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ARTICLE

Statistical optimization of conditions for protease production from *Bacillus* sp.

Rajshree Saxena[1], Rajni Singh[2]*

[1]Amity Institute of Biotechnology, Amity University, Sector-125, Noida, Uttar Pradesh, India, [2]Amity Institute of Microbial Biotechnology, Amity University, Sector-125, Noida, Uttar Pradesh, India

ABSTRACT Response surface methodology was employed for the optimization of different nutritional and physical parameters for the production of protease by a soil isolated *Bacillus* strain in submerged fermentation. Initial screening of production parameters as carbon (glucose) and nitrogen source (soybean) were optimized together with four variables K_2HPO_4, NaCl, $MgSO_4.7H_2O$, $CaCl_2 2H_2O$ and four physical parameters including agitation, inoculum size, pH and time was performed using Plackett Burman design and the variables with statistically significant effect on the protease production identified. These variables were selected for further optimization studies using central composite design in RSM. The protease activity under unoptimized conditions was 330 U/ml. Under the final optimized conditions, the predicted response for protease production was 449 U/ml, and the observed validated experimental value was 577 U/ml. The statistical optimization by response surface methodology resulted in about two fold increase in the production of the enzyme by the selected bacterial strain

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Proteases, also known as peptidyl-peptide hydrolases (EC 3.4.21-24 and 99) are industrially useful enzymes which catalyze the hydrolysis of a peptide bond in a protein molecule. Microbial proteases, especially from *Bacillus* sp. have traditionally held the predominant share of the industrial enzyme market of the worldwide enzyme sales with major application in food and feed, leather, detergent, pharmaceutical, silk, and recovery of silver from photographic films (Anisworth 1994; Outtrup et al. 1995; Inhs et al. 1999).

Protease production by microorganism is highly influenced by media components as carbon, nitrogen ratio, presence of some easily metabolizable sugars such as glucose (Gupta et al. 2002; Beg et al. 2002; Ferrero et al. 1996) and metal ions (Varela et al. 1996). Besides this several other factors such as aeration, inoculums, density, pH, temperature and incubation time also affect the amount of protease produced (Nehete et al. 1985; Hameed et al. 1999). Process optimization is a topic of central importance in industrial production processes with particular regard to biotechnology (Reddy et al. 2008). Optimization of medium by the classical method involves changing one independent variable while maintaining all others at a fixed level is extremely time consuming and expensive when a large number of variables are evaluated. To overcome this difficulty, experimental factorial design and response surface methodology can be employed to optimize the medium components.

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*Corresponding author. E-mail: rsingh3@amity.edu
rajni_vishal@yahoo.com

The Plackett–Burman factorial designs allow for the screening of main factors from a large number of process variables, and these designs are thus quite useful in preliminary studies in which the principal objective is to select variables that can be fixed or eliminated in further optimization processes as response surface methodology (RSM). RSM is a collection of statistical and mathematical techniques useful for the modeling and analysis of problems in which a response of interest is influenced by several variables, with the objective being to optimize this response (Montgomery, 2001). Response surface methodology has eliminated the drawbacks of classical methods and has proved to be powerful and useful for the optimization of the target metabolites production (Deepak et al. 2008; Liu and Wang 2007; Sayyad et al. 2007). Second-order models like Central Composite Box–Behnken and Doehlert designs are widely used in RSM as they can take on a wide variety of functional forms, and this flexibility allows them to more closely approximate the true response surface (Srinivas et al. 1994; Carvalho et al. 1997; Adinarayan and Elliah 2002; Rahman and Gomes 2003; Li et al. 2007; Xiao et al. 2007). Moreover, it is easy to estimate the parameters in a second-order model using the method of least squares. RSM has been recently used for the modelling and optimization of several bioprocesses, including fermentations (Sen 1997) enzymatic reactions (Ferreira et al. 1998) product recovery (Annadurai et al. 1996) and enzyme immobilization techniques (Zhao et al. 2007; Chang et al. 2007). The application of experimental design and response surface methodology in fermentations process can result in improved

Table 1. Design Placket Burman.

Std Run	Factor 1 A: Glucose %	Factor 2 B: Soybean %	Factor 3 C: K_2HPO_4 %	Factor 4 D: $MgSO_4$ %	Factor 5 E: NaCl %	Factor 6 F: $CaCl_2$ %	Factor 7 G: Agitation RPM	Factor 8 H: Inoculum %	Factor 9 J: pH	Factor 10 K: Time h	Response 1 Protease Activity U/ml
1	2	5	0.05	0.5	0.5	0.2	120	1	8	48	187.6
2	1	5	0.5	0.01	0.5	0.2	180	1	8	24	218.4
3	2	2	0.5	0.5	0.05	0.2	180	3	8	24	228.2
4	1	5	0.05	0.5	0.5	0.01	180	3	10	24	234.4
5	1	2	0.5	0.01	0.5	0.2	120	3	10	48	200.2
6	1	2	0.05	0.5	0.05	0.2	180	1	10	48	238
7	2	2	0.05	0.01	0.5	0.01	180	3	8	48	330.2
8	2	5	0.05	0.01	0.05	0.2	120	3	10	24	166.6
9	2	5	0.5	0.01	0.05	0.01	180	1	10	48	231.2
10	1	5	0.5	0.5	0.05	0.01	120	3	8	48	240.8
11	2	2	0.5	0.5	0.5	0.01	120	1	10	24	35.2
12	1	2	0.05	0.01	0.05	0.01	120	1	8	24	134.2

product yields, reduced process variability and development time and over all costs (Rao et al. 2000).

This paper aims to optimize the composition of the production medium and the cultivation parameters of the bioprocess for obtaining protease by *Bacillus* strain using the response surface methodology

Materials and Methods

Isolation and screening of protease producing strain

About 45 strains were isolated from soil collected from various sites. Primary screening of the isolates for protease production was done by milk agar plate method. The strain exhibiting largest zone of hydrolysis was selected for further experimentation. Stock culture of the organism was maintained at -20°C in 50% glycerol.

Protease production

Enzyme production was studied in media containing (g/l) glucose 10.0, soybean 10.0, K_2HPO_4 5.0, $MgSO_4$.$7H_2O$ 0.5, NaCl 0.5 and $CaCl_2$ 0.5. Batch mode shake flask experiments were conducted at 37°C and 120 rpm for 24 h in100-ml Erlenmeyer flasks containing 50 ml of the media. The production media was inoculated with 1% of inoculums and incubated in a shaking incubator. The fermentation broth was then centrifuged at 10,000 g for 10 min in a centrifuge, and the total protease activity in the cell-free supernatant was determined.

Enzyme assay

Protease activity was measured using casein as substrate. The enzyme extract suitably diluted, was mixed with 50 mM glycine–NaOH buffer (pH 8) to make 1 ml volume. 1ml of 1% casein was added and incubated for 10 min at 60°C. The reaction was stopped by addition of 0.5 ml TCA (20%, w/v). The mixture was allowed to stand at room temperature for 30 min to 1 h and then filtered to remove the precipitate. 1 ml of the filtrate was mixed with 5 ml of 0.5 M Na_2CO_3 solution. 0.5 ml of Folin Ciocalteu's (phenol reagent) reagent was added and the mixture was incubated in dark to develop the blue colour. The blue coloured solution was then was estimated spectrophotometrically at 660 nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg tyrosine per millilitre in 1 min under the experimental conditions used. The experiments were carried out in triplicates and standard error was calculated.

Optimization of media

The optimization of media for enzyme production was carried out using statistical design of experiments in two steps. In the first step the screening of variables which was done by Plackett- Burman design. The second step involved the optimization of significant variables by RSM employing the central composite design. Design Expert® 8.0.2.0 (Stat-Ease, Inc., Minneapolis, MN, USA) was used to design and analyze both the experiments.

Selection of significant variables by Plackett–Burman design

Plackett–Burman design is an effective method for screening for significant medium components that influence enzyme production. It allows the evaluation of N variables in the N+1 experiments; each variable is examined at two levels: -1 for a low level and +1 for a high level (Plackett and Burman 1946; Rama et al. 1999; Ghanem et al. 2000). In this part, the selected carbon (glucose) and nitrogen source (soybean) were optimized together with four variables: K_2HPO_4, NaCl, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$ and four physical parameters including agitation, inoculum size, pH and time. These vari-

Table 2 a. Ranking of the variables investigated in the Plackett–Burman design.

Variable	Component	M_i^+–	M_i^-	$E(x_i)$	Absolute $E(x_i)$	Ranking
A	Glucose	1179	1266	-14.5	14.5	8
B	Soyabean	1279	1166	18.8333	18.8333	7
C	K_2HPO_4	1154	1291	-22.833	22.833	5
D	$MgSO_4$	1164.2	1280.8	-19.433	19.433	6
E	NaCl	1206	1239	-5.5	5.5	9
F	$CaCl_2$	1239	1206	5.5	5.5	10
G	Agitation	1480.4	964.6	85.9667	85.9667	1
H	Inoculum	1400.4	1044.6	59.3	59.3	4
I	pH	905.4	1339.4	-72.333	72.333	2
J	Time	1428	1017	68.5	68.5	3

Table 2 b. ANOVA for Placket Burman.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	55925.61	10	5592.561	373.751	0.0402	significant
A-Glucose	630.75	1	630.75	42.15304	0.0973	
B-Soybean	1064.083	1	1064.083	71.11272	0.0751	
C-K_2HPO_4	1564.083	1	1564.083	104.5277	0.0621	
D-$MgSO_4$	1132.963	1	1132.963	75.71597	0.0728	
E-NaCl	90.75	1	90.75	6.064825	0.2456	
F-$CaCl_2$	90.75	1	90.75	6.064825	0.2456	
G-Agitation	22170.8	1	22170.8	1481.675	0.0165	
H-Inoculum	10549.47	1	10549.47	705.0214	0.0240	
J-pH	4555.203	1	4555.203	304.4244	0.0364	
K-Time	14076.75	1	14076.75	940.7496	0.0207	
Residual	14.96333	1	14.96333			
Cor Total	55940.57	11				

Std. Dev: 3.87; R-Squared: 0.9997; Adj R-Squared: 0.9971, Pred R-Squared: 0.9615; PRESS: 2154.72; Adeq Precision: 79.653.

ables screened with a twelve-run Plackett–Burman design are shown in Table 1. The effect of each variable was determined by the following equation: $E(x_i) = 2(\sum M_i^+ - M_i^-) / N$ (1) where $E(x_i)$ is the concentration effect of the tested variable, M_i^+ and M_i^- are the total production from the trials where the measured variable (x_i) was present at high and low concentrations, respectively; and N is the number of trials.

Central composite design

The next step in the optimization was to determine the optimum levels of significant variables screened by Placket Burman design. For this purpose, RSM, using a central composite design (CCD) was adopted. The CCD is a statistical experimental design where each numeric factor is varied over 5 levels- alpha points (-1.682, +1.682), 1 factor (-1, +1) and one centre point resulting in a total of 20 experiments. Three significant variables (glucose, soybean K_2HPO_4,) were chosen for the experiment. The 20 experiments were conducted in duplicates. The design is shown in Table 3 (a and b).

Statistical analysis and Modelling

The statistical analysis of the data obtained from RSM for protease production was subjected to analysis of variance (ANOVA). A second order polynomial equation (1) can be used to represent the function in the range of interest.

$$Y = \beta o + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

Where Y is the measured response, β_0 is the intercept term, β_1, β_2, β_3 are linear coefficient, β_{11}, β_{22}, β_{33} are quadratic coefficient, β_{12}, β_{13}, β_{23} are interaction coefficient and X_1, X_2 X_3 are coded independent variables.

Results and Discussion

The selected isolate was a *Bacillus* strain as identified on the basis of microscopic and biochemical analysis.

Table 3 a. Design Summary CCD.

Study Type			Response Surface			Runs			20	
Design Type			Central Composite			Blocks			No Blocks	
Design Model			Quadratic			Build Time (ms)			4.67898	
Factor	Name	Units	Type	Subtype	Minimum	Maximum	-1 Actual	+1 Actual	Mean	Std. Dev.
A	Glucose	%	Numeric	Continuous	0.659104	2.340896	1	2	1.5	0.413171
B	Soybean	%	Numeric	Continuous	0.977311	6.022689	2	5	3.5	1.239514
C	K_2HPO_4	%	Numeric	Continuous	-0.20681	1.306807	0.1	1	0.55	0.371854
Response Name	Units	Obs	Analysis	Minimum	Maximum	Mean	Std. Dev.	Ratio	Trans	Model
Protease Activity	U/ml	20	Polynomial	199.65	456.64	358.8005	92.7528	2.287203	None	Quadratic

Table 3 b. Central composite design matrix for the experimental design and predicted responses for protease activity.

Std Run	Factor 1	Factor 2	Factor 3	Response 1 Protease Activity (U/ml)			
	A:Glucose %	B:Soybean %	C:K_2HPO_4 %	Actual Value	Predicted Value	Residual value	Validation Value
1	1	2	0.1	214.9	218.8342	-3.93	291.06
2	2	2	0.1	199.65	195.1977	4.452	148.5
3	1	5	0.1	309.17	306.0927	3.077	380.16
4	2	5	0.1	288.75	296.2112	-7.46	249.99
5	1	2	1	356.4	350.9927	5.407	406.89
6	2	2	1	298.65	303.7812	-5.13	317.81
7	1	5	1	440.87	447.3762	-6.51	482.24
8	2	5	1	415.8	413.9197	1.88	449.4
9	0.66	3.5	0.55	372.9	372.7273	0.173	420.76
10	2.34	3.5	0.55	327.45	324.718	2.732	361.12
11	1.5	0.98	0.55	216.98	218.4424	-1.46	252.66
12	1.5	6.02	0.55	388.8	384.4329	4.367	337.24
13	1.5	3.5	-0.21	218.6	217.2916	1.308	233.89
14	1.5	3.5	1.31	429	427.4038	1.596	500.48
15	1.5	3.5	0.55	448.45	449.7647	-1.31	577.67
16	1.5	3.5	0.55	456.64	449.7647	6.875	577.67
17	1.5	3.5	0.55	448.23	449.7647	-1.53	577.67
18	1.5	3.5	0.55	449.64	449.7647	-0.12	577.67
19	1.5	3.5	0.55	442.68	449.7647	-7.08	577.67
20	1.5	3.5	0.55	452.45	449.7647	2.685	577.67

Screening of significant variables using Plackett–Burman design

A total of ten variables were analyzed with regard to their effects on protease production using a Plackett–Burman design. The design matrix selected for the screening of significant variables for protease production and the corresponding responses are shown in Table 1. Table 2a represents the $E(x_i)$ value and ranking of the variables investigated in the Plackett–Burman design. A large $E(x_i)$ coefficient, either positive or negative, indicates a large impact on response; while a coefficient close to zero indicates little or no effect. Agitation, inoculum size and time along with chemical factors soybean and $CaCl_2$ had a positive effect on the production, while pH, glucose, K_2HPO_4, $MgSO_4$ and NaCl negatively affected the production. The adequacy of the model was calculated, and the variables evidencing statistically significant effects were screened via Student's t-test for ANOVA (Table 2b). The Model F-value of 373.75 implies the model is significant. There is only a 4.02% chance that a Model F-Value this large could occur due to noise. Values of Prob > F less than 0.0500 indicated that the model terms are significant. In this case factors G, H, J and K are significant model terms. The Predicted R-Squared of 0.9615 is in reasonable agreement with the Adjusted R-Squared of 0.9971. Adequate Precision measures

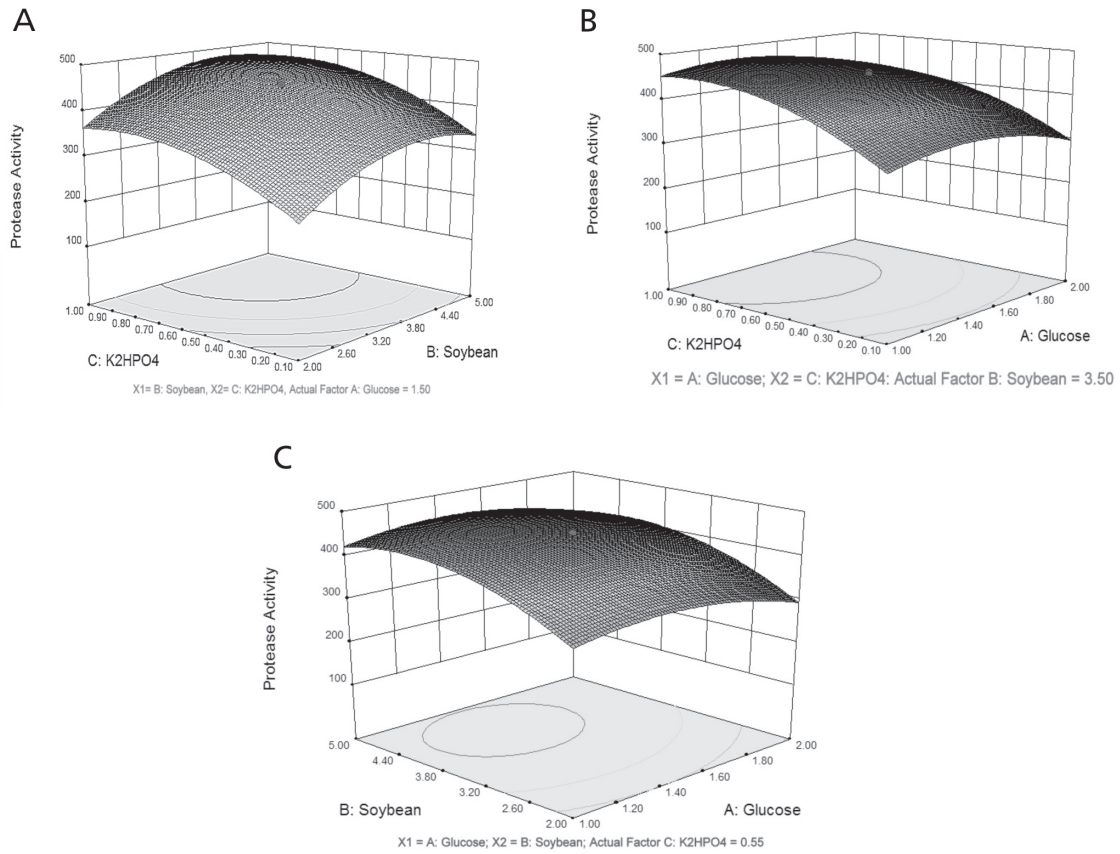

Figure 1. Contour plots of enzyme activity as a function of the interactions of two variables by keeping the other at centre level: interactions of soybean and K_2HPO_4 with glucose at 1.5% (a), interactions of glucose and K_2HPO_4 with soybean at 3.5% (b), and interactions of glucose and soybean with K_2HPO_4 at 0.55% (c).

the signal to noise ratio. A ratio greater than 4 is desirable. In our model the ratio of 79.653 indicates an adequate signal. This model can be used to navigate the design space.

Optimization of significant variables using response surface methodology

A quadratic model consisting of twenty trials experiments was designed (Table 3a). The design matrix and the corresponding results of RSM experiments to determine the effects of three independent variables (glucose, soybean and K_2HPO_4), along with the mean predicted values and the residual value are shown in Table 3b.

The ANOVA analysis of the optimization study is given in table 4. The Model F-value of 527.45 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. The model was found to be highly significant and sufficient to represent the actual relationship between the response and the significant variables as indicated by the small model P-value (<0.0001), large lack-of-fit P-value (0.2089). The "Lack of Fit F-value" of 2.16 implies that it is non significant relative to the pure error. Non-significant lack of fit is good.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.In this case A, B, C, AC, A^2, B^2, C^2 are significant model terms.

The regression equation coefficients were calculated and the data was fitted to a second-order polynomial equation. Thus the response (Y), (in terms of coded factors) protease production by the selected *Bacillus* sp. can be expressed in terms of the following regression equation:

$$\text{Protease Activity} = +449.76 - 14.27A + 49.35B + 62.47C + 3.44AB - 5.89AC + 2.28BC - 35.72A^2 - 52.44B^2 - 45.05C^2$$

Where A is glucose, B is soybean and C is K_2HPO_4.

The regression equation obtained from the ANOVA showed that the R^2 (multiple correlation coefficient) was 0.9979 (a value >0.75 indicates fitness of the model). This is an estimate of the fraction of overall variation in the data accounted by the model, indicating that the model is capable of explaining 99.79% of the variation in response. The adjusted R^2 is 0.9960. The pred R^2 of 0.9864 is in reasonable agreement with the ad-

Table 4. Analysis of variance table (ANOVA for Response Surface Quadratic Model - CCD).

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	163114.9	9	18123.88	527.4532	< 0.0001	significant
A-Glucose	2782.246	1	2782.246	80.97074	< 0.0001	
B-Soybean	33259.21	1	33259.21	967.9314	< 0.0001	
C-K_2HPO_4	53290.33	1	53290.33	1550.89	< 0.0001	
AB	94.60001	1	94.60001	2.753112	0.1281	
AC	277.8903	1	277.8903	8.087346	0.0174	
BC	41.63281	1	41.63281	1.211625	0.2968	
A^2	18391.5	1	18391.5	535.2416	< 0.0001	
B^2	39632.7	1	39632.7	1153.417	< 0.0001	
C^2	29246.1	1	29246.1	851.1393	< 0.0001	
Residual	343.6112	10	34.36 112			
Lack of Fit	234.8798	5	46.97595	2.160182	0.2089	not significant
Pure Error	108.7315	5	21.7463			
Cor Total	163458.6	19				

Std. Dev: 5.86; R-Squared: 0.9979; Adj R-Squared: 0.9960; Pred R-Squared: 0.9864; Adeq Precision: 61.416.

justed R^2 of 0.9960. For a good statistical model, the R^2 value should be in the range of 0–1.0, and the values as obtained in the data analysis indicates that the model is good. The 'adequate precision value' of the present model was 61.416 which indicates an adequate signal and that the model can be used to navigate the design space.

The optimal levels of each variable for maximum protease production were determined by the three-dimensional response surface plots which were constructed by plotting the response (protease production) on the Z-axis against any two independent variables, while maintaining other variables at their optimal levels. As is shown in Fig. 1 A, a increase in protease production was observed when the concentrations of soybean and K_2HPO_4 increased initially but a concomitant decline was observed when concentration of both the components were further increased at a constant level of glucose. Similar results was observed when concentrations of glucose and K_2HPO_4 (Fig. 1B) and glucose and soybean (Fig.1C) were increased at a single concentration of soybean and K_2HPO_4 respectively. All the three figures show a fairly strong degree of curvature of 3D surface where the optimum can easily be determined.

Thus the maximum protease production was 452 U/ml after 48h when the levels of glucose, soybean and K_2HPO_4 were at their central value of 1.5, 3.5 and 0.55 % respectively

Validation of the model equation in shake flask culture

The validation of the statistically optimized condition for the production of protease by the selected Bacillus strain was verified by carrying out shake flask fermentation at bench scale in the laboratory. The model was verified for the three variables within the design space. 15 production combinations were prepared and tested for the protease production (Table 7). The protease activity under unoptimized conditions was 330 U/ml. Under the final optimized conditions, the predicted response for protease production was 449U/ml, and the observed validated experimental value was 577U/ml. These results confirmed the validity of the model, and the experimental were found to be quite close to the predicted values. The optimization of the media led to a two fold increase in the production value.

Conclusion

The use of Plackett–Burman design and Central Composite Design in Response Surface Methodolgy for determination of optimal medium composition for protease production is demonstrated in the present study. The optimization resulted in about two fold increase in the production of the enzyme by the selected bacterial strain. The results show the effect of various factors on the enzyme production. The results of ANOVA and regression of the second-order model showed that the effects of glucose, soybean and K_2HPO_4 and the interactive effects of all variables are more significant for protease production.

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