 mg fibrin, 0.5 ml of Tris-HCl buffer (0.2 M, pH 8.0) and 0.5 ml of diluted enzyme was incubated at 40 °C for 30 min. The reaction was stopped with 1.0 ml of 10% trichloroacetic acid and the contents were centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was subjected to color reaction using Folin ciocalteu's phenol reagent. Optical density was measured at 650 nm with L-tyrosine as a standard curve. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μg of L- tyrosine from fibrin per min under assay conditions.

The protein content of the enzyme preparation was determined according to Ohnisitti and Bar (1978) using bovine serum albumin as a standard.

2.6. Molecular identification of the tested bacterium

2.6.1. DNA extraction and PCR amplification

Grown bacterial culture in NB was centrifuged at 4000 rpm for 10 min. Extraction of DNA was carried out using GeneJet genomic DNA purification Kit (Thermo K0721). Total extracted DNA used as template and amplified by PCR with the aid of oligonucleotide primers 16S rDNA forward primer PF: (5-TTGCTCCCTGATGTTAGCGG-3), and reverse primer PR: (5-TGCGGAAGATTCCCTACTGC-3) with the thermal-cycling conditions consisted of denaturation at 94 °C for 10 min and subsequent 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 60 s, and extension at 72 °C for 2 min followed by final extension at 72 °C for 10 min.

2.6.2. Agarose gel electrophoresis

Ten microliters of PCR product was applied to a 1% agarose gel and run for approximately 4 h at 100 V. TAE buffer was the running buffer (0.001 M EDTA, pH 8.0; 0.04 M Tris, pH 8.0; 0.02 M acetic acid). The gel was stained in a solution of ethidium bromide (1 μg/ml) for approximately 15 min and washed in water for about 30 min. Gel images were recorded in Gel Doc XR+ Bio RAD with Image Lab Software.

2.6.3. 16S rDNA sequencing and data analysis

Sequencing analysis was performed on a 516 bp PCR product. The sequence analysis was performed using the ABI 3130 genetic analyzer and Big Dye Terminator version 3.1 cycle sequencing kit. The 16S rDNA sequence was aligned and compared with other partial 16S rDNA gene sequences in the GenBank by using the NCBI Basic Local alignment search tools BLAST-n program (http://www.ncbi.nlm.nih.gov/BLAST). Multiple alignments of sequences and nucleotide sequence statistics and variability were performed using DNAMAN software (Wisconsin, Madison, USA) and calustalow (ver. 1.74) program (Thompson et al., 1994). Phylogenetic relationships among Egyptian *Bacillus subtilis* isolate (*Bacillus subtilis* Egy) compared with other international isolates registered in NCBI site using Un weighted Pair Group Method with Arithmetic Mean (UPGMA) thought DNAMAN software and Neighbor joining (NJ) implemented thought Molecular Evolutionary Genetics Analysis (MEGA) software (ver. 4.0) (Tamura et al., 2007).

The 16S rDNA sequence has been deposited to GenBank to assign accession number by NCBI.

2.7. Statistical design for fibrinolytic enzyme production

Central composite was employed for the design of experiments (CCD), between seven independent variables (Table 1); namely fodder yeast concentration (A), yeast extract concentration (B), medium initial pH (C), incubation temperature (D), incubation period (E), aeration

Table 1
Summary of central composite design (CCD).

Factor	Name	Units	Actual levels		Mean
			Low	High	
A	Fodder yeast concentration	%	3	7	5
B	Yeast extract concentration	%	0.4	0.8	0.6
C	Initial pH	Unit	7	8	7.5
D	Temperature	°C	30	40	35
E	Incubation period	Days	2	4	3
F	Aeration	%	20	40	30
G	Inoculum size	%	1.2	3.2	2.2

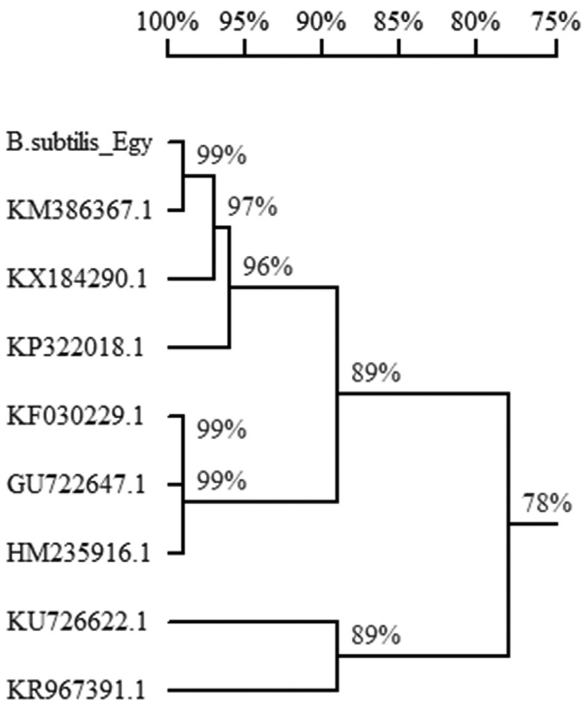

Fig. 1. Phylogenetic tree of *Bacillus subtilis* Egy.

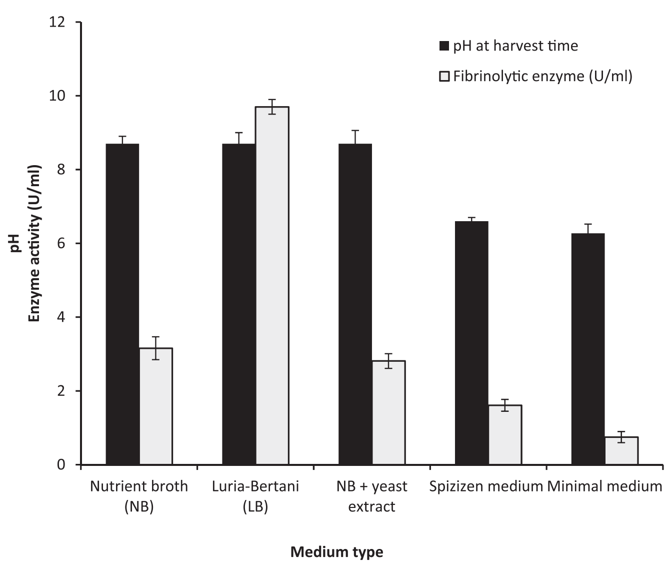

Fig. 2. Effect of different standard bacterial media on production of fibrinolytic enzyme from *Bacillus subtilis* Egy.

level (F) and inoculum size (G), using Design-Expert software (Stat-Ease Inc., Minneapolis, MN, USA, ver 7.0.0). Statistical evaluation of the produced data, optimization and prediction of the optimum conditions were performed using analysis of variance (ANOVA) and regression analysis. The significance of each parameter and variable was estimated by *F*-test (calculated p-value). Coefficients with a p-value < 0.05 were considered significant.

2.7.1. Optimization of variables levels and model validation

The independent variables were theoretically optimized, and the result was numerically predicted using Design-Expert software. The statistical model based on CCD was validated by the experimental application of theoretical results and comparing it with the theoretical values.

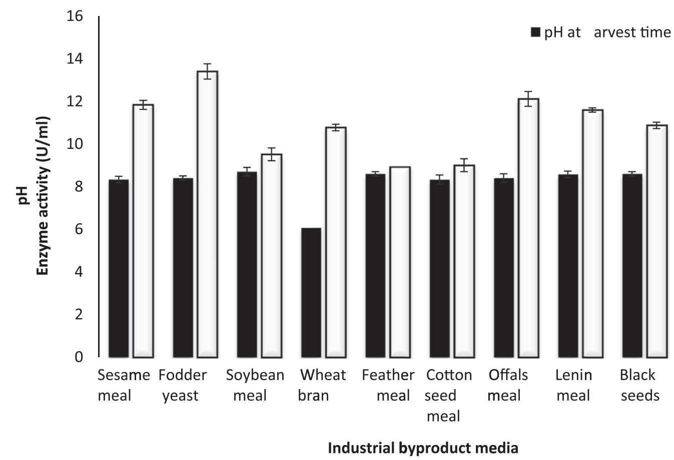

Fig. 3. Effect of some industrial byproducts (3%) on production of fibrinolytic enzyme under submerged fermentation conditions.

2.8. In vitro blood clot degradation

In vitro fibrinolytic activity was determined by artificial blood clot degradation method. Blood clot was formed by spontaneous coagulation of freshly collected human blood in a glass tube. After 1 h, artificial blood clot was rinsed out thoroughly and dipped in 10 ml of phosphate-buffered saline containing either crude or ethanol precipitated fibrinolytic enzyme and incubated at room temperature for clot dissolution (Mahajan et al., 2010). Phosphate-buffered saline was the negative control and Clexane drug used as a positive control

3. Results

3.1. Identification of tested bacterial isolate

Among 126 bacterial isolates from Egyptian soil, isolate B-26 gave the highest fibrinolytic activity. Thus, it was subjected to molecular identification.

3.1.1. 16S rDNA gene sequence identification

Molecular identification of the isolated bacterial strain B-26 was carried out based on 16S rDNA sequence analysis. The results showed that 16S rDNA gene partial sequence is about 516 bp long and is composed of both variable and conserved regions. The resulted sequence was compared with bacterial species recorded in the GenBank data base using DNAMAN program and identified as *Bacillus subtilis* with the highest similarity of 99%. It has been assigned with NCBI accession number KY703635.1.

Phylogenetic tree was mapped using the neighbor joining method and showed eight clusters were generated in comparison with different eight Gen Bank 16S rDNA partial sequences for *Bacillus subtilis* strains. The highest homologous were found between *Bacillus subtilis* Egy and KM386367.1 with similarity 99%. Minimum similarity percentage (78%) observed between *Bacillus subtilis* Egy and other strains have accession no. KU726622.1 and KR967391.1, so it was represented as a separate cluster as shown in Fig. 1.

3.2. Selection of fibrinolytic production medium

Figs. 2 and 3 showed the effect of the medium type on the fibrinolytic enzyme production yield by the tested organism. They revealed that 3% fodder yeast gave the highest enzyme activity under submerged fermentation conditions. Therefore, fodder yeast was chosen as the production medium through this study.

Table 2
Experimental results and predicted values based on CCD.

Run order	Enzyme activity (U/ml)		
	Residual	Predicted value	Actual value
1	7.07	7.20	−0.13
2	9.66	8.92	0.74
3	11.4	11.62	−0.22
4	11.02	11.00	0.02
5	5.9	6.70	−0.80
6	10.07	10.36	−0.29
7	10.2	10.73	−0.53
8	11.8	13.08	−1.28
9	9.64	9.34	0.30
10	10.35	11.02	−0.67
11	9.3	8.74	0.56
12	9.2	9.34	−0.14
13	10.2	11.14	−0.94
14	8.4	8.67	−0.27
15	13	13.48	−0.48
16	15.3	14.77	0.53
17	8	7.88	0.12
18	10.64	10.13	0.51
19	5.4	4.77	0.63
20	18.2	17.96	0.24
21	15.1	14.83	0.27
22	7.17	7.28	−0.11
23	13.5	12.88	0.62
24	4	3.94	0.06
25	10.4	9.43	0.97
26	6	6.90	−0.90
27	8	8.57	−0.57
28	7.4	6.20	1.20
29	8.71	7.97	0.74
30	4.68	4.09	0.59
31	8.46	8.64	−0.18
32	15.6	15.45	0.15
33	18.9	18.88	0.02
34	10	10.73	−0.73
35	10.8	10.90	−0.10
36	7.68	7.71	−0.03
37	3.02	3.79	−0.77
38	13.6	13.57	0.03
39	8.3	8.61	−0.31
40	9.48	9.99	−0.51
41	6.4	6.07	0.33
42	6.04	5.99	0.05
43	7.43	7.45	−0.02
44	5.02	5.21	−0.19
45	17.6	17.37	0.23
46	6.15	6.33	−0.18
47	8	7.72	0.28
48	10.5	9.00	1.50
49	9.84	9.28	0.56
50	13.43	13.27	0.16
51	6.2	6.80	−0.60
52	12	12.41	−0.41
53	11.28	11.22	0.06
54	2	1.9	0.09
55	5.25	5.46	−0.21
56	10.3	9.00	1.30
57	10	9.82	0.18
58	5.13	7.17	−2.04
59	4	4.18	−0.18
60	8.3	8.76	−0.46
61	5.25	6.11	−0.86
62	7.66	7.89	−0.23
63	4.1	4.77	−0.67
64	9.84	9.50	0.34
65	8.6	9.37	−0.77
66	9.48	7.76	1.72
67	4.66	3.60	1.06
68	16.6	15.95	0.65
69	9.22	8.63	0.59
70	6.15	6.25	−0.10
71	13.17	13.13	0.04
72	7.56	7.46	0.10
73	6.66	6.57	0.09
74	3.33	4.19	−0.86
75	8.2	8.18	0.02
76	3.07	4.00	−0.93
77	8.35	8.14	0.21
78	15.3	15.61	−0.31
79	9.07	9.70	−0.63
80	8.25	8.34	−0.09
81	10.7	9.40	1.30
82	4.35	4.19	0.16
83	13.4	13.55	−0.15
84	10.58	10.07	0.51
85	7.68	8.93	−1.25
86	13.07	13.05	0.02
87	17.84	17.70	0.14
88	11.02	10.30	0.72
89	10.3	10.23	0.07
90	7.68	7.44	0.24
91	10.2	10.23	−0.03
92	11.17	11.03	0.14
93	10	9.84	0.16
94	7.43	7.84	−0.41
95	13.97	14.29	−0.32
96	9.9	9.97	−0.07
97	2.64	3.02	−0.38
98	17.84	17.45	0.39
99	12.3	12.34	−0.04
100	9.35	8.80	0.55
101	8.86	9.49	−0.63
102	6.92	6.12	0.80
103	15.8	15.44	0.36
104	10.1	10.21	−0.11
105	7.76	8.20	−0.44
106	10.84	10.34	0.50
107	9.2	7.61	1.59
108	3.33	4.36	−1.03
109	13.68	13.20	0.48
110	7.2	7.33	−0.13
111	14.6	16.33	−1.73
112	7	7.43	−0.43
113	3.84	4.24	−0.40
114	8.38	8.18	0.20
115	6.15	6.82	−0.67
116	13.25	13.00	0.25
117	10.8	10.56	0.24
118	15.3	15.40	−0.10
119	9.78	11.43	−1.65
120	6	6.11	−0.11
121	12.5	12.43	0.07
122	5.71	5.26	0.45
123	8.3	8.27	0.03
124	9.84	9.25	0.59
125	8.4	8.28	0.12
126	8.38	8.09	0.29
127	10.1	10.21	−0.11
128	11.2	11.76	−0.56
129	3.58	3.68	−0.10
130	9.33	9.29	0.04
131	7.94	6.06	1.88
132	10.76	9.92	0.84
133	9.4	9.57	−0.17
134	17.3	17.83	−0.53
135	9.82	9.78	0.04
136	9.48	9.18	0.30
137	10	10.30	−0.30
138	2.56	2.39	0.17
139	10.3	10.30	0.00
140	11.02	10.64	0.38
141	7.25	7.93	−0.68
142	10.6	10.30	0.30
143	10.2	10.30	−0.10
144	12.9	12.66	0.24
145	9.9	10.25	−0.35

(continued on next page)

Table 2 (*continued*)

Run order	Enzyme activity (U/ml)		
	Residual	Predicted value	Actual value
146	10	10.40	−0.40
147	10.2	10.34	−0.14
148	9.5	9.36	0.14
149	9.5	9.05	0.45
150	9.8	9.95	−0.15
151	12	11.55	0.45
152	17.6	17.40	0.20
153	9.8	10.20	−0.40
154	6.66	6.49	0.17

3.3. Optimization of fibrinolytic enzyme production using CCD

For statistical optimization of different factors affecting production physiology of fibrinolytic enzyme by *Bacillus subtilis* Egy, CCD was employed consisting of seven critical parameters viz. fodder yeast concentration (A), yeast extract concentration (B), medium initial pH (C), incubation temperature (D), incubation period (E), aeration level (F) and inoculum size (G). A total sum of 154 run with different combinations of these seven selected parameters were carried out as shown in Table 2. The enzyme activity ranged from 2 U/ml (run 54) to 18.9 U/ml (run 33). The interactive effects of the seven parameters were deduced by analysis of variance (ANOVA) of the results, regression coefficient, *F* values, *P* values of variables as shown in Table 3. The analysis revealed that both fodder yeast concentration, incubation temperature, aeration level followed by yeast extract concentration and incubation period are significant factors in enzyme yield. On the other hand, initial pH of the medium and inoculum size were found to be non-significant factors as P value > 0.05.

ANOVA of the data obtained from CCD showed that the model is significant as *F*-value was 119.80 and A, B, D, E, F, AB, AC, AD, AE, AF, CD, CE, CG, DE, DG, EF, EG, A^2, D^2, BDF, A^2D, A^2E are all significant model terms as Prob > F values were < 0.05. The model R^2 was 0.969206 and the predicted R^2 of 0.942293 was in reasonable agreement with the adjusted R^2 of 0.961116. In addition, adequate precision, which measures the signal to noise ratio, was 50.90804. According to these results, this model can be used to navigate the design space.

Thus, the final equation in terms of actual factors is:

$$\begin{aligned}
&\textbf{Fibrinolytic Enzyme activity (U/ml)}\\
&= -135.7294321 - 1.967738995*A - 36.00288373*B\\
&+ 14.82546094*C + 5.138562979*D + 15.57749609*E\\
&- 1.152900965*F + 7.388037684*G\\
&+ 0.781445313*A*B - 1.185625*A*C\\
&+ 0.32971875*A*D - 2.0496875*A*E\\
&+ 0.068878906*A*F + 2.3015625*B*C\\
&+ 0.43078125*B*D + 1.287148438*B*F\\
&- 0.24496875*C*D - 1.45421875*C*E\\
&+ 0.06796875*C*F - 0.66578125*C*G\\
&+ 0.026515625*D*E + 0.006834375*D*F\\
&- 0.125203125*D*G - 0.012242187*E*F\\
&+ 0.654609375*E*G - 0.640759878*A^2\\
&- 0.058555955*D^2 - 0.111796875*B*C*F\\
&- 0.013867188*B*D*F + 0.167851563*A^2*C\\
&- 0.026558594*A^2*D + 0.168066406*A^2*E
\end{aligned}$$

Table 3
Analysis of variance (ANOVA) of the results based on CCD.

Source	Sum of squares	Df	Mean square	F-Value	p-value* Prob > F
Block	60.62171	4	15.15543		
Model	1766.249	31	56.97578	119.8035	< 0.0001
A-Fodder yeast concentration (%)	550.3786	1	550.3786	1157.286	< 0.0001
B-Yeast extract concentration (%)	3.995265	1	3.995265	8.40088	0.0045
C-Initial pH	0.02	1	0.02	0.042054	0.8379
D-Temperature (°C)	8.405	1	8.405	17.67327	< 0.0001
E-Incubation period	3.125	1	3.125	6.570965	0.0116
F-Aeration level (%)	190.2406	1	190.2406	400.0206	< 0.0001
G-Inoculum size (%)	0.075765	1	0.075765	0.159313	0.6905
AB	12.50625	1	12.50625	26.297	< 0.0001
AC	31.09647	1	31.09647	65.38682	< 0.0001
AD	52.64663	1	52.64663	110.7005	< 0.0001
AE	69.72329	1	69.72329	146.6078	< 0.0001
AF	242.9084	1	242.9084	510.7655	< 0.0001
BC	1.417507	1	1.417507	2.980605	0.0869
BD	0.027907	1	0.027907	0.05868	0.8090
BF	0.688845	1	0.688845	1.448439	0.2312
CD	48.00775	1	48.00775	100.9463	< 0.0001
CE	67.67207	1	67.67207	142.2947	< 0.0001
CF	0.002538	1	0.002538	0.005337	0.9419
CG	14.18447	1	14.18447	29.82581	< 0.0001
DE	2.249851	1	2.249851	4.730781	0.0316
DF	0.706563	1	0.706563	1.485697	0.2253
DG	50.16263	1	50.16263	105.4774	< 0.0001
EF	1.918351	1	1.918351	4.033733	0.0469
EG	54.84972	1	54.84972	115.333	< 0.0001
A^2	21.12893	1	21.12893	44.42799	< 0.0001
D^2	76.15125	1	76.15125	160.1239	< 0.0001
BCF	1.599813	1	1.599813	3.363942	0.0692
BDF	2.461426	1	2.461426	5.175662	0.0247
A^2C	0.848539	1	0.848539	1.78423	0.1842
A^2D	2.124375	1	2.124375	4.466942	0.0367
A^2E	3.40285	1	3.40285	7.155204	0.0085
Residual	56.11809	118	0.475577		
Lack of Fit	55.88559	111	0.503474	15.15835	0.0005
Pure Error	0.2325	7	0.033214		
Cor Total	1882.989	153			

* p-value < 0.05 was considered significant.

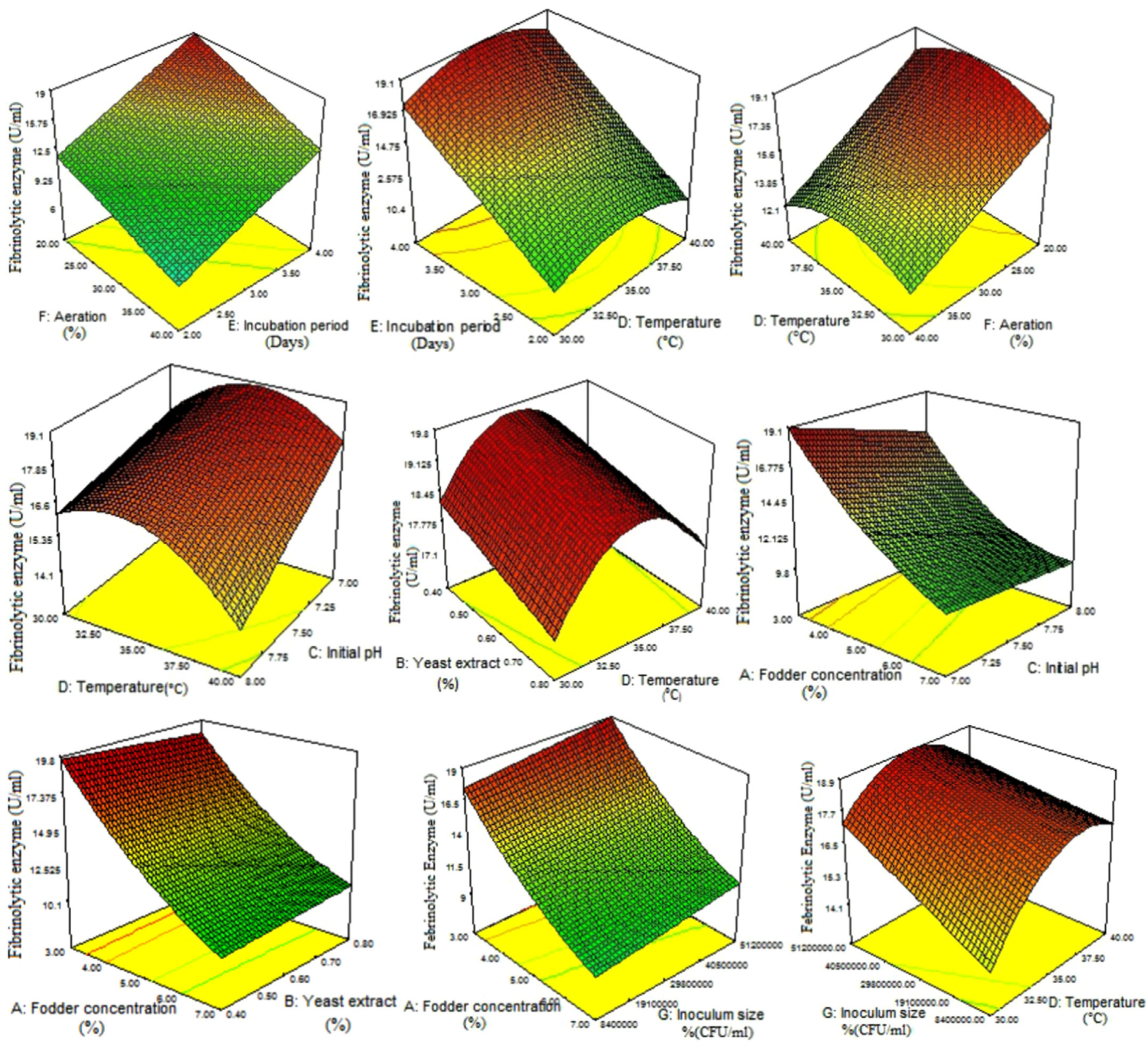

Fig. 4. Effect of different variables on production of fibrinolytic enzyme by *Bacillus subtilis* Egy.

Where, A: fodder yeast concentration (%), B: yeast extract concentration (%), C: initial pH, D: incubation temperature (°C), E: incubation period (days), F: aeration level (%) and G: inoculum size (%).

3.3.1. Optimization of variables levels and model validation

The optimum levels of the different variables were fodder yeast concentration, 3.05%; yeast extract concentration, 0.71%; initial pH, 7; incubation temperature, 36.7 °C; incubation period, 4 days; aeration level, 20% and inoculum size, 3.2%. At these conditions the theoretical fibrinolytic enzyme activity was 18.9 U/ml. The practical application of these conditions produced 16.6 U/ml. Comparing the practical and theoretical results showed that the model was valid by 87.83%. The effect of different levels and the interactions of the used variables on the productivity of fibrinolytic enzyme by Bacillus subtilis Egy was illustrated by Fig. 4.

3.4. In vitro blood clot degradation

Thrombolytic activity of the fibrinolytic enzyme produced by *Bacillus subtilis* Egy was assayed in vitro by human blood clot degradation with Clexane Drug as a positive control and phosphate-buffer saline as negative control. The obtained results are highly promising as the crude and ethanol precipitated enzyme can significantly digest the fibrin net of the human blood clot and they were more efficient than the Clexane drug as shown in Fig. 5. It is worth mentioning that ethanol precipitated fibrinolytic enzyme achieved more clot degradation than the crude one.

4. Discussion

In recent decades thrombosis is considered as the cause of lethal medical complications. Microbial fibrinolytic enzymes can dissolve these clots and help to solve these problems in more efficient and safer way. In this study, among 126 bacterial isolates from Egyptian soil

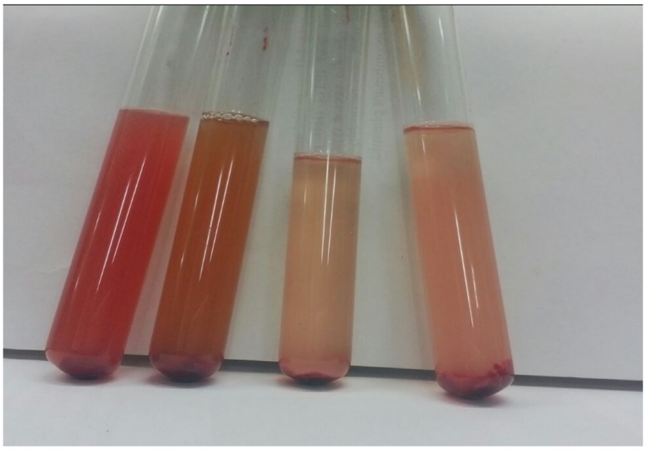

Fig. 5. *In vitro* blood clot degradation. **P:**Ethanol precipitated fibrinolytic enzyme of *Bacillus subtilis* Egy. **C:**Crude fibrinolytic enzyme of *Bacillus subtilis* Egy. **S:**Phosphate-buffer saline solution as a negative control. **D:**Clexane drug (injection solution) as a positive control.

Bacillus subtilis Egy showed the highest fibrinolytic activity. *Bacillus subtilis* is an organism of choice for industrial applications due to its high suitability for genetic engineering and its large-scale fermentation (Harwood, 1992). The most *Bacillus subtilis* strains which produce fibrinolytic enzymes were isolated from natto, a common Japanese fermented soybean food as reported by Dabbagh et al. (2014) and Nagata et al. (2016). Other strains which produce fibrinolytic enzymes were found in other fermented foods, including *Bacillus subtilis* DC33,*Bacillus amyloliquefaciens* LSSE-62, *B. subtilis* MX-6 from Chinese foods (Wang et al., 2006a,b; Wei et al., 2012; Man et al., 2018); *Bacillus* cereus from rust (Vaithilingam et al., 2016); *Bacillus sp*. DJ-4, *Bacillus sp*. CK11-4, *Bacillus vallismortis* Aceo2 from traditional Korean condiment doen-jang and chungkook-jang (Kim and Choi, 2000; Anh et al., 2015); *Bacillus megaterium* KSK-07 from Egyptian kishk (Kotb, 2015). Other sources of this enzyme were investigated such as *Pseudomonas* sp. and some marine organisms (Mohanasrinivasan et al., 2017). In this study, Bacillus subtilis Egy, a strain with high fibrinolytic enzyme production, was isolated from Egyptian soil.

An ideal thrombolytic drug should have available cost. Thus, in this study a cost effective medium for production of fibrinolytic enzyme was introduced. The current investigation is the first report about using a byproduct, fodder yeast as the production medium instead of highly expensive standard bacterial growth media for production of fibrinolytic enzyme from *Bacillus subtilis* Egy. Fodder yeast is a byproduct of ethanol production from *Saccharomyces cerevisiae*. It characterized by its high protein content (40–46%), amino acids and minerals so that it can be served as carbon and nitrogen source for production of the microbial metabolites. Central Composite Design as an economic strategy was employed to optimize and investigate interactions between critical variables. The results revealed that fodder yeast concentration, incubation temperature, aeration level followed by yeast extract concentration and incubation period are significant factors in the fibrinolytic yield production. On the other hand, initial pH of the medium and inoculum size were found to be not significant factors as *P* value > 0.05. The practical application of the optimal conditions produced 16.6 U/ml of the enzyme under study. It was reported that use of CCD for optimization the growth conditions was economic way because it gives enormous information in less experimental runs and less time consuming (Box and Draper, 1959; Box et al., 1978; Deepak et al., 2010). Results obtained from CCD depicted the significant and non-significant interactions among the different parameters influencing fibrinolytic enzyme activity. It is worth to notice that the three-dimensional response surface plots which graphically represent the regression equation are very simple and provide an essential contribution in understanding the interactions between two variables and to find their optimum levels (Surwase et al., 2012). Model validation revealed that experimental and predicted values were very close and experimental design is effective towards process optimization. Ku et al. (2009) reported 13.78 SU/ml of Subtilisin NAT under submerged cultivation using response surface methodology. Fibrinolytic activity from fermented soybean paste was found to be 14.7 U/ml as reported by Ko et al. (2008). While it was reported to be about 20.8 to 207 U/ mg by Cho et al. (2004). Recently, Kumar et al. (2018) optimized production of a fibrinolytic enzyme from newly isolated marine bacterium *Pseudomonas aeruginosa* KU1 using Box-Benhken design with 1.32-fold increase of one factor at a time optimization.

In vitro testing of human blood clot degradation by the ethanol precipitated and the crude enzyme preparations revealed obvious digestion of the fibrin net of the blood clot indicating positive activity as a thrombolytic agent for cardiovascular diseases treatment. The same findings were reported by Vijayaraghavan and Vincent (2014) and Zaman et al. (2016) for *Bacillus cereus* INDI and *Bacillus licheniformis* EMS250-O-1, respectively.

5. Conclusion

Search for new, safe and cost-effective fibrinolytic enzyme producer is encourage. A potent fibrinolytic enzyme producing bacteria *Bacillus subtilis* Egy was isolated from Egyptian soil. Recycling of agro-industrial by products as a mean of cost-effective production medium was applied. Fodder yeast was chosen as an efficient growth medium for tested enzyme production. Improved production of fibrinolytic enzyme was achieved by employing Central Composite Design. Using this statistical approach not only reduces the time course of the bioprocess but also the cost of enzyme production. This study introduces a new and economic candidate for fibrinolytic enzyme production and it can be used for medical application.

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Conflict of interests

The authors have declared no conflict of interests.

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