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STATISTICAL OPTIMIZATION FOR PRODUCTION OF THERMOSTABLE AND ALKALI TOLERANT SUPEROXIDE DISMUTASE BY *ANOXYBACILLUS GONENSIS* KA 55 ISOLATED FROM THERMAL HOT SPRINGS OF NORTH WESTERN HIMALAYAS

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ABSTRACT

Thermostable superoxide dismutase containing bacterium was isolated from water sample collected from Manikaran hot water spring in the Kullu district of Himachal Pradesh. Bacterium was identified by 16S rDNA analysis and found 99% similarity with *Anoxybacillus gonensis*. To enhance the production of superoxide dismutase from the bacterium various nutritional and reaction parameters were optimized using Central Composite design of response surface methodology. Experimental data was analysed using ANOVA. Enzyme production was optimized by submerged fermentation. Relatively high activity (530.6 U/mL) of superoxide dismutase was obtained in a production medium containing 1 % peptone, 1 % meat extract, 0.5 % NaCl and 0.03 % K_2HPO_4 at a pH of 9 and a cell volume of 100 µl. A 3.98-fold increase in superoxide dismutase activity was observed under optimal conditions. Enzyme was stable up to a temperature of 70°C and showed striking stability at a pH ranging from 7 to 10. *A. gonensis* KA 55 is a novel source for the production of thermostable superoxide dismutase enzyme.

Keywords: Superoxide dismutase, *Anoxybacillus gonensis* KA 55, Central composite design, Response surface methodology

INTRODUCTION

Superoxide dismutases (SODs, EC 1.15.1.1) are a class of antioxidant enzymes that convert superoxide radical into oxygen and hydrogen peroxide [1]. Four different types of SODs have been found based on the type of metal ion present. Till date, four different varieties of SOD have been found, differing in the type of the metal present whether its iron, manganese, copper-zinc or nickel [2-5]. SOD play crucial role in preventing the organism from reactive oxygen species (ROS). SOD is having wide applications in medical field as well as pharmaceutical industry. Numerous studies have shown safety of superoxide dismutases drugs in man and animals. Use of SOD as an anti-inflammatory agent has been established in many studies [6]. *In vivo* use of lecithinized SOD for the treatment of ulcerative colitis has been reported in many studies [7]. The topical application of SOD cream has been proved to be effective against various ailments like; systemic lupus erythematosus, progressive systemic sclerosis, Behcet's disease, burns and herpes simplex [8]. Different *in vivo* and *in vitro* studies have shown decrease in the concentration of superoxide dismutase in tumour cells [9] which shows its significance in the field of oncology. Keeping in view the importance of SOD in both pharmaceutical and medical field, present study has been carried out on the isolation and optimization of superoxide dismutase production from thermophilic bacterium.

For hyper production of thermostable superoxide dismutase from *A. gonensis* KA 55 for industrial applications, an attempt was made to optimize production and reaction parameters through statistical approach using OVAT (one variable at a time) and RSM (response surface methodology).

MATERIALS AND METHODS

Chemicals

All the chemicals used were of analytical grade and procured from Merck and Hi-Media, India.

Enzyme assay

Assay method was based on the ability of superoxide dismutase to inhibit the reduction of Nitroblue tetrazolium chloride (NBT) by superoxide radical [10]. One unit of enzyme activity is defined as the enzyme concentration required to inhibit the optical density at 560nm of chromogen production by 50% in one minute under standard conditions.

Isolation of superoxide dismutase producing bacteria

In this study, thermophilic bacteria producing superoxide dismutase were isolated from hot water springs of

Himachal Pradesh namely Manikaran (Kullu), Tattapani (Shimla), Vashisht (Kullu) and Tatwani (Kangra). Superoxide dismutase producing bacteria were isolated on the nutrient agar at a pH of 7 and were incubated at different temperatures ranging from 50°C to 70°C. The isolates were screened for superoxide dismutase activity by the assay method given by Kakkar et al. 1984. Isolate which was having maximum SOD activity and thermostability was identified and characterized.

Identification of the isolate

Bacterial isolate was characterized by observing colony morphology, color, margin, elevation and Gram staining. Biochemical characterization was also done. The bacterium was identified by isolating 16S rDNA. DNA was isolated and amplified using universal primers (8F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-ACGGTTACCTTGTTACGACTT-3') [11].

Optimization by one variable at a time method

For increasing the production of SOD from *A. gonensis* KA 55, optimization of various nutritional and reaction parameters was carried out including temperature, pH, carbon sources, nitrogen sources, sodium chloride concentration, potassium hydrogen phosphate concentration, reaction temperature, cell volume, buffer, pH of buffer and thermostability by one variable at a time method. Factors which were crucial for superoxide dismutase production were used in designing the experiments for CCD.

Study of the enzyme stability

The cells of *A. gonensis* KA 55 were preincubated at temperature of 50°C, 60°C, 70°C and 80°C, enzyme assay was performed after an interval of 1 h and the enzyme activity was calculated.

Optimization of medium components using RSM

The preliminary study showed that peptone, meat extract, sodium chloride and K_2HPO_4 were significant variables for SOD production. A CCD of RSM (Stat-Ease, Inc Design Expert software, trial version, 10.0.3.0 (32 bit) Minneapolis, USA) using these factors was designed to optimize the production of SOD. The CCD was studied at five levels and consisted of total of 21 runs, out of which 5 were centre points, 8 axial and 8 factorial. Each variable was studied at three different levels (-1, 0, +1).

Optimization of reaction conditions using RSM

Factors that were taken into consideration were pH of the buffer, reaction temperature and cell volume. The experimental design consisted of 20 experiments. All the experiments were performed in triplicates and average superoxide dismutase activity obtained was taken as dependent variable or response. Each variable was studied at three different levels (-1, 0, +1).

RESULTS

Isolation and selection of SOD producing bacterium

A total of 70 isolates were obtained after isolation from hot water springs. Eight isolates showed appreciable SOD activity. Out of these eight isolates one isolate which gave the maximum activity was selected and designated as M560.

Identification of the isolate

The morphological and biochemical analysis of isolate KA 55 revealed that it had characteristics similar to genus *Bacillus.* The 16S rRNA sequencing was also performed and this isolate showed maximum similarity (99%) with *Anoxybacillus gonensis.* Hence it was designated as *Anoxybacillus gonensis* KA 55.

Optimization by one variable method

In order to increase the yield of superoxide dismutase, its production was optimized by one variable at a time method. A remarkable increase in the superoxide dismutase activity, i.e., from 133 U/mL to 512 U/mL, was achieved under following optimized conditions viz., 55°C incubation temperature, 1.0 % peptone, 1.0 % meat extract, 0.5 % NaCl, 0.03 % K_2HPO_4, 9.0 buffer pH, 40°C reaction temperature and 100 µl cell volume.

The stability of SOD produced by *A. gonensis* KA 55

To determine the thermostability of SOD produced by *A. gonensis* KA 55, the resting cells were suspended in sodium tetrapyrophosphate buffer (0.05M, pH 9.0) at different temperatures 50°C, 60°C, 70°C and 80°C (Figure 1). The enzyme assay was performed after every 1 h to observe the effect of temperature on the stability of enzyme. It was evident that this enzyme was stable up to 70°C. A very rapid denaturation of enzyme was observed at a temperature of 80°C.

Optimization of the medium components using RSM

The effect of medium components on SOD production was investigated. Table 1 shows independent variables that were investigated and their range and levels and Table 2 shows Central composite design and the levels of each variable and enzyme activity (U/mL) as response. The quadratic model expressed by equation given below represents SOD activity (Y) as a function of peptone (A), meat extract (B) NaCl (C) and K_2HPO_4 (D).

Y (Enzyme activity U/mL) = +473.95 + 9.80peptone + 9.05meat extract + 1.57NaCl + 2.55K_2HPO_4 + 1.41peptone meat extract + 7.51peptoneNaCl + 1.12peptone K_2HPO_4 + 2.76meat extract NaCl + 6.81meat extract K_2HPO_4+ 6.57NaCl K_2HPO_4 -7.67peptone^2

- 5.55meat $extract^2$ - $7.30NaCl^2$- 4.80 $K_2HPO_4^2$.

F test was used to check the statistical significance of the polynomial equation. Analysis of variance (ANOVA) for response surface quadratic model is shown in the Table 3. The Model F-value of 25.84 indicated the model was significant. Values of "Prob > F" less than 0.0500 indicated model terms were significant. In this case A, B, AC, BD, CD, A^2, B^2, C^2, D^2 were significant model terms. However C, D, AB, AD and BC had negative effects. The model was highly significant as the *p* value is less than 0.001 and the coefficient determination (R^2) was 0.9837, indicating that 98.37% of the variability in the response could be explained by the model and only less than 2% of the total variations were not explained by the model. The predicted determination coefficient value (predicted R^2 = 0.8387) was within reasonable agreement with the adjusted R^2 = 0.9456 which also indicated the significance of the model.

The maximum enzyme activity was obtained when 1% peptone, 1% meat extract, 0.5 % NaCl and 0.03% K_2HPO_4 were added to the production medium. 3D curves (Figure 2) showed the interaction between different factors. Significance of the interactions between the factors was determined by the shape of the curves. The spherical curves in Figure 2a–f indicated that the interactions between peptone, meat extract, NaCl and K_2HPO_4 were significant. Therefore, this model could be used for the prediction of SOD activity from *A. gonensis* KA 55.

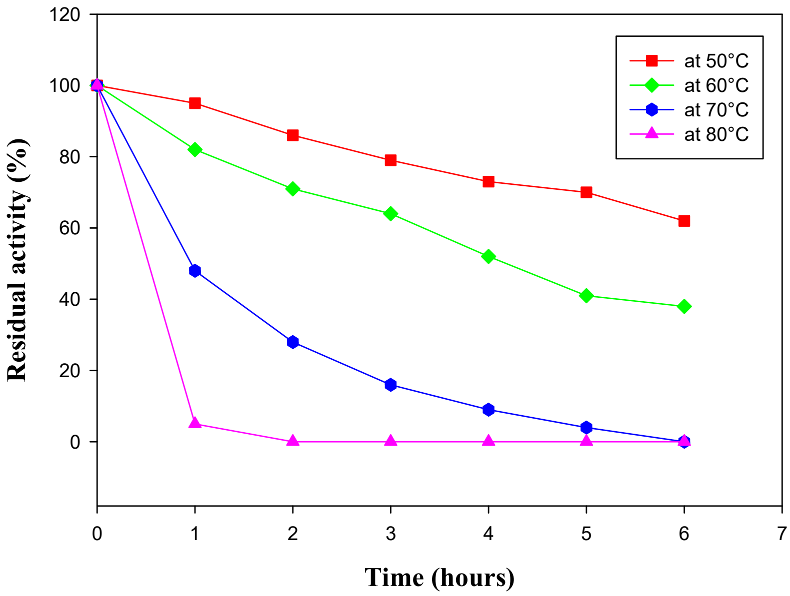


Figure 1: Stability of enzyme at different temperatures

Table 1: Independent variables and their range and levels

	Name	Units	Low	High	-alpha	+alpha
A	Peptone	Percent	0.8	1.2	0.6	1.4
B	Meat extract	Percent	0.8	1.2	0.6	1.4
C	NaCl	Percent	0.4	0.6	0.3	0.7
D	K_2HPO_4	Percent	0.025	0.035	0.02	0.04

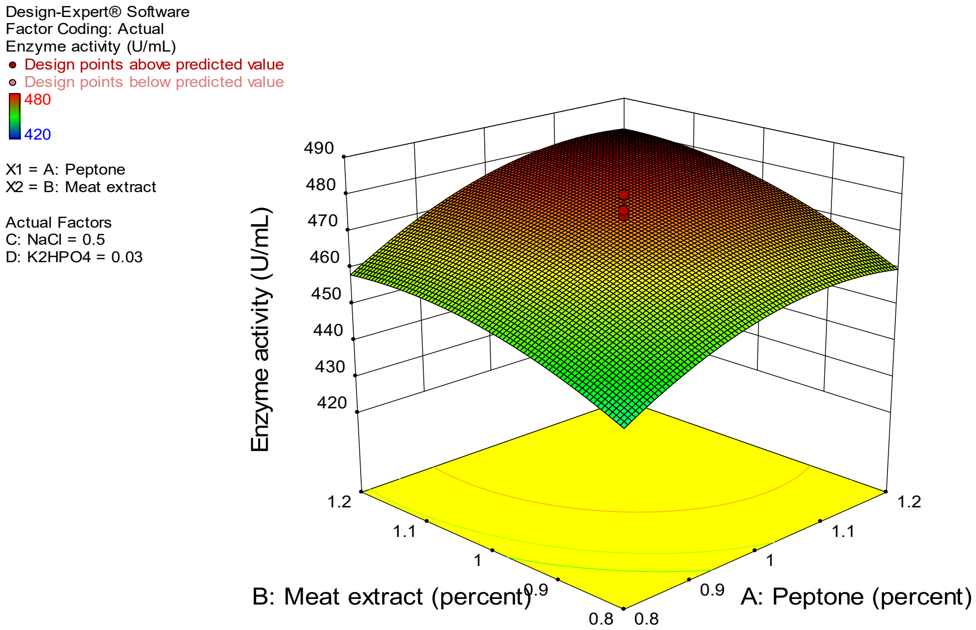
Table 2: Experimental design matrix with enzyme activity as a response

		Factor 1	Factor 2	Factor 3	Factor 4	Response 1	
Std	Run	A:Peptone percent	B:Meat extract percent	C:NaCl percent	D:K2HPO4 percent	Enzyme activity U/mL	
						Predicted value	Actual value
8	1	0.8	0.8	0.4	0.025	451.85	452.5
4	2	0.8	1.2	0.4	0.035	451.35	452.0
17	3	1	1	0.5	0.03	447.908	480.0
13	4	1	1	0.3	0.03	441.613	440.0
7	5	0.8	1.2	0.6	0.035	458.111	456.8
11	6	1	0.6	0.5	0.03	433.669	434.0
18	7	1	1	0.5	0.03	473.954	474.3
2	8	1.2	1.2	0.4	0.025	453.15	453.8
5	9	1.2	0.8	0.4	0.035	431.95	432.6
3	10	1.2	0.8	0.6	0.035	457.741	456.43
10	11	1.4	1	0.5	0.03	462.869	463.2
12	12	1	1.4	0.5	0.03	469.869	470.2
1	13	1.2	1.2	0.6	0.025	463.691	462.38
20	14	1	1	0.5	0.03	473.954	470.67
6	15	0.8	0.8	0.6	0.025	421.311	420.0
19	16	1	1	0.5	0.03	473.954	469.0
14	17	1	1	0.7	0.03	447.908	450.2
9	18	0.6	1	0.5	0.03	423.669	424.0
16	19	1	1	0.5	0.04	459.869	460.2
21	20	1	1	0.5	0.03	473.954	475.8
15	21	1	1	0.5	0.02	449.669	450.0

Table 3: ANOVA for Response Surface Quadratic model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	5580.41	14	398.60	25.84	0.0003	significant
A-Peptone	768.32	1	768.32	49.81	0.0004	
B-Meat extract	655.22	1	655.22	42.48	0.0006	
C-NaCl	39.41	1	39.41	2.55	0.1611	
D-K2HPO4	52.02	1	52.02	3.37	0.1159	
AB	7.91	1	7.91	0.51	0.5008	
AC	451.65	1	451.65	29.28	0.0016	
AD	5.01	1	5.01	0.32	0.5895	
BC	60.78	1	60.78	3.94	0.0944	
BD	185.57	1	185.57	12.03	0.0133	
CD	345.19	1	345.19	22.38	0.0032	
A^2	1476.94	1	1476.94	95.76	< 0.0001	
B^2	772.01	1	772.01	50.05	0.0004	
C^2	1336.07	1	1336.07	86.62	< 0.0001	
D^2	577.33	1	577.33	37.43	0.0009	
Residual	92.54	6	15.42			
Lack of Fit	17.14	2	8.57	0.45	0.6640	not significant
Pure Error	75.41	4	18.85			
Cor Total	5672.96	20				

(a)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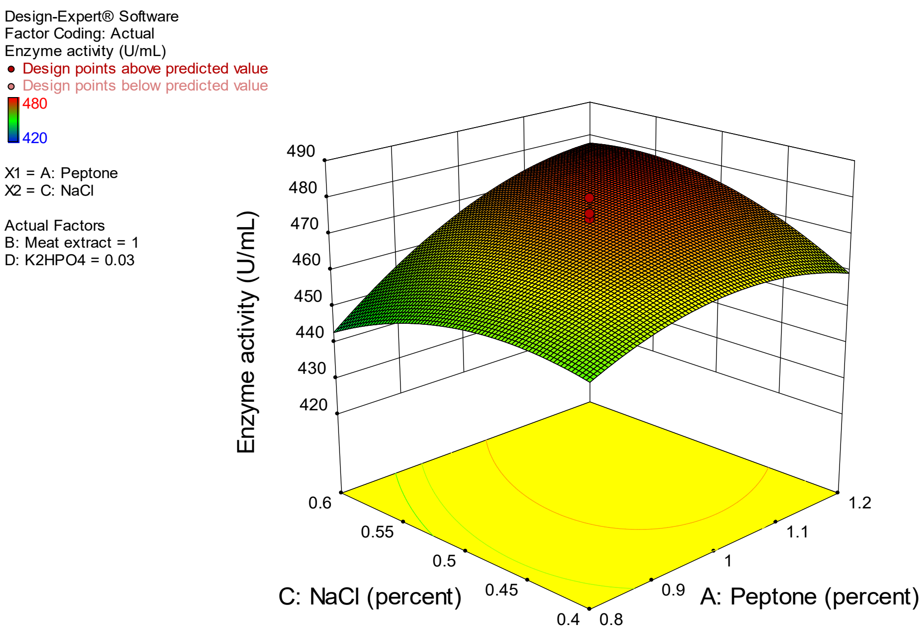
Factor Coding: Actual
Enzyme activity (U/mL)
Design points above predicted value
Design points below predicted value
480
420
X1 = A: Peptone
X2 = B: Meat extract
Actual Factors
C: NaCl = 0.5
D: K2HPO4 = 0.03
Enzyme activity (U/mL)
490
480
470
460
450
440
430
420
B: Meat extract (percent)
A: Peptone (percent)
1.2
1.1
1
0.9
0.8

(b)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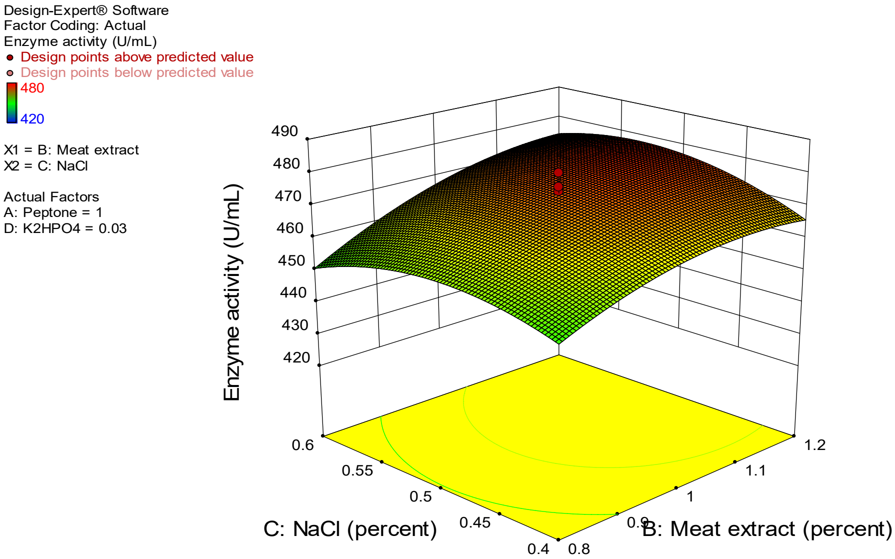
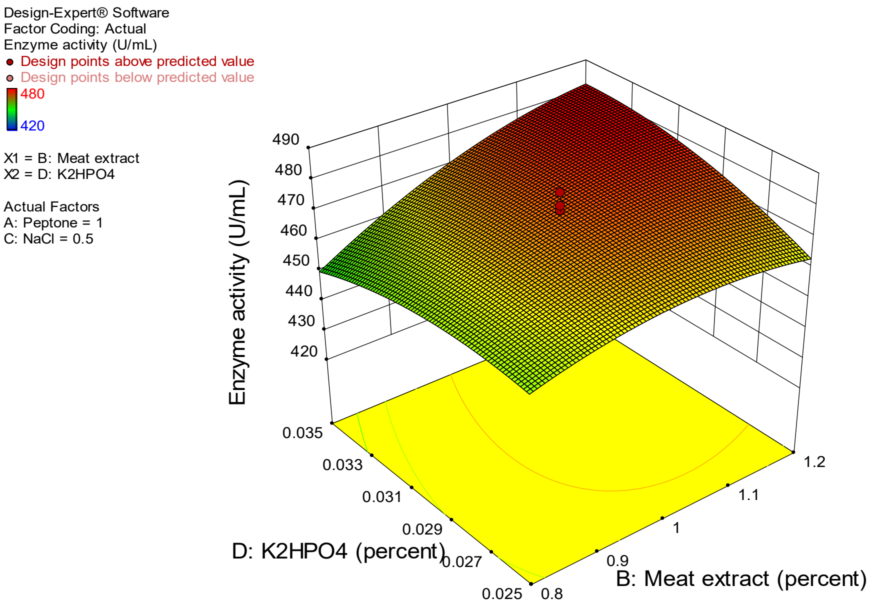
Enzyme activity (U/mL)
Design points above predicted value
Design points below predicted value
480
420
X1 = A: Peptone
X2 = C: NaCl
Actual Factors
B: Meat extract = 1
D: K2HPO4 = 0.03
Enzyme activity (U/mL)
490
480
470
460
450
440
430
420
C: NaCl (percent)
0.6
0.55
0.5
0.45
0.4
A: Peptone (percent)
1.2
1.1
1
0.9
0.8

(c)



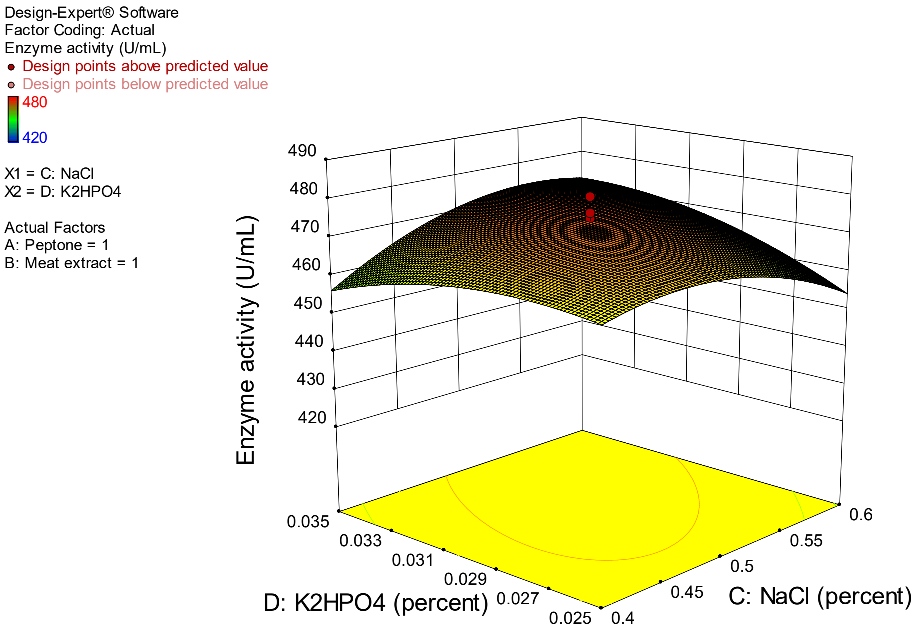
Design-Expert® Software
Factor Coding: Actual
Enzyme activity (U/mL)
Design points above predicted value
Design points below predicted value
480
420
X1 = B: Meat extract
X2 = C: NaCl
Actual Factors
A: Peptone = 1
D: K2HPO4 = 0.03
Enzyme activity (U/mL)
490
480
470
460
450
440
430
420
C: NaCl (percent)
0.6
0.55
0.5
0.45
0.4
B: Meat extract (percent)
0.8
0.9
1
1.1
1.2

(d)



Design-Expert® Software
Factor Coding: Actual
Enzyme activity (U/mL)
Design points above predicted value
Design points below predicted value
480
420
X1 = B: Meat extract
X2 = D: K2HPO4
Actual Factors
A: Peptone = 1
C: NaCl = 0.5
Enzyme activity (U/mL)
490
480
470
460
450
440
430
420
D: K2HPO4 (percent)
0.035
0.033
0.031
0.029
0.027
0.025
B: Meat extract (percent)
0.8
0.9
1
1.1
1.2

(e)



(f)

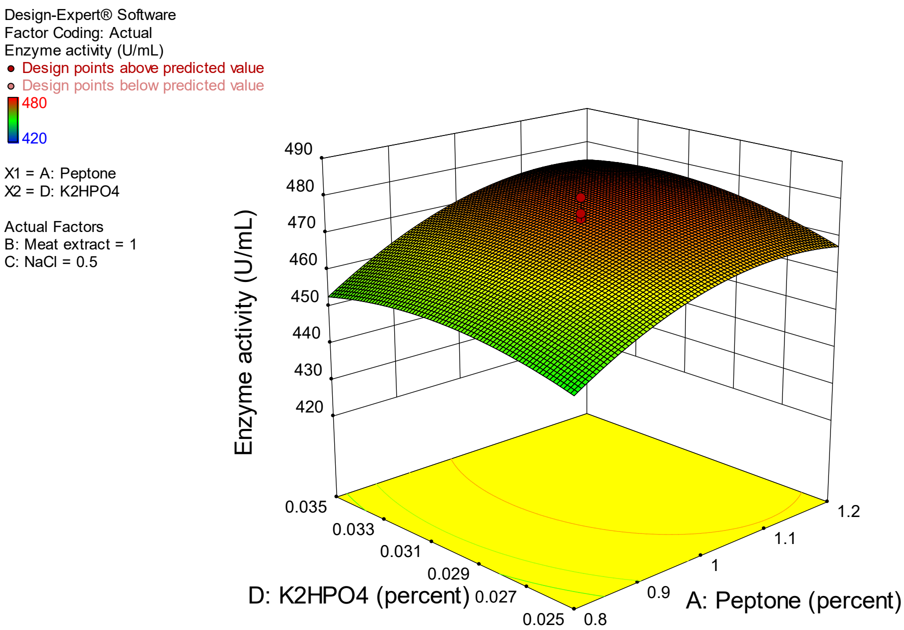


Figure 2: Three dimensional plots (3D) showing combined effect of peptone concentration, meat extract concentration, NaCl and K_2HPO_4 concentration on SOD production. (a) showing the effect of meat extract and peptone (b) NaCl and peptone (c) NaCl and meat extract (d) K_2HPO_4 and meat extract (e) K_2HPO_4 and NaCl (f) K_2HPO_4 and peptone on SOD production from *A. gonensis* KA 55.

Optimization of the reaction conditions

Based on a one variable at a time approach pH, temperature and cell volume were identified as significant factors that were enhancing the SOD activity. Therefore, a CCD was designed to investigate the optimum levels of these factors. Independent variables and their range and levels are shown in Table 4. Experimental design and enzyme activity as a response is shown in Table 5. In order to show the relationship between the enzyme activity and these factors a quadratic polynomial equation was devised.

Y (Enzyme activity U/mL) = +531.54 + 7.79 pH - 7.30temperature + 18.27cell volume -14.05 pH temperature -17.59 pH cell volume - 3.04 temperature cell volume -11.04 pH^2-13.22 $temperature^2$-27.02 cell $volume^2$.

Table 4: Independent variables and their range and levels

	Name	Units	Low	High	-alpha	+alpha
A	pH		8	10	7	11
B	Temperature	°C	30	50	20	60
C	Cell volume	µl	70	130	40	160

Table 5: Experimental design matrix with enzyme activity as a response

Std	Run	Factor 1	Factor 2	Factor 3	Response 1	
		A:pH	B:temperature degree celcius	C:cell volume microlitre	Enzyme activity U/mL	
					Predicted value	Actual value
6	1	10	30	130	513.119	510.6
18	2	9	40	100	531.54	534.0
19	3	9	40	100	531.54	528.6
11	4	9	20	100	493.26	498.4
14	5	9	40	160	459.986	457.4
3	6	8	50	70	446.339	451.8
13	7	9	40	40	386.896	386.6
8	8	10	50	130	464.324	469.0
12	9	9	60	100	464.046	456.0
5	10	8	30	130	504.619	504.56
17	11	9	40	100	531.54	525.68
9	12	7	40	100	471.811	468.5
4	13	10	50	70	469.059	472.0
7	14	8	50	130	512.044	518.0
15	15	9	40	100	531.54	530.4
20	16	9	40	100	531.54	538.2
2	17	10	30	70	505.674	502.6
16	18	9	40	100	531.54	529.48
10	19	11	40	100	502.971	503.4
1	20	8	30	70	426.974	425.0

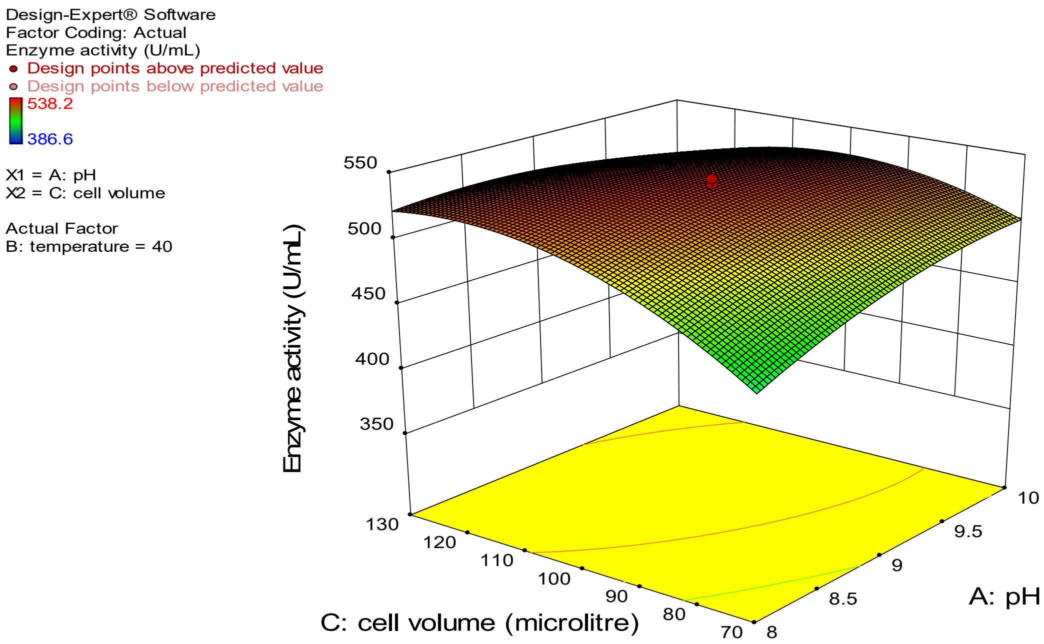
Table 6: ANOVA for Response Surface Quadratic model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	31536.46	9	3504.05	108.67	< 0.0001	significant
A-pH	970.95	1	970.95	30.11	0.0003	
B-temperature	852.06	1	852.06	26.42	0.0004	
C-cell volume	5342.15	1	5342.15	165.67	< 0.0001	
AB	1580.34	1	1580.34	49.01	< 0.0001	

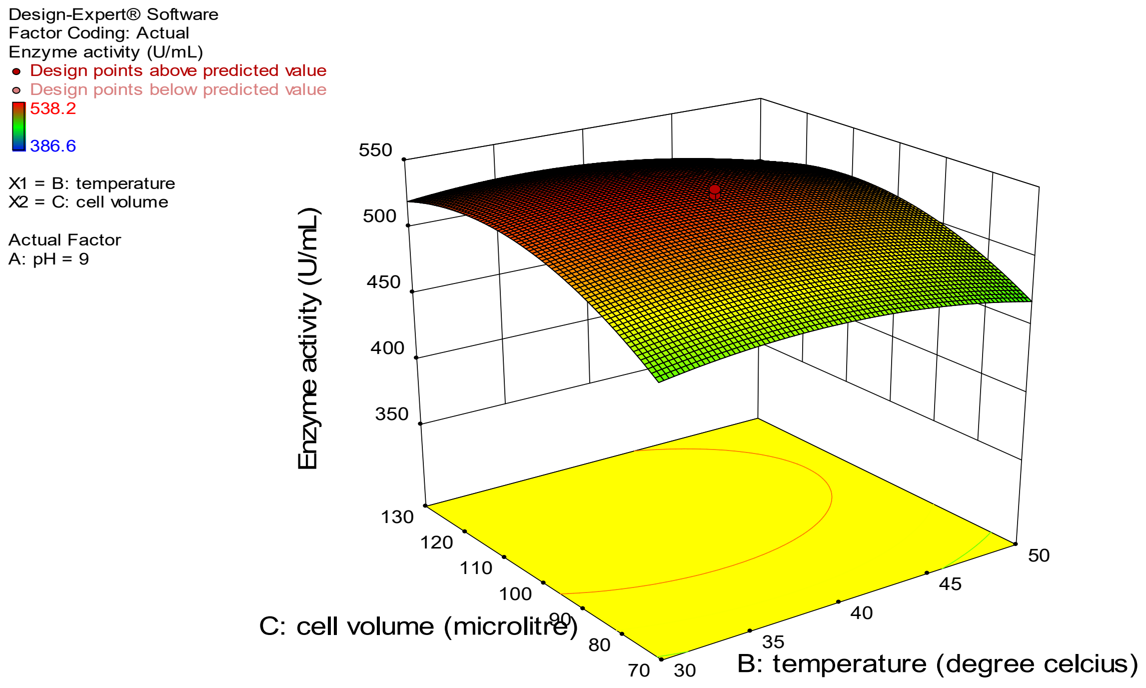
AC	2476.67	1	2476.67	76.81	< 0.0001	
BC	74.18	1	74.18	2.30	0.1603	
A^2	3062.94	1	3062.94	94.99	< 0.0001	
B^2	4397.35	1	4397.35	136.37	< 0.0001	
C^2	18362.79	1	18362.79	569.47	< 0.0001	
Residual	322.45	10	32.25			
Lack of Fit	224.90	5	44.98	2.31	0.1903	not significant
Pure Error	97.55	5	19.51			
Cor Total	31858.91	19				

The analysis of variance for the response surface quadratic model is shown in Table 6. The p value for the lack of fit was 0.1903 implied the adequacy of the model. The coefficient determination (R^2) was 0.9899, indicating that 98.99% of the variability in the response could be explained by the quadratic model and only less than 2% of the total variations were not explained by the model. The predicted determination coefficient value (predicted R^2 = 0.9370) was within reasonable agreement with the adjusted R^2 =0.9808 and it also indicated the significance of the model. The maximum enzyme activity of 531.54 U/mL was obtained at a pH of 9, reaction temperature of 40°C and a cell volume of 100 µl. The Model F-value of 108.67 implied the model was significant. Values of "Prob > F" less than 0.0500 indicated model terms were significant. In this case A, B, C, AB, AC, A^2, B^2, C^2 were significant model terms. 3D curves (Figure 3a-c) showed that interactions between pH of buffer, reaction temperature and cell volume were significant.

(a)



(b)



(c)

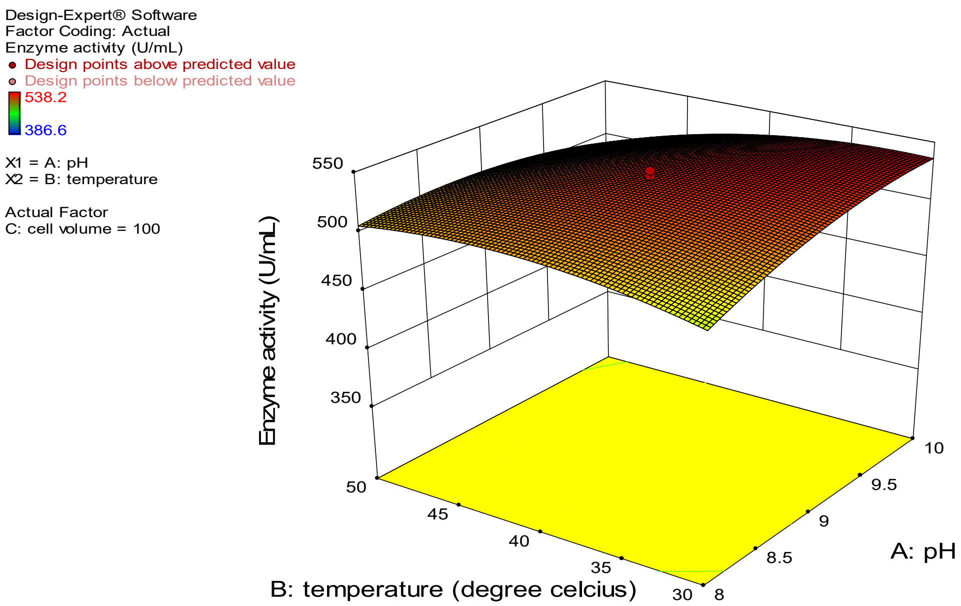


Figure: 3 Three dimensional plots (3D) showing combined effect of reaction temperature, buffer pH and cell volume on SOD production (a) showing the effect of cell volume and pH (b) cell volume and reaction temperature (c) reaction temperature and buffer pH

Experimental validation of the optimized factors

To verify the accuracy of the model an experiment was conducted with optimized factors predicted by RSM. The SOD activity was found to be 530.6 U/mL after optimizing all the factors which was very close to the 531.54 U/mL as predicted by the model. The congruity between the experimental and estimated responses verified the validity and accuracy of the model in terms of predicting the enzyme production by *A. gonensis* KA 55. A 3.98-fold increase in enzyme activity was observed after optimizing various parameters.

DISCUSSION

Thermophiles are group of microorganisms which are capable of growing at temperatures of 55°C or higher. They find wide applications in industrial area as well as in therapeutics due to their high specific activities and stability at high temperature. Previously many thermostable enzyme producing bacterial strains have been isolated from hot springs of Himachal Pradesh like lipase from *Bacillus licheniformis* [12], xylanse from *Bacillus aestuarii* [13] and cellulase from *Geobacillus thermodenitrificans* [14]. The SODs from thermophilic microorganisms are suitable models for the study of protein adaptation at high temperatures. SOD has been isolated from prokaryotic sources like *Thermothrix* sp. [15], *Deinococcus radiophilus* [16], *Bacillus* sp. MHS47 [17]. For industrial applications, it is important that enzyme should be thermostable, as high temperature causes the denaturation of the enzyme. Due to this various attempts are being made for the isolation of SODs from thermophiles and hyper thermophiles [18].

In the present study, *A. gonensis* KA 55 was isolated from hot water spring at Manikaran (Kullu) which was found to have good SOD activity. SOD was found to have optimum activity at a pH of 9 although appreciable activity was also observed at pH 10. Boyadzhieva et al. [19] and Seatovic et al. [15] have reported pH 8 and pH 7.8 as the optimum pH for SOD activity respectively. SOD from *A. gonensis* KA 55 was stable up to a temperature of 70°C however SOD from *Desulphovibrio gigas* was stable up to a temperature of 50°C [20].

There are a number of reports [21-22] on optimization of medium components and reaction conditions on various microbial enzymes using statistical approach. Thermophilic microbial sources of SOD enzyme have not been explored extensively therefore various growth and process parameters of *A. gonensis* KA 55 have been analyzed statistically to acquire a better perspective of this new source of SOD enzyme.

Maximum enzyme activity was observed in the medium containing peptone and meat extract as the nitrogen source. Earlier studies by Seatovic et al. [15] have also reported peptone and meat extract the optimum nitrogen source for SOD production from *Thermothrix* sp. NaCl (0.5%, w/v) and K_2HPO_4 (0.03, w/v) also played significant role for the production of SOD from *A. gonensis* KA 55. A 3.98-fold increase in SOD activity was observed after optimization through response surface methodology.

CONCLUSION

It can be concluded from the present study that *A. gonensis* KA 55 is a novel source of highly thermostable and alkali tolerant SOD. SOD production has been optimized to give higher yield. Mostly commercial SODs are obtained from animal tissues. The enzymes from microbial sources are more active and stable than the enzymes produced by plants and animals. Moreover it is easy to culture microorganisms in large quantities in short duration of time by fermentation due to their biochemical diversity and vulnerability to gene manipulation. Hence *A. gonensis* KA 55 is a promising candidate for industrial production of SOD enzyme.

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