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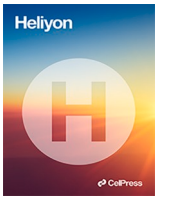

Research article

Modeling and *in-vivo* evaluation of fibrinolytic enzyme produced by *Bacillus subtilis* Egy under solid state fermentation

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A R T I C L E I N F O

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A B S T R A C T

Blood clot formation increases cases of myocardial infarction (AMI) and stroke, thus urges directing much research works for treatment and prevention of the causes. One of these directions is the microbial production of fibrinolytic enzymes as thrombolytic agents. In the current work, *Bacillus subtilis* Egy has been used for enzyme production under solid state fermentation. Among twelve nutrient meals in addition to wheat bran as a control fodder yeast yielded the highest enzyme activity reaching 114U/g. Applying statistical model for optimization of enzyme production revealed that 3.6%, fodder yeast; 40%, moisture content; 6 days, incubation period and 2%, inoculum size were the optimum conditions for maximum fibrinolytic enzyme production (141.02 U/g) by *Bacillus subtilis* Egy under solid-state fermentation The model was significant and data were experimentally validated. The produced fibrinolytic enzyme was evaluated for *in vitro* and *in vivo* cytotoxicity. *In-vivo* examination of the enzyme resulted in no mortality during the first 24 h after *treatment*. After 14 days, the results revealed no significant changes detected in hematological parameters (RBCs, MCV, hemoglobin except WBCs which showed an increase for both sexes. Histopathological examination of liver and kidney of rats received oral and subcutaneous treatments showed normal architecture. The data showed the applicability of the produced enzyme for the treatment of blood clot with no significant effect on living cells or on physiological functions.

1. Introduction

The blood clot is the blood transformation from liquid state into a gel-like or semisolid. It takes place as a protective phenomenon of the body form excess bleeding due to injury. However, clot formation inside the blood stream hinders or blocks the blood flow causing myocardial infarction (AMI) and stroke which is a life-threatening situation [1,2]. Fibrin is the main protein component of the blood clot, generated from fibrinogen by the catalysis of thrombin [3–5]. Fibrinolytic enzymes are the ideal therapy for cases with hyper

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coagulable disorder (known as excessive clotting disorder or thrombophilia) to avoid mortality and morbidity [6,7].

Fibrinolytic enzymes from microbial sources have been selected as a safer and more effective alternative for thrombolytic therapy which has many limitations including high production cost and undesirable side effects such as excessive bleeding [8]. Fibrinolytic enzymes formulated as a drug to prevent hyper coagulable disorder.

The role of microorganisms (i.e., *Bacillus*) in fibrinolytic enzymes production can never be under-estimated as it has attracted the attention for their outstanding productivity [9,10]. However, for more economic bioprocess based on cost/benefit ratio an optimization process is usually conducted using the various biotechnological methods to reduce the production cost [10,11]. Statistical modeling using factorial design and/or response surface method is an emerging method for more effective optimization of the bioprocesses and a successful alternative of the traditional optimization method (One-Factor-At-A time) [12]. Statistical modeling of biological processes has proven to be important and reliable tool in biotechnology [13,14].

The current study aims to produce and optimize different physiological parameters of fibrinolytic enzyme from a microbial origin; *Bacillus subtilis* Egy under solid state fermentation. The produced enzyme was evaluated in-vivo using rats for application in cases of hypercoagulable disorder.

2. Materials and methods

2.1. Chemicals, reagents and media

Fibrin was purchased from MP Biochemicals (https://www.mpbio.com/), Germany. L-tyrosine was purchased from BDH (https://copens-sci.com/bdh-uk/), England. All chemicals were of analytical grade. Nutrient Broth medium (0.5% peptone, 0.3% beef extract) from Oxoid used for culture preservation. Luria-Bertani (LB) medium (1% tryptone, 1% sodium chloride and 0.5% yeast extract) was used for culture preparation at 30 °C overnight.

2.2. Microorganism

Bacillus subtilis Egy is the focus of the current study. It was isolated from a slaughter-house soil sample (El-Sharkia, Egypt) and molecularly identified and got the accession number of "KY703635.1" in GenBank data base. The production of fibrinolytic enzyme by *Bacillus subtilis* Egy was reported previously [15,16].

2.3. Inoculum preparation

Bacterial Inoculum was prepared by inoculating sterile nutrient broth medium (5 gm peptone, and 3 gm meat extract per liter) in 250 ml conical flasks. These flasks were incubated on orbital rotary shaker (100 rpm) for 24 h.

2.4. Screening of agro-industrial byproducts for fibrinolytic enzyme production

In this experiment, twelve meals compared with fodder yeast and a control test (containing only wheat bran as a carrier) were tested (at 6%) for their ability to produce fibrinolytic enzyme under solid state fermentation. The twelve meals are: Sesame meal, Olive meal, Lenin meal, Gatrova meal, Jojoba meal, *Nigella sativa* meal, Wheat germ meal, Peanut meal, Soy bean meal, Checkin offal meal, Feather meal and Cotton seed meal. All these meals were dried at 60 °C for 1 h and grinded before use. Each of them was added separately to 10 gm wheat bran at final concentration 6% in 250 conical flask. After autoclaving for 30 min at 121 °C, these flasks were inoculated by the bacterial inoculum and incubated for 6 days under static growth conditions at 30 °C.

2.5. Enzyme extraction

After incubation period, the enzyme was extracted from the solid fermented biomass. By distilled water (1: 10 w/v) under shaking conditions at 120 rpm for 2 h at 30 °C. The obtained filtrate was centrifuged at 4000 rpm for 10 min. The supernatant was used as crude enzyme source.

Table (1)
Summary of the CCD design.

Variable code	Variable	Units	Levels	
			Min	Max
A	Fodder yeast	%	1.8	9
B	Moisture	%	30	70
C	Incubation period	Days	3	7
D	Inoculum size	%	1	5

2.6. Design of experiment (DOE) for fibrinolytic enzyme production modeling and optimization

For solid-state production modeling and optimization of fibrinolytic enzyme by the isolate *Bacillus subtilis* Egy, four variables have been selected; fodder yeast, moisture content, incubation period and inoculum size. Design of experiment was used to study the effect of each variable and factor-factor interactions on enzyme production. For this purpose, central composite design (CCD) has been employed with the aid of Design-Expert software **(Stat-Ease Inc., Minneapolis, MN, USA, ver 7.0.0).** Summary of the conducted CCD model design has been shown in (Table 1).

Erlenmeyer conical flask (250 ml) with wheat bran (10 g) has been used for this experiment. Data were analyzed using regression analysis and analysis of variance (ANOVA), where terms with *p*-value less than 0.05 were considered as significant for the enzyme production.

2.7. Optimization of enzyme production and model validation

Fibrinolytic maximum enzyme production by ***Bacillus subtilis* Egy** has been theoretically obtained by numerical optimization of the variables. The predicted values have been practically applied and the obtained activity was compared with the theoretical result.

2.8. Fibrinolytic enzyme assay

Fibrinolytic enzyme activity was determined by using fibrin as the substrate as shown in Ref. [16]. Assay mixture contains 0.5 ml of enzyme and 0.5 ml of Tris- HCl buffer (0.2 M, pH 8) in a test tube containing 10 mg of fibrin. The reaction mixture was incubated at 40 °C for 30 min. Then it was stopped by 1.0 ml of 10% trichloroacetic acid (TCA). After centrifugation at 4000 rpm for 10 min at 4 °C supernatant was subjected to color reaction using Folin ciocalteus 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.

2.9. Safety studies of fibrinolytic enzyme from Bacillus subtilis Egy

2.9.1. In vitro cytotoxicity on human normal retina cell line (RPE1)

In vitro bioassay on human normal cell line was conducted and determined by the Bioassay-Cell Culture Laboratory, National Research Centre, El-Tahrir St., Dokki, Cairo, Egypt. Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan [17].

Procedure: All the following procedures were done in a sterile area using a Laminar flow cabinet biosafety class II level (Baker, SG403INT, Sanford, ME, USA). Cells were suspended in DMEM-F12 medium, 1% antibiotic-antimycotic mixture (10,000U/ml Potassium Penicillin, 10,000 μg/ml Streptomycin Sulfate and 25 μg/ml Amphotericin B) and 1% L-glutamine at 37 °C under 5% CO2.

Cells were batch cultured for 10 days, then seeded at concentration of 10′4 cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37 °C for 24 h under 5% CO2 using a water jacketed Carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). Media were aspirated, fresh medium (without serum) was added and cells were incubated either alone (negative control) or with different concentrations of sample to give a final concentration of (50-25-12.5-6.25-3.125- 1.56 and 0.78 μL/ml). After 48 h of incubation, medium was aspirated, 40 μl MTT salt (2.5 μg/ml) were added to each well and incubated for further 4 h at 37 °C under 5% CO2. To stop the reaction and dissolving the formed crystals, 200 μL of 10% Sodium dodecyl sulfate (SDS) in deionized water was added to each well and incubated overnight at 37 °C. Doxorubicin (DOX) were used as positive control at 100 μg/ml gives 100% lethality under the same conditions [18].

The absorbance was then measured using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595 nm and a reference wavelength of 620 nm. A statistical significance was tested between samples and negative control (cells with vehicle) using independent *t*-test by SPSS 11 program. DMSO is the vehicle used in final concentration less than 0.2%. The percentage of change in viability was calculated according to the formula:

((Reading of extract/Reading of negative control) −1) x 100.

2.9.2. Acute toxicity study in vivo

2.9.2.1. Animals. Adult male Wister albino rats weighing 120–150 g were purchased from the animal house colony of the National Research Centre (Dokki, Cairo, Egypt) and were kept at room temperature 25 °C ± 2 and with a 12 h on/off the light program. During the whole experiment, standard food and water were submitted to animals under conventional laboratory conditions. Experiments were carried out according to the Ethical Committee of the Medical Research of the National Research Centre, Egypt.

2.9.2.2. Experimental design. The enzyme toxicity was evaluated in thirty six male and female Wister albino rats. Rats were randomly assigned to six experimental groups each having 6 animals as follows: Groups 1 &2: Male and female rats were received normal saline orally and served as control groups. Groups 3 &4: Male and female rats were injected subcutaneously with tested enzyme, once (80 U/kg). Groups 5 &6: Male and female rats received tested enzyme orally, once (80 U/kg) [19]. The percentage of rat's mortality was recorded for the first 24 h. For 14 days, rats were inspected for any changes in the skin, respiratory, circulatory, autonomic and central

nervous systems, somatomotor activity and behavioral pattern. Particular attention has been given for the observation of tremors, convulsions, salivation, diarrhea, lethargy, sleep, and coma [20]. At the end of the 14 days, blood samples were collected from retro-orbital venous plexus of anesthetized rats and blood was allowed to coagulate and then centrifuged at 3000 rpm for 15 min [21]. The obtained serum was used for assessment of liver (ALT, AST) and kidney (creatinine and BUN) functions [22]. For hematological analysis, blood was taken in EDTA tube then centrifuged [23]. Data represented as (Mean ± Standard Error (S.E.)

2.9.3. Histological examination of liver and kidney

At the end of the experimental period, the animals were sacrificed by decapitation [24]. Liver and kidney tissues were fixed in formalin saline solution (10%) and placed in molten paraffin wax and allowed to solidify. Tissues were then cut into 5 μm thick sections. The sections were stained on glass slides using Hx (Hemotoxaline) & E (Eosin) and examined under light microscope.

2.10. Statistical analysis

Data are expressed as mean ± S.E. (Stander Error). Multiple comparisons of means were done by One-way analysis of variance (ANOVA) followed by least significant test at $p < 0.05$. Data were analyzed statistically using SPSS Version 12.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Screening for the optimum agro-industrial byproducts for fibrinolytic enzyme production under SSF conditions

According the data shown in Table (2), fodder yeast resulted in the highest enzyme productivity followed by sesame meal and olive meal. Thus, fodder yeast was chosen as a nutrient source for enzyme production under SSF conditions.

3.2. Statistical optimization of fibrinolytic enzyme production under solid state fermentation

For modeling of fibrinolytic enzyme production by *Bacillus subtilis* Egy, central composite design (CCD) has been applied, results were registered, evaluated and compared with the model predicted results. Data represented an excellent correlation between actual and predicted values ($R^2 = 0.9922$) as shown in Table (3) and Figure (1).

Furthermore, CCD model has been evaluated using Analysis of variance statistical methods (Table 4). ANOVA shows that the produced model is significant as the model F-value was 32.99. In addition, B, C, D, BD, B^2, C^2, D^2, ABD, ACD, BCD, A^2C, A^2D, ABCD, A^2B^2 are all significant model terms (p-value < 0.05). The model R^2 and the adjusted-R^2 were 0.992154 and 0.96208, respectively. The model signal to noise ratio is 23.903 which indicate model adequate precision. The experimental results showed good fitting to the model data (predicted) as the model lack of fit was not significant (p-value >0.05). Accordingly, the model can be used to navigate the design space. The statistical model of fibrinolytic enzyme production by *Bacillus subtilis* Egy under solid-state fermentation can be expressed by the following equation as a function of fodder yeast (%), moisture content (%), incubation period (days) and inoculum size (%):

Fibrinolytic enzyme production (U/g) = −2383.717658 + 530.2850726*A + 31.38478604*B + 563.2267793*C + 726.1075676*D - 4.780416667*A*B - 91.39027778*A*C - 117.4319444*A*D - 9.2335*B*C - 11.72*B*D - 169.235*C*D −21.00385455*A^2 + 0.07005214*B^2 + 2.158322072*C^2 + 9.527072072*D^2 + 1.034097222*A*B*C + 1.1575*A*B*D + 20.67291667*A*C*D + 2.9795*B*C*D - 0.149982274*A^2*B + 2.617283951*A^2*C + 3.613040123*A^2*D - 0.329583333*A*B*C*D + 0.002558079*A^2*B^2

Table (2)
Screening for the optimum agro-industrial byproducts for fibrinolytic enzyme production.

Nutrient meal	Fibrinolytic enzyme (U/g)
Wheat bran **(Control)**	80
Sesame meal	109.4
Olive meal	107.4
Lenin meal	98.5
Gatrova meal	69.4
Jojoba meal	95
Nigella sativa meal	85.7
Wheat germ meal	102.5
Peanuts meal	104
Soy bean meal	84.6
Offal meal	103
Feather meal	98.4
Cotton seed meal	93.5
Fodder yeast	**114**

Table (3)
Relation between actual and predicted fibrinolytic enzyme production according to Central Composite Design (CCD).

Run order	Enzyme activity (U/g)		
	Actual	Predicted	Residual
1	70.3	70.3	0.0000
2	71.8	71.9208	−0.1210
3	92.3	92.4208	−0.1210
4	74.35	74.2292	0.1208
5	89.74	89.6192	0.1208
6	25.65	25.65	0.0000
7	51.5	53.7333	−2.2333
8	84.6	84.6	0.0000
9	58.97	58.8492	0.1210
10	74.4	74.5208	−0.1210
11	100	100.121	−0.1208
12	102.5	102.621	−0.1208
13	115.38	115.501	−0.1210
14	110.2	110.2	0.0000
15	120.5	120.379	0.1210
16	51.2	53.7333	−2.5333
17	56.4	56.4	0.0000
18	60.7	61.1833	−0.4833
19	53.8	53.7333	0.0667
20	56.4	56.4	0.0000
21	55.3	53.7333	1.5700
22	89.7	89.8208	−0.1210
23	100.2	100.079	0.1208
24	64.1	53.7333	10.3667
25	51.2	50.7167	0.4830
26	94.8	94.6792	0.1210
27	120.5	120.379	0.1210
28	**141.02**	140.899	0.1208
29	110.2	110.321	−0.1210
30	46.5	53.7333	−7.2333

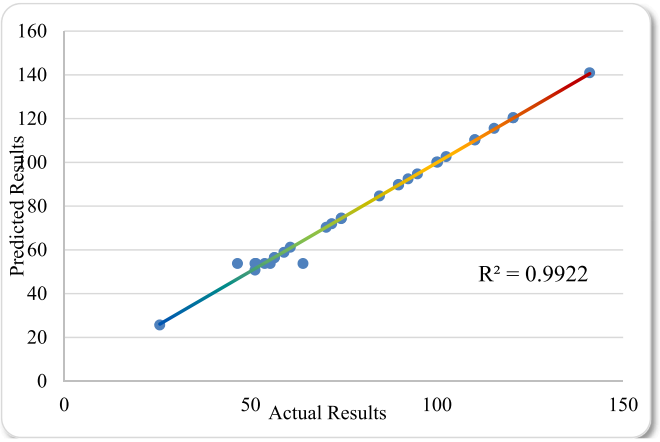

Figure (1). Relation between the actual and predicted fibrinolytic enzyme results.

3.3. Optimization of fibrinolytic enzyme production and model validation

Numerical optimization of the variables for maximum fibrinolytic enzyme production (141.899 U/g) under solid state fermentation showed that can be obtained at 3.6%, fodder yeast; 40%, moisture content; 6 days, incubation period; and 2%, inoculum size. These conditions were practically applied and resulted in (141.02 U/g) fibrinolytic enzyme production. The theoretical data of the model revealed (99.38%) validation when compared with practical results. The effect of the values of each variable and the interactions between these variables on fibrinolytic enzyme production by *Bacillus subtilis* Egy under solid-state fermentation are shown in figure (2) where each panel represents inter action between two factors (X1 and X2) on fibrinolytic activity (Y axis).

Table (4)
ANOVA of CCD model for fibrinolytic enzyme production.

Source	Sum of Squares	Df	Mean Square	F-Value	*p*-value[a] Prob > F
Model	22048.96	23	958.6503	32.98976	0.0001
A-Fodder Yeast	164.3267	1	164.3267	5.654927	0.0549
B-Moisture	796.005	1	796.005	27.39269	0.0020
C-Incubation period	472.7813	1	472.7813	16.26969	0.0069
D-Inoculum size	397.62	1	397.62	13.68318	0.0101
AB	19.75802	1	19.75802	0.679927	0.4412
AC	70.0569	1	70.0569	2.410848	0.1715
AD	9.703225	1	9.703225	0.333914	0.5844
BC	4.020025	1	4.020025	0.13834	0.7227
BD	448.1689	1	448.1689	15.42271	0.0077
CD	91.10702	1	91.10702	3.13524	0.1270
A^2	7.370417	1	7.370417	0.253636	0.6325
B^2	2000.2	1	2000.2	68.83232	0.0002
C^2	242.2526	1	242.2526	8.336569	0.0278
D^2	421.6817	1	421.6817	14.51121	0.0089
ABC	10.66023	1	10.66023	0.366847	0.5669
ABD	1246.796	1	1246.796	42.90564	0.0006
ACD	911.738	1	911.738	31.37538	0.0014
BCD	2303.04	1	2303.04	79.25386	0.0001
A^2B	62.70041	1	62.70041	2.157691	0.1922
A^2C	383.5221	1	383.5221	13.19804	0.0109
A^2D	730.8602	1	730.8602	25.15088	0.0024
ABCD	563.1129	1	563.1129	19.37824	0.0046
A^2B^2	5752.254	1	5752.254	197.9507	<0.0001
Residual	174.3542	6	29.05903		
Lack of Fit	0.700833	1	0.700833	0.020179	0.8926
Pure Error	173.6533	5	34.73067		
Cor Total	22223.31	29			

[a] Value of ("Prob > F″) < 0.05 indicates that the model term is significant.

3.4. Safety studies of fibrinolytic enzyme from Bacillus subtilis egy

3.4.1. In vitro cytotoxicity on human normal retina cell line (RPE1)

Sample range between 0.78 and 50 μL (ie up to 7.05 U/ml) from enzyme/ml using MTT assay gave no toxic effect on the used normal retina cell line. The obtained results (Table 5) proved the safety of the enzyme *in vitro* on normal human cell line.

3.4.2. In vivo acute toxicity study

The *in vivo* examination of the produced fibrinolytic enzyme resulted in no mortality during the first 24 h after taking the dose.

Following up, the dosed rats revealed no physical nor behavioral changes during the 14 investigation days. However, hematological analysis revealed an increase in WBCs for both sexes compared with controls as shown in Table (6).

The liver and kidney functions in both the tested groups showed no significant changes from the control group, except for serum AST in male rats of the subcutaneous dosed group and serum creatinine in female rats of the oral dosed group (Table 7).

4. 5. histopathological examination of liver

The livers of the rats from control group (male & female) showed normal hepatic cells with well-preserved cytoplasm, prominent nucleus and central veins with blood sinusoids (Fig. 3 A& B). Histopathological examination of liver from rats (male & female) received oral fibrinolytic enzyme showed a normal architecture with few inflammatory cells, congestion of hepatic sinusoids lined with endothelial and activated Kupfer cells in male, whereas, with few pyknotic cells, congestion of hepatic sinusoids and activated Kupfer cells in female (Fig. 3C & D).

Moreover, histopathological examination of liver from rats (male & female) received subcutaneous enzyme showed a nearly normal liver architecture, congestion of central vein, with few pyknotic cells and activated Kupfer cells in male, whereas, with few necrotic cells, congestion of hepatic sinusoids and activated Kupfer cells in female (Fig. 3 E & F).

4.1. Histopathological examination of kidney

Kidney section of control albino rats from (male & female) showed normal glomerulus surrounded by Bowman's capsule (Urinary space), proximal and distal convoluted tubules (Fig. 4 A & B). Histopathological Examination of kidney from rats (male & female) received oral enzyme showed a normal architecture with minimal interstitial hemorrhage (Fig. 4C & D). Moreover, sections of kidneys of rats (male & female) received subcutaneous enzyme showed nearly normal kidney architecture with slight wide of urinary space, with minimal interstitial hemorrhage and few pyknotic nuclei (Fig. 4 E & F).

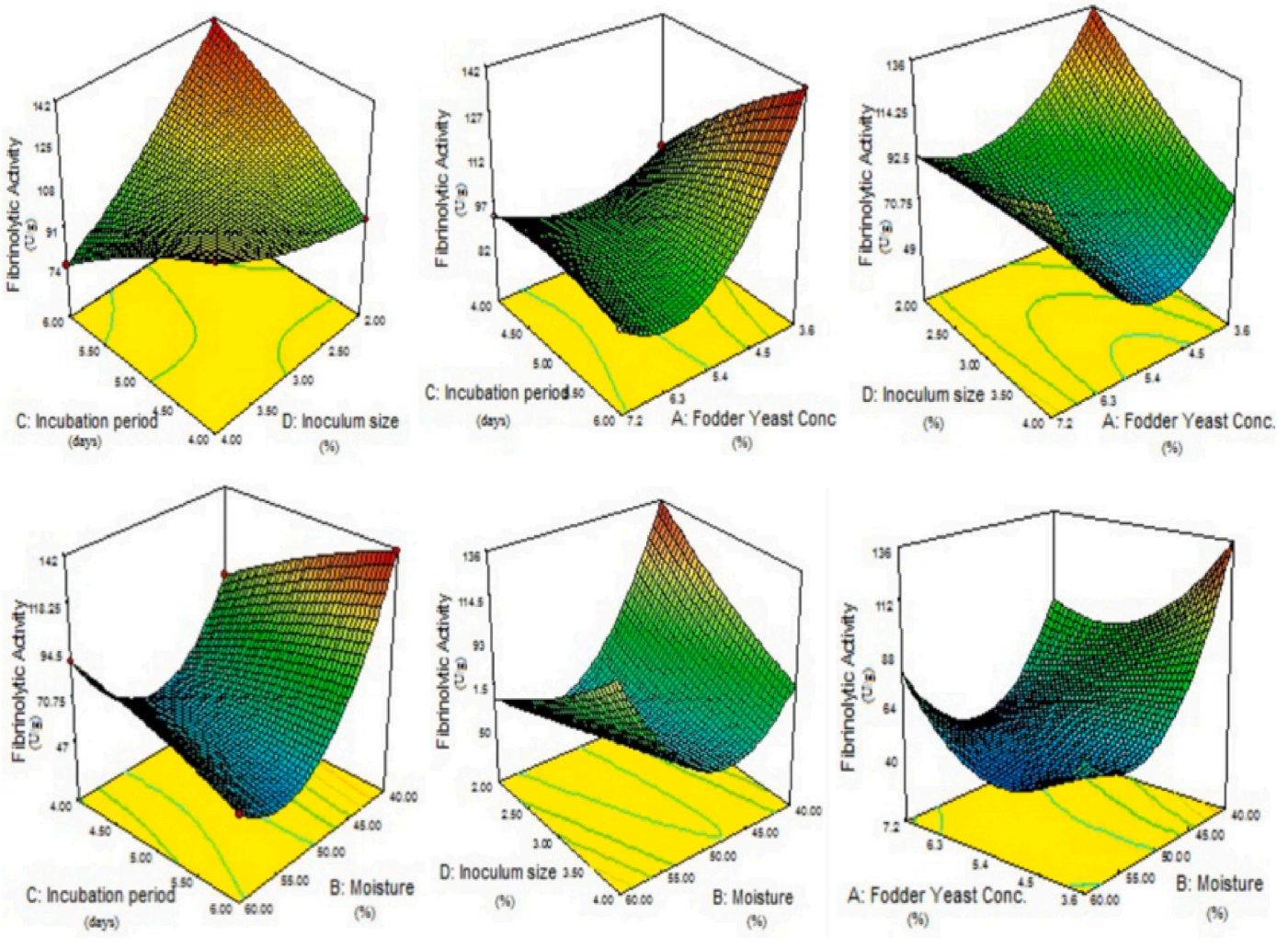

Figure (2). 3D representation of the effect of the different factors and factor-factor interactions on fibrinolytic enzyme production. A: Fodder yeast conc. B: Moisture, C: Incubation period (days), and D: Inoculum size (%).

Table (5)
In vitro cytotoxicity on human normal cell line.

Sample Code	IC_{50} (μg/ml)	IC_{90} (μg/ml)	Remarks
Fibrinolytic Enzyme	————	————	3.6% at 50 μL from enzyme/ml
DMSO	–	–	5% at 100 ppm
Negative control	–	–	0%

IC_{50}: Lethal concentration of the sample which causes the death of 50% of cells in 48 h IC_{90}:Lethal concentration of the sample which causes the death of 90% of cells in 48 h.

Table (6)
Effect of the subcutaneous and oral dosed fibrinolytic enzyme on rats'

Hematological Parameters	Control		Subcutaneous		Oral	
	Male	Female	Male	Female	Male	Female
RBCs (million cells/ul)	6.61 ± 0.27^a	6.4 ± 0.13^a	6.05 ± 0.22^a	6.05 ± 0.12^a	6.04 ± 0.04^a	6.07 ± 0.31^a
HB%	12.68 ± 0.30^a	12.30 ± 0.31^a	11.96 ± 0.30^a	12.06 ± 0.40^a	11.40 ± 0.10^a	11.48 ± 0.44^a
MCV (fL)	55.64 ± 0.11^a	58.76 ± 0.60^a	54.08 ± 1.80^a	55.82 ± 0.81^a	55.88 ± 0.93^a	55.00 ± 2.13
WBCs ($\times 10^9$/L)	9.280 ± 0.75	10.24 ± 0.55	12.20 ± 1.95	13.06 ± 0.68	13.68 ± 1.08	13.460 ± 0.90

Data are expressed as mean ± S.E. (Stander Error). Multiple comparisons of means were done by One-way analysis of variance (ANOVA) followed by least significant test at $p < 0.05$. Same letter means non-significant difference, while different letter means significant difference at $p < 0.05$.

Table (7)
Effect of the subcutaneous and oral dosed enzyme on rats' liver and kidney functions[2].

Functions		Control		Subcutaneous		Oral	
		Male	Female	Male	Female	Male	Female
Liver functions	AST (U/L)	64.55 ± 0.22^a	64.35 ± 0.94^a	61.18 ± 0.20^b	63.61 ± 0.17^a	63.21 ± 0.02^a	64.35 ± 0.20^a
	ALT (U/L)	43.85 ± 0.82^a	43.45 ± 0.65^a	45.18 ± 0.89^a	42.28 ± 0.32^a	44.54 ± 2.75^a	44.30 ± 0.12^a
Kidney functions	Serum creatinine (mg/dl)	3.01 ± 0.02^a	2.69 ± 0.12^a	2.79 ± 0.02^a	2.55 ± 0.06^a	2.80 ± 0.05^a	2.28 ± 0.02^b
	Urea (BUN) (g/dl)	5.80 ± 0.09^a	4.77 ± 0.10^a	6.23 ± 0.13^a	4.52 ± 0.29^a	6.22 ± 0.01^a	5.33 ± 0.15^a

Data are expressed as mean ± S.E. (Stander Error). Multiple comparisons of means were done by One-way analysis of variance (ANOVA) followed by least significant test at $p < 0.05$. Same letter means non-significant difference, while different letter means significant difference at $p < 0.05$.

5. Discussion

Solid State Fermentation (SSF) is the process in which microorganisms grow in an environment without or with very low free water content. This process guarantees very low handling and treatment of wastewater. In addition, SSF requires less energy for sterilization due to low water activity, allows the use of solid agro-industrial wastes as a substrate and/or energy source in their natural form allowing solid waste management. Furthermore, SSF is less susceptible to bacterial contamination, gives higher enzymatic productivity (compared with submerged fermentation) for many enzymes. The higher enzyme productivity may be attributed to less susceptible to substrate inhibition. Solid state fermentation has been used for production of biochemicals, pharmaceuticals, biofuels, food products and many other industries [25,26]. In this consideration twelve meals in additional to fodder yeast were tested for their ability to produce fibrinolytic enzyme under solid state fermentation using wheat bran as a carrier. Each tested nutrient was applied separately in conical flask with 6% final concentration. Fodder yeast revealed the highest enzyme productivity followed by sesame meal and olive meal. Thus, fodder yeast was chosen as a nutrient source for enzyme production under SSF conditions.

Fodder yeast products are composed of high concentrations of carbohydrates, proteins, amino acids, vitamins, micronutrients and many other valuable substances and have been used for decades in animal chicken, poultry and fish feed [27]. This explains the comparative and higher productivity of the enzyme. Chen-Tien et al. [28], reported high fibrinolytic enzyme production by *Bacillus subtilis* under solid-state fermentation using wheat bran as medium. In contrarily, Tuan et al. [29], reported that using Chickpeas as a substrate for the production of fibrinolytic enzyme by *B. amyloliquefaciens* under solid state fermentation yielded 39.28 fibrin degradation units per gram (FU)/g. Apple pomace was the optimum waste for production of fibrinolytic enzyme by the mutant *Bacillus cereus* GD55, under solid state fermentation in a study by Raju and Divakar [30]. In another study by Hua et al. [31], soybean meal was the optimum for fibrinolytic enzyme production by *Bacillus subtilis* XZI125. Biji et al. [32] used cuttle fish waste and cow dung substrate under solid state fermentation for production of fibrinolytic enzyme from *Bacillus cereus* IND5 [33].found that Sucrose as the carbon source and shrimp shell powder (SSP) as the nitrogen source expressed Nattokinase activity of 1721 U mL^{-1} and 2524 U mL by *pseudomonas aeruginosa* CMSS. Recently [34], studied the production of Nattokinase by *Bacillus subtilis* VITMS 2 on 2% soy bean meal as agroindustrial by product.

Central composite design (CCD) has been formed and applied to construct a statistical production model for fibrinolytic enzyme by *Bacillus subtilis* Egy. The analysis of the produced results based on ANOVA and (p-value <0.05) resulted in a significant model with good fitting and correlation among the predicted and actual data. The design resulted in a model that can navigate the design space.

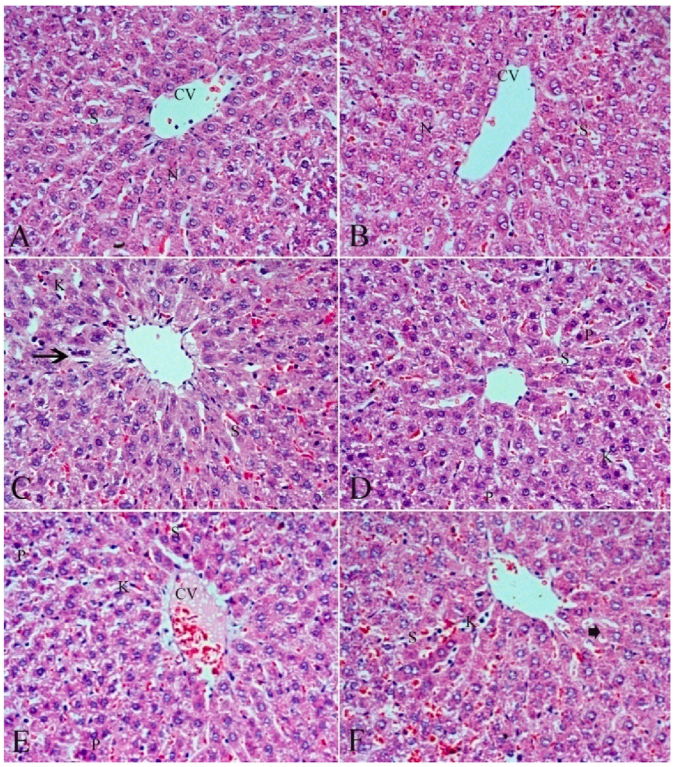

Figure (3). Photomicrograph (X400) of the liver sections stained with Hemotoxaline and Eosin. (**A**): Control liver of male showing normal hepatocytes architecture with normal central vein, sinusoids and nuclei; (**B**): Control liver of female showing normal hepatocytes architecture with normal central vein, sinusoids and nuclei; (**C**): Group male treated with purified enzyme showing normal architecture with few inflammatory cells (arrow), congestion of hepatic sinusoids and activated Kupfer cells; (**D**): Group female treated with purified enzyme showing normal architecture few pyknotic cells, congestion of hepatic sinusoids and activated Kupfer cells; (**E**): Group male treated with crude enzyme showing nearly normal liver architecture, congestion of central vein, with few pyknotic cells and activated Kupfer cells; (**F**): Group female treated with crude enzyme showing nearly normal liver architecture few necrotic cells (arrowhead), congestion of hepatic sinusoids and activated Kupfer cells.

Further investigations have been conducted to find out the optimum production conditions and to validate the results. Numerical optimization revealed that 3.6%, fodder yeast; 40%, moisture content; 6 days, incubation period and 2%, inoculum size were the optimum conditions for maximum fibrinolytic enzyme production (141.9 U/g) by *Bacillus subtilis* Egy under solid-state fermentation. The numerical data has been applied and resulted in (141.02 U/g) productivity which indicates 99.38% accuracy of the model. The obtained productivity is highly competitive and exceeds that obtained in many researches. Biji et al. [32] have utilized central composite design (Response Surface Method) to improve the productivity of fibrinolytic enzyme by *Bacillus cereus* IND5 and results in 2.5-fold excess production under solid state fermentation. Furthermore, CCD has been used by Vijayaraghavan et al. [35], for modeling and optimization of the fibrinolytic enzyme production by *Bacillus halodurans* IND18 where medium contained wheat bran, 1%, peptone and 80%, moisture content. Tuan et al., [29] obtained maximum enzyme productivity (39.28 U/g) at 50%, moisture content using Chickpeas as the substrate. Raju and Divakar [30], obtained maximum fibrinolytic enzyme (52.20 U/g) in a less economic process containing: peptone, NH_4NO_3 at 70%, moisture content; 2% inoculum size, for 4 days at 35 °C. The optimum inoculum size obtained by Raju and Divakar [30], is in agreement with that in the current study. Huy et al. [36], reported maximum fibrinolytic enzyme production at (77.9 FU/g) by *Bacillus amyloliquefaciens* under solid state fermentation.

The obtained results exhibited no mortality in rat groups after 24 h of subcutaneous or orally administered fibrinolytic enzyme. After 14 days, the results revealed no significant changes in hematological parameters (RBCs, MCV, hemoglobin except WBCs). WBCs showed significant increase due to both subcutaneous and oral enzyme intake. On the other hand, liver functions (serum ALT and AST) and kidney functions (serum creatinine and BUN) showed no significant changes except for serum creatinine, which decreased after oral administration in female rats as compared to female normal control, and serum AST, which decreased after subcutaneous administration of the enzyme in male rats as compared with male normal control. Based on our team study, we can conclude that both oral and subcutaneous administration are safe as a thrombolytic agent. These results are in agreement with that obtained by Lampe and English [19], who evaluated the safety of nattokinase *in vitro* and reported that no adverse effects were observed in 28-day and 90-day sub-chronic toxicity studies conducted in rats at doses up to 167 mg/kg-day and 1000 mg/kg-day, respectively. Mice inoculated with 7.55×10^8 CFU of the enzyme-producing bacterial strain showed no signs of toxicity or residual tissue concentrations of viable bacteria.

The *in vitro* assay conducted by Wang et al. [37], revealed significant blood clot lysis of the fibrinolytic enzyme produced by *Bacillus*

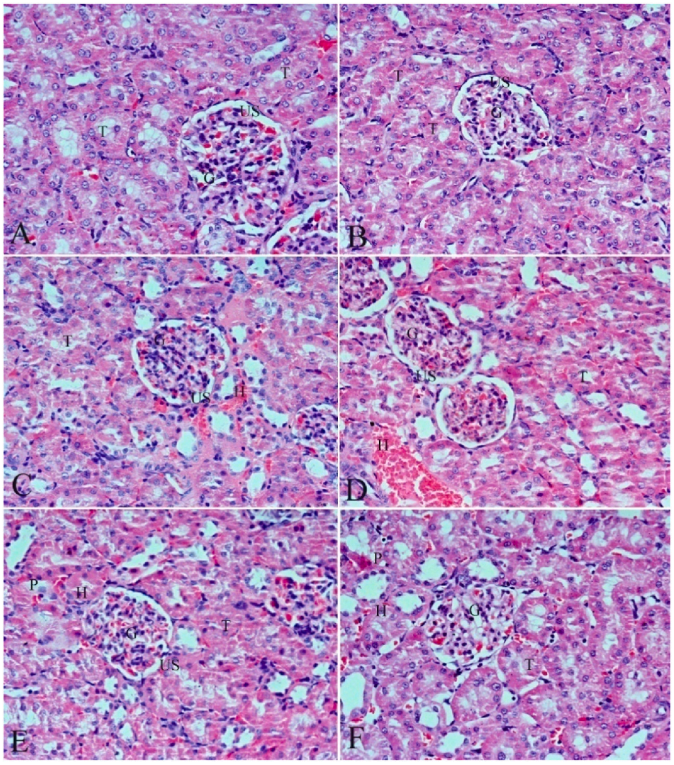

Figure (4). Photomicrograph (X400) of the kidney sections stained with Hemotoxaline and Eosin. (**A**): Control kidney of male showing normal glomerulus surrounded by Bowman's capsule, proximal and distal convoluted tubules; (**B**): Control kidney of female showed normal glomerulus surrounded by Bowman's capsule, proximal and distal convoluted tubules; (**C**): Male group treated with oral enzyme showed normal architecture with minimal interstitial hemorrhage; (**D**): Female group treated with oral enzyme showed normal architecture with minimal interstitial hemorrhage; (**E**): Male group treated with subcutaneous enzyme showed nearly normal kidney architecture with slight wide of urinary space, with minimal interstitial hemorrhage and few pyknotic nuclei; (**F**): Female group treated with subcutaneous enzyme showed, in generally, normal kidney architecture with slight wide of urinary space, with minimal interstitial hemorrhage and few pyknotic nuclei.

subtilis LD-8 [38] reported the non-toxicity and efficiency of fibrinolytic enzyme isolated from *Bacillus amyloliquefaciens* FCF-11 based on *in vitro* and *in vivo* assays. The produced enzyme showed *in vitro* direct action on blood clots and prolonged the blood clotting time to 4.1-fold. Research conducted by Yogesh and Halami [39], revealed an interesting characteristic feature of the native isolate *Bacillus subtilis* BR21, for its ability to produce multiple proteases acting on fibrin and fibrinogen. The fibrinolytic ability of multiple proteases together may help in developing cheaper and effective orally administrable thrombolytic preparations. Recently, Krishnamurthy et al. [40] reviewed the available methods to assess therapeutic potential of the microbially produced fibrinolytic enzymes. They discussed the different assay techniques, *in vitro* trials and *in vivo* models for evaluating efficacy of potential drug candidate. The investigations conducted by Omura et al. [41] on a purified protein, referred to as NKCP obtained from *B. subtilis natto* fermentation, showed direct degradation of artificial blood clot and suggested that NKCP is considered as safe for clinical use and a fibrinolytic effect similar to heparin. In 2010, Mahajan et al. [42], had investigated blood clot degradation using nattokinase produced by *B. natto* NRRL 3666. The results obtained by Vijayaraghavan et al. [35], are in agreement with our results and where the purified fibrinolytic enzyme was capable to degrade the fibrin net of blood clot and was suggested as a potentially effective thrombolytic agent. Zhou et al. [43], assessed therapeutic efficacy of fibrinolytic enzyme from marine *Bacillus velezensis* Z01 *in vivo*. They reported that a dose of 0.22–0.88 mg/kg could effectively prevent mouse tail thrombosis.

6. Conclusion

The current work is a part of bigger project for production of fibrinolytic enzyme from microbial source using the biotechnological methods. *Bacillus subtilis* Egy has been used for production of fibrinolytic enzyme under solid state fermentation. Central Composite Design revealed an interactive and promising tool in biotechnology for modeling and optimization of the bioprocesses. Fibrinolytic enzyme produced by *Bacillus subtilis* Egy showed biological safety *in vitro* on human normal cell line and *in vivo* examination using groups of rats. Finally, fibrinolytic enzyme produced by *Bacillus subtilis* Egy could be safely and effectively applied as thrombolytic agent.

Author contribution statement

Maysa E. Moharam: Conceived and designed the experiments; Contributed reagents, materials analysis tools or data; Wrote the paper.

Magda A. El- Bendary: Analyzed and interpreted the data; Contributed reagents, materials analysis tools or data.

Mostafa M. Abo Elsoud: Conceived and designed the experiments; Contributed reagents, materials analysis tools; Analyzed and interpreted the data; Wrote the paper.

Fawkia El- Beih: Contributed reagents, materials analysis tools or data.

Saadia M. Hassnin: Analyzed and interpreted the data.

Abeer Salama, Nora N. Elgamal: Performed the experiments; Wrote the paper.

Enayat A. Omara: Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

The data that has been used is confidential.

Declaration of competing interest

The authors declare that they have no competing interests.

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