https://doi.org/10.33472/AFJBS.2.2.2020.25-39 ISSN: 2663-2187

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Statistical augmentation of thermostable Superoxide dismutase (SOD) production from *Bacillus licheniformis* SPB-13 of Himalayan ranges

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Article Info

Volume 2, Issue 2, April 2020
Received : 20 September 2019
Accepted : 18 December 2019
Published : 06 April 2020
doi: 10.33472/AFJBS.2.2.2020.25-39

Abstract

Background: Aging is a natural phenomenon in all organisms. The pace of aging and related manifestations depends on balance of formation and dismutation of reactive oxygen species (ROS) in the cell. Superoxide dismutase (SOD; EC 1.15.1.1) is an antioxidative enzyme that prevents the aerobic organisms from oxidative damage caused by free radicals that are generated during oxygen metabolism. This enzyme system is the key biomolecule of the cells having antiaging properties and increases the life span of organisms. **Objective:** The aim of present research was to isolate thermostable SOD producing bacterial strain and to increase its activity by optimizing the production and assay parameters for thermophilic bacterium using response Surface Methodology (RSM) and its identification and phylogenetic analysis using molecular techniques. **Materials and Methods:** The water and soil samples were collected from thermal springs of different regions of Western Himalayas of India. Out of total 40 isolates, *Bacillus* sp. SPB-13 was screened for production of SOD on the basis of highest activity and identified using 16s rRNA gene sequencing. One Variable at a Time (OVAT) approach followed by central composite design was employed to analyze the effect of different media and reaction components, i.e., Glucose, casein, yeast extract, NaCl, buffer system, reaction temperature and enzyme concentration on SOD production. **Results:** One hyperproducer of SOD was isolated and found to be *Bacillus licheniformis* SPB-13 (NCBI Accn. No. KX185731). Enzyme activity was enhanced from 80 U/mL to 120 U/mL after production and reaction process optimization. A significant increase in SOD activity of 40 U/mL was obtained after classical and statistical optimization. SOD remained fully active at 70°C and after metallotyping, found to be a member of Fe/Mn SOD family. **Conclusion:** *Bacillus licheniformis* SPB-13 produces a thermostable SOD with very good antioxidant activity. The production and reaction parameters have been optimized completely using classical and analytical methods to cut the production cost and to increase the yield of SOD. The antiaging potential of this enzyme can be exploited in cosmetics and pharma industry for antioxidative formulations and topical ointments.

Keywords: *Antiaging potential, Bacillus licheniformis SPB-13, Free radicals, Response Surface Methodology (RSM), Superoxide dismutase (SOD).*

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1. Introduction

Aging is a genetically programmed process that affects each and every living entity. There are so many intrinsic and extrinsic factors that enhance the process of aging. Extrinsic factors are environmentally induced, but intrinsic factors include various substances and chemical species that lead to fast aging process. On the fore front of this list are Reactive Oxygen Species (ROS) or free radicals. These chemical species are generated during oxygen metabolism in aerobic or facultative aerobic organisms. Superoxide dismutase (SOD) is an enzyme that protects the organisms from the lethal effects of these ROS. Superoxide dismutases (SOD, EC 1.15.1.1) belong to a class of enzymes that catalyze the dismutation of superoxides into oxygen and hydrogen peroxide. These enzymes contain metal cofactors like Fe, Zn, Cu, Mn and Ni and on the basis of these cofactors, three major families of SOD, i.e., Cu/Zn, Fe/Mn and Ni SOD exist. Cu/Zn SOD is mostly found in all the eukaryotes as homodimeric form while Fe/Mn SOD and Ni SOD are present in most of the prokaryotes. In prokaryotes, Fe SOD is synthesized constitutively while Mn SOD is whereas manganese SOD is inducible and its concentration is linked to the metabolism of oxygen in the organisms (Hassan and Fridovich, 1977). Currently, the main focus of researchers worldwide is to establish the link between SOD expression and a variety of diseases like amyotropic lateral sclerosis, cancer and parkinson's disease (Al-Chalabi *et al.*, 2010; Conwit, 2006; and Deng *et al.*, 1993). Aging is directly related to SOD expression and functioning. So, SOD has wider applications in cosmetics and pharmaceutical industry and thermally stable SOD can prove a boon for these industries. Presently, commercial SOD is obtained from animals and plant sources and bacterial sources are very less explored for its large scale production. SOD interrupts the inflammatory cascades and limits the further disease progression (Riedl *et al.*, 2005). SODs are reported for the treatment of skin ulcer lesions, especially due to burn and wound (Niwa 1989). Researchers have reported that SOD ameliorates radiation induced side effects such as radiation-induced sclerosis (Menander-Huber *et al.*, 1978; and Emerit *et al.*, 1983). The topical application of SOD cream is effective for rapidly improving Progressive Systemic Sclerosis (PSS), herpes simplex and burns (Mizushima *et al.*,1991). To fill the lacunas of a thermostable SOD for commercial purpose, the current research work has been carried out. Different thermophilic bacterial strains possessing SOD activity, from different hot water springs of Himalayas have been isolated and screened for hyperproducer strain and their production and reaction conditions have been optimized using statistical tools for maximum enzyme production. One Variable at a Time (OVAT) approach was used and it was followed by Composite Central Design (CCD) for maximum enzyme production. Then 16s rRNA sequencing was employed to identify the strain and SOD metallotyping was also done to find out the type of SOD on the basis of metal cofactor present. By this piece of work, we have been able to find out a thermostable SOD from a thermophilic bacterium that can be used in antioxidative and antiaging industrial products involving elevated temperature dependent processing. The optimization studies can give a cutting edge to simulations of parameters for cosmetics products formulation at bulk scale.

2. Objective

The aging process and free radicals damage to the cells of skin and other organs can be reversed and controlled using SOD application both as topical and oral administration. The need of the cosmetics and pharmaceutical industry is to have a thermostable SOD which can retain the antioxidative potential at high temperature during industrial processes. The western Himalayan region was explored for novel microbial sources of SOD as there are no published reports of SOD from thermal springs from western Himalayan ranges and no study has been carried out for its production process optimization and typing.

3. Materials and methods

3.1. Chemicals

Unless stated other way round, inorganic salts and media components were purchased from HiMedia (Mumbai, India) and they all were of analytical grade. Nitroblue tetrazolium (NBT) and Phenazine methosulphate (PMS) were obtained from Sigma Aldrich (USA) and NADH was purchased from Merck (Germany).

3.2. Sample collection

Soil and water samples for the isolation of SOD producing microorganisms were collected from different hot water springs of Kullu and Mandi districts of Himachal Pradesh, India. The collected samples were brought to the laboratory and stored at 4 ^{o}C till further studies.

3.3. Isolation of SOD producing microorganisms

One ml of water sample or 1 gram of soil sample was added to Erlenmeyer flask (250 ml) containing 50 ml sterile medium containing (%, w/v) (peptone 0.5, yeast extract 0.5, glucose 1.0, agar 2.0,

K_2HPO_4 0.5, NaCl 0.1, $MgSO_4$ .001, $FeSO_4$ .001 and incubated at 55 °C for 24 h under shaking conditions for enrichment.

3.4. Screening of hyperproducers microorganisms for SOD

One ml of enriched sample was serially diluted and 100 μl of diluted samples were spread over the medium containing (%, w/v) (peptone 0.5, yeast extract 0.5, glucose 1.0, agar 2.0, K_2HPO_4 0.5, NaCl 0.1, $MgSO_4$ 0.001 and $FeSO_4$ 0.001; pH 7.5) and incubated at 55 °C for 24 h. Colonies were picked up depending upon different shape, size and color, and pure cultures were established by repeatedly streaking single colonies on the medium described above. A total of 40 isolates showed SOD activity, out of which 13 isolates showed good SOD activity. Out of these 13 isolates, one hyperproducer strain of SOD was selected due to its ability to produce increased intracellular SOD in the liquid medium in comparison to the rest of the strains isolated.

3.5. Enzyme production

The medium containing peptone 5.0 g/l, yeast extract 5.0 g/l, D-glucose 10 g/l, K_2HPO_4 5.0 g/l, NaCl 1.0 g/l, $MgSO_4$ 10.0 mg/l and $FeSO_4$ 10 mg/l was used for growing the isolated strains at pH 7.5. The seed culture was prepared by growing the bacterial strains in 50 ml of seed medium at 55 °C in an incubator with continuous agitation (150 rpm). After 24 h 8% (v/v) inoculum from the seed culture was inoculated into 50 ml of production medium and incubated at 55 °C for 24 h in an incubator shaker (150 rpm). The culture contents were centrifuged at 10,000 g for 10 min, at 0-4 °C and pellet thus collected was further assayed for SOD activity.

3.6. Superoxide dismutase assay

SOD activity was assayed using the modified spectrophotometric assay (Kakkar *et al*., 1984). To 1.2 ml of Sodium pyrophosphate buffer, 0.1 ml of Phenazine methosulphate (PMS) and 0.3 ml of Nitroblue Tetrazolium (NBT) were added. 0.2 ml of enzyme preparation and 1.0 ml of distilled water was added to reaction volume. The reaction was initiated by addition of 0.2 ml of NADH. Total volume of reaction mixture was made to 3.0 ml and was incubated for a time of 90 sec at 30 °C. Reaction was stopped by addition of 1.0 ml of concentrated glacial acetic acid. Then reaction mixture was shaken with 4.0 ml of butanol and allowed to stand for 10 min. Then mixture was centrifuged at 5,000 g for 5 min. Intensity of chromogen in butanol layer was taken and measured at 560 nm. The enzyme activity was expressed in terms of international units. One unit (U) of enzyme activity is defined as the amount of enzyme required to inhibit the optical density at 560 nm of chromogen production under assay conditions and expressed as U/mL for crude and U/mg for purified enzyme.

3.7. Identification of the isolate

Primarily, the morphological study was carried out using Gram's staining. Then we moved towards biochemical characterization of the isolate. Finally 16s rRNA gene sequencing was also performed in which genomic DNA of the strain was isolated and its 16s rRNA encoding gene was amplified and its sequencing was done using Sanger dideoxy method (Sanger *et al*., 1977). The sequence obtained thus was subjected to multiple sequence alignment using BLAST.

3.8. Optimization of culture conditions for SOD production

The production of SOD enzyme was investigated with One Factor-At-a-Time (OFAT) method of designing experiments to observe the possible optimum levels of the conditions by changing one value alone. 10 different growth media were taken for growth of isolate at 55 °C with 150 rpm for 24 h with pH 7.5. The medium with maximum SOD production was considered for individual components optimization like carbon source, nitrogen source, growth supplements and their concentration, pH, incubation temperature, NaCl concentration and inoculums size. Growth and enzyme production profiling was also done. The results thus obtained were used in statistical combination designing for SOD activity enhancement.

3.9. Variables interaction analysis for production factors

Central composite design (CCD) component of Response Surface Methodology (RSM) statistical tool (Stat-Ease, Inc Design Expert software, version, 10.0, Minneapolis, USA) was used to study the effect of different variables interaction on growth influenced SOD activity. Four crucial production parameters were chosen and subjected to CCD. For these independent factors, i.e., glucose (A, %), casein (B, %), yeast extract (C, %) and NaCl (D, %), each at five levels, five central, eight axial and eight factorial points were found in CCD. The experimental planning consisted of 21 runs. The correlation between the four parameters and the response (SOD activity) was described by the following quadratic polynomial equation:

$$Y = \beta_0 + \Sigma\, \beta_i X_i + \Sigma\, \beta_{ii} X_{i2} + \Sigma\, \beta_{ij} X_i X_j$$

Here, Y is the predicted response [SOD activity (U/mL)], β_0 is the constant term, β_i the linear coefficients, β_{ii} the squared coefficients, and β_{ij} the interaction coefficients. A triple experimentation method was followed and ANOVA was used for analysis of variance. Standard deviation was also mentioned and only significant values were taken into consideration.

3.10. Assay parameters optimization for maximum SOD activity

Different reaction conditions such as buffer, pH, reaction time, reaction temperature, enzyme concentration, metal ions, etc. have crucial effect on enzyme activity. To get maximum enzyme activity, a range of the each parameter was investigated in different level by changing the value alone and optimized value was used in next experiment (OVAT). The thermostability of SOD was also analyzed at different temperatures. The findings were exposed to CCD system of RSM statistical tool.

3.11. Variable interaction analysis for reaction parameters

The SOD assay works on free radicals generation and their subsequent dismutation by enzyme thereby leading to prevention of reduction of NBT dye. Different factors affect the antioxidative activity of SOD in assay, so after each factor optimization using OVAT method, interactive effect of reaction variables was also studied for maximum SOD activity. A total of four significant factors, i.e., Tris- HCl concentration (A, mM), buffer pH (B), reaction temperature (C, °C) and enzyme concentration (μl) were taken for interaction analysis using CCD at five levels with five central, eight factorial and eight axial. 21 runs were made out of total combinations. ANOVA was used for variance analysis.

3.12. Validation of the model

The model generated through CCD of RSM was validated for actual versus predicted responses. The results thus obtained were compared with predicted responses for optimization.

3.13. SOD typing

The cells containing SOD were incubated with various types of chemical inhibitors. Three SOD inhibitors, i.e., H_2O_2, NaN3 and EDTA at a concentration of 10 mM, were used to treat the cells of *Bacillus licheniformis* SPB-13 containing SOD for 90 min. The SOD activity of treated cells was assayed to analyze the type of SOD.

4. Results

4.1. Isolation and screening of superoxide dismutase producing bacteria

A total of 40 isolates were obtained from samples of hot water springs of Himalayan region. Out of these, 13 isolates showed reasonable SOD activity. Isolate number 13 exhibited maximum intracellular SOD activity (53.4% inhibition/80.10 U/ml). This isolate was finally selected and named as SPB-13. Firstly, growth associated SOD production profiling of this isolate was done for 96 h. The best growth was recorded at 84-88 h whereas maximum enzyme activity was recorded after 24 h of incubation at 55 °C (101.91 U/mL). The growth seized after four days (Figure 1).

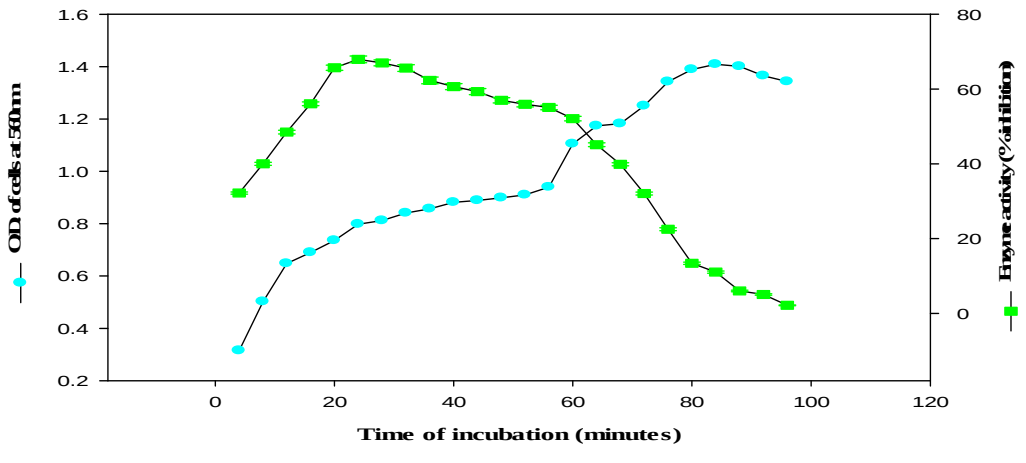

Figure 1: Growth profile of *Bacillus licheniformis* SPB-13 showing incubation time (min), optical density of cells at 560 nm and enzyme activity (% inhibition of NBT reduction)

4.2. Identification of the isolate

Identification of isolate was started with Gram's staining and it was found to be Gram's positive rods. The colonies were light red in color with rough margins. Biochemical characterization was also done using HIMEDIA kit having 12 tests that showed its relatedness to genus Bacillus.

Then the 16s rRNA gene sequence obtained after sequencing was aligned with available data using BLAST and it showed maximum relatedness (99%) with *Bacillus licheniformis* strain DGB and few non-identities in the gene sequence showed the uniqueness of this strain. The strain was named as *Bacillus licheniformis* SPB-13 and its sequence was submitted in NCBI and assigned with accession number KX185731. The phylogenetic tree of strain was also constructed using MEGA-6 tool (Figure 2).

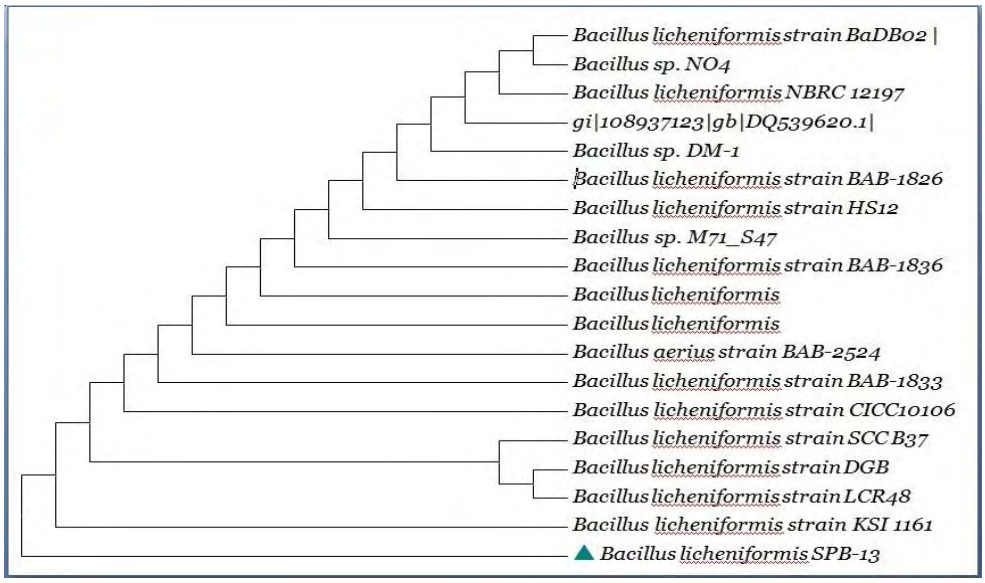

Figure 2: Phylogenetic tree of *Bacillus licheniformis* SPB-13

4.3. Optimization of culture conditions for SOD production

Growth induced SOD production was optimized by taking different parameters into consideration. Out of 10 different growth media used, SPM-1 medium (Glucose-10.0; Yeast extract- 5.0; Peptone-5.0; $MgSO_4$-0.001; NaCl-1.0; K_2HPO_4- 5.0; $FeSO_4$-0.001) [4] proved to be best for SOD production. Glucose at a concentration of 60 mM along with casein as nitrogen source at a concentration of 1.5% showed maximum enzyme production. The optimum pH of growth medium was 7.5 at incubation temperature of 55 °C for SOD production. Yeast extract along with NaCl at concentrations of 0.5% and 0.15% respectively induced maximum SOD production by *Bacillus licheniformis* SPB-13. A 5 mL of inoculums size was optimum for maximum enzyme production. At this stage, enzyme activity of 101 U/ml was obtained.

4.4. Variables interaction analysis for production factors

After optimization of production conditions for *Bacillus licheniformis* SPB-13 using OVAT approach, CCD analysis was done for four important production variables, i.e., glucose (A, %), casein (B, %), yeast extract (C, %) and NaCl (D, %). After the run, five central, eight axial and eight factorial points were found in CCD (Figure 3 a-f). The research design and predictive responses are mentioned in Table 1. After the application of various regression analysis on the experimental data, a quadratic polynomial equation was constructed to elaborate the correlation between enzyme activity and the four significant factors as follows:

$$R = +101.00 - 0.25 * A + 2.75 * B - 2.81 * C + 5.50 * D + 1.13 * AB + 1.63 * AC + 1.87 * AD + 2.13 * BC - 1.13 * BD + 2.12 * CD - 5.58 * A^2 - 7.08 * B^2 - 3.20 * C^2 - 3.20 * D^2$$

Here, R is response (SOD activity), A, B, C and D were coded readings for glucose, casein, yeast extract and NaCl respectively. After RSM application, SOD activity increased from 101 U/mL to 105 U/mL. The variance analysis for the given response surface quadratic model is shown in Table 2. The value of $p < 0.0021$ indicated that the linear, interactive and squared factors had a significant impact on SOD activity. The p-value for the lack of fit was 0.5805 that indicated that model properly fits into the data.

4.5. Assay parameters optimization for maximum SOD activity

The components of assay mixture exhibit significant effect on the activity of enzyme system. So, various parameters of reaction were optimized on OVAT basis. Tris-HCl buffer at a concentration of 60 mM at pH 8.0

Table 1: Central composite design (CCD) with predicted and actual responses

Std	Sr. No.	Glucose (mM)	Casein (%)	Yeast Extract (%)	NaCl (%)	Predicted response(U/mL)	Actual response (U/mL)
20	1	60	1.5	1.5	0.15	102	102.06
3	2	80	1	2	0.18	85	85.28
11	3	60	0.5	1.5	0.15	68	67.50
16	4	60	1.5	1.5	0.21	76.65	76.19
19	5	60	1.5	1.5	0.15	104.68	102.5
12	6	60	2.5	1.5	0.15	85	84.54
8	7	40	1	1	0.12	78.5	79.1
6	8	40	1	2	0.12	67	67.2
7	9	40	2	2	0.18	74.68	74.96
2	10	80	2	1	0.12	79	79.2
18	11	60	1.5	1.5	0.15	102.87	102.05
4	12	40	2	1	0.18	85	84.67
5	13	80	1	1	0.18	76.9	77.5
17	14	60	1.5	1.5	0.15	100.78	102.06
10	15	100	1.5	1.5	0.15	84.68	84.22
9	16	20	1.5	1.5	0.15	80	79.54
14	17	60	1.5	2.5	0.15	82.67	82.55
1	18	80	2	2	0.12	80.96	81.24
13	19	60	1.5	0.5	0.15	89.9	89.10
15	20	60	1.5	1.5	0.09	67.43	66.97
21	21	60	1.5	1.5	0.15	100	102.06

Table 2: Analysis of variance of response surface methodology (RSM) for production factors

Source	Sum of Squares	df	Mean Square	*F*-Value	*p*-Value	Prob. > *F*
Model	2756.69	14	196.91	68.76	< 0.0001	Significant
A-Glucose	10.95	1	10.95	3.82	0.0983	
B-Casein	144.50	1	144.50	50.46	0.0004	
C-Yeast extract	42.97	1	42.97	15.00	0.0082	
D-NaCl	42.50	1	42.50	14.84	0.0084	
AB	0.34	1	0.34	0.12	0.7435	

Table 2 (Cont.)

Source	Sum of Squares	df	Mean Square	*F*-Value	*p*-Value	Prob. > *F*
AC	127.04	1	127.04	44.36	0.0006	
AD	29.59	1	29.59	10.33	0.0183	
BC	3.08	1	3.08	1.07	0.3400	
BD	3.35	1	3.35	1.17	0.3211	
CD	6.70	1	6.70	2.34	0.1771	
A^2	638.90	1	638.90	223.09	< 0.0001	
B^2	1062.16	s1	1062.16	370.89	< 0.0001	
C^2	413.53	1	413.53	144.40	< 0.0001	
D^2	1457.46	1	1457.46	508.92	< 0.0001	
Residual	17.18	6	2.86			
Lack of Fit	3.78	2	1.89	0.56	0.6087	Non significant
Pure Error	13.41	4	3.35			
Cor Total	**2773.88**					

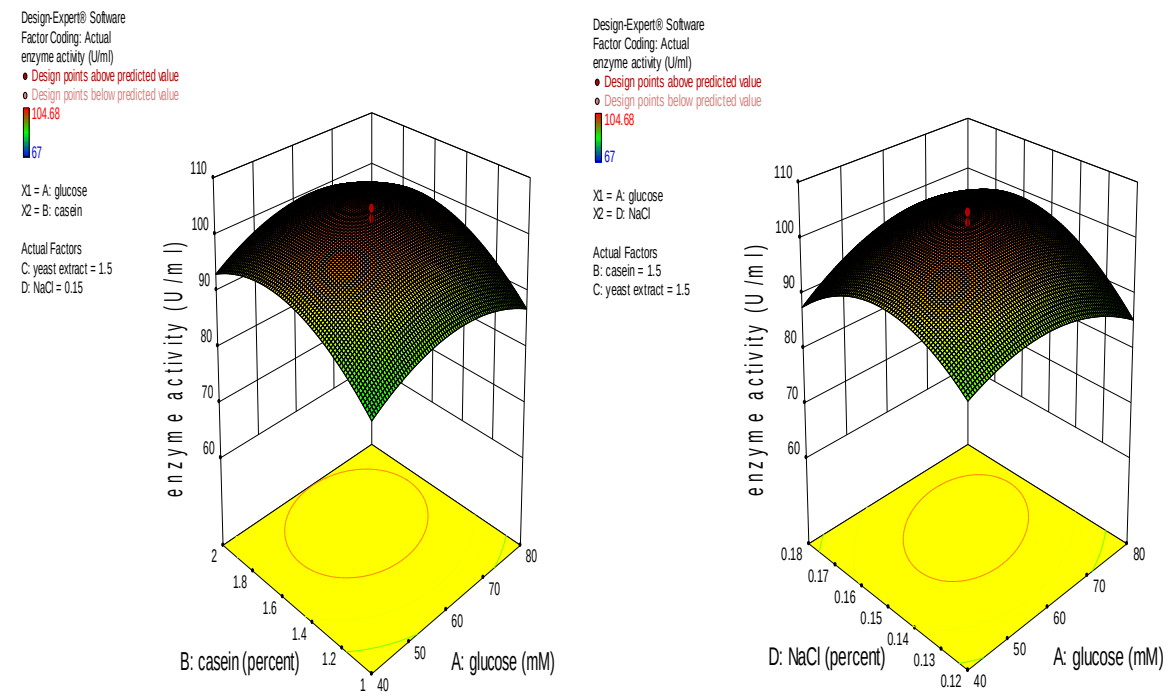

Figure 3: 3D response surface plots elucidating the effect of interactions of (a) **NaCl and Yeast extract** (b) **NaCl and Glucose** (c) **Casein and Glucose** (d) **Yeast extract and Glucose** (e) **NaCl & casein and** (f) **Yeast extract and Casein on superoxide dismutase production from *B. licheniformis* SPB-13**

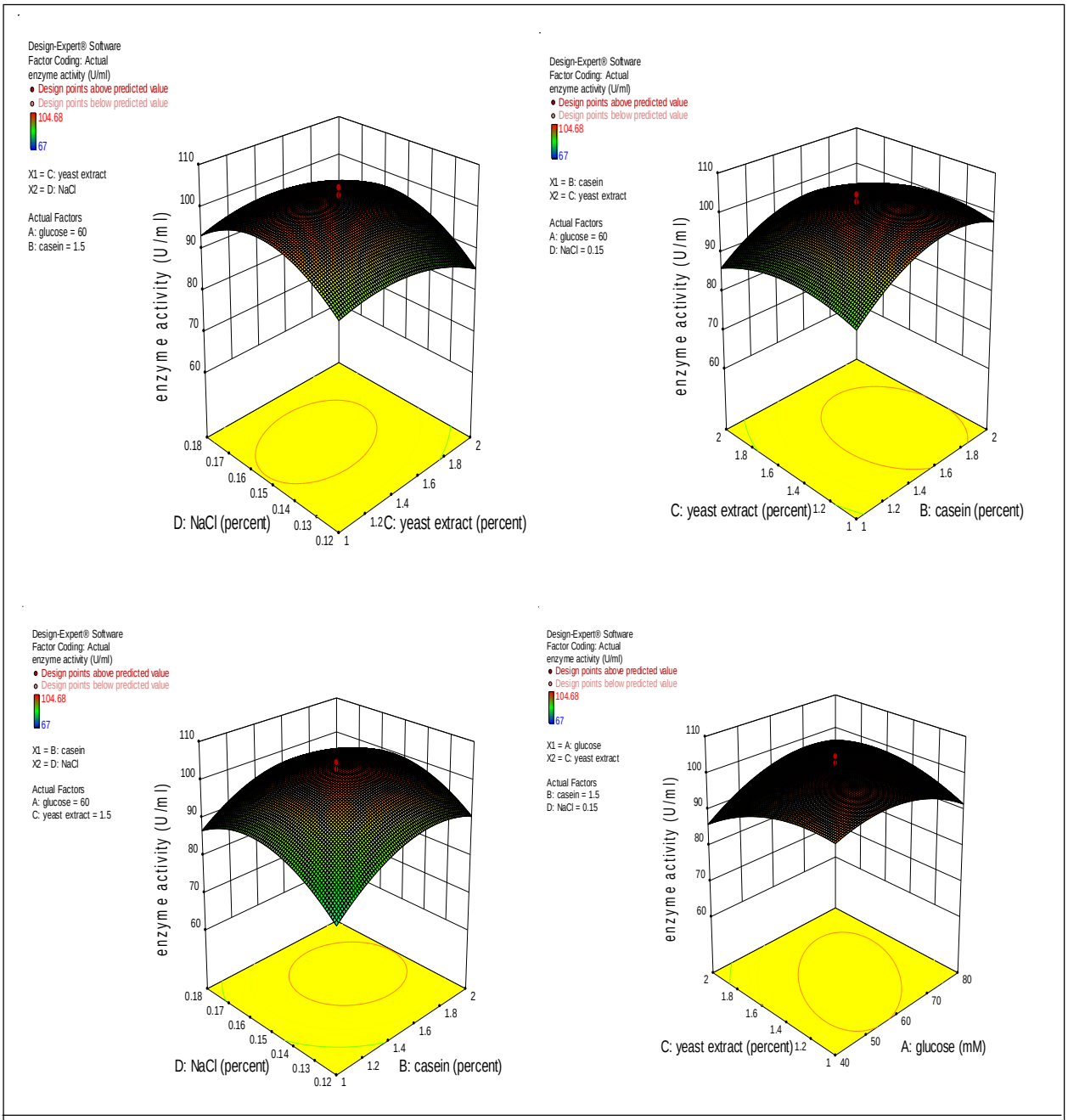

Figure 3 (Cont.)

proved to be best buffer system for SOD activity of *Bacillus licheniformis* SPB-13. SOD has very high turnover number (k_{cat}), so incubation time of reaction mixture was optimized and found to be 2 min at 40 °C and the enzyme remained stable till 60 °C (Figure 4). Enzyme concentration of 200 μL along with $MnCl_2.2H_2O$ and $FeCl_3$ (1 mM) as metal ions gave maximum enzyme activity in reaction mixture. For thermostability profiling, enzyme was preincubated at 40 °C, 50 °C, 60 °C, 70 °C and 80 °C for varying duration and then assay was performed. Enzyme remained fully active till 70 °C for 30 min (Figure 5).

4.6. Variable interaction analysis for reaction parameters

For reaction variables, OVAT approach was used followed by statistical tool application for analyzing variables interaction for maximum SOD activity in assay system. Four significant factors, i.e., buffer molarity (A, mM), buffer pH (B), reaction temperature (C, °C) and enzyme concentration (D, μL) were chosen for CCD analysis. After the run, five central, eight axial and eight factorial points were found in CCD (Figure 6 a-f). The research design and predictive responses are mentioned in Table 3. After the application of various regression analysis on the experimental data, a quadratic polynomial equation was constructed to elaborate the correlation between enzyme activity and the four significant factors as follows:

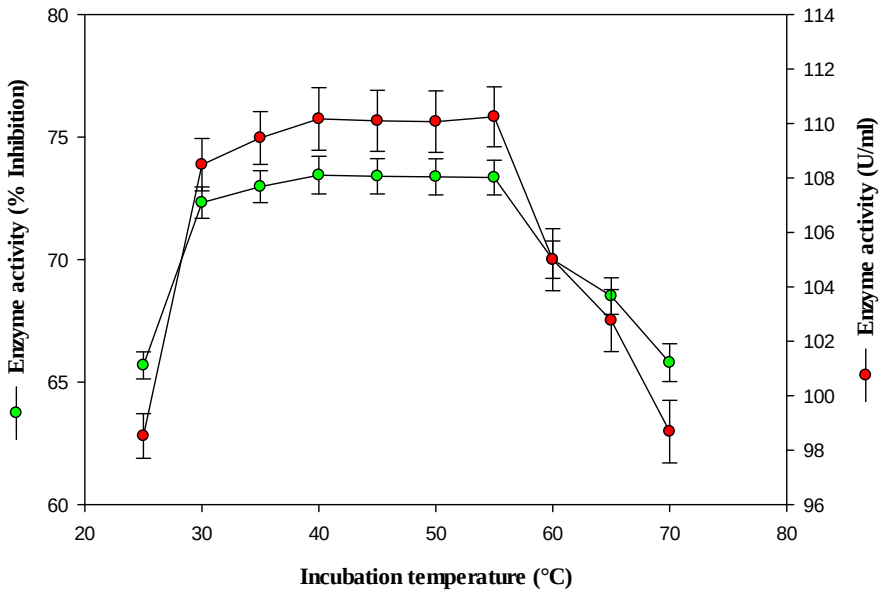

Figure 4: Effect of reaction temperature of SOD activity

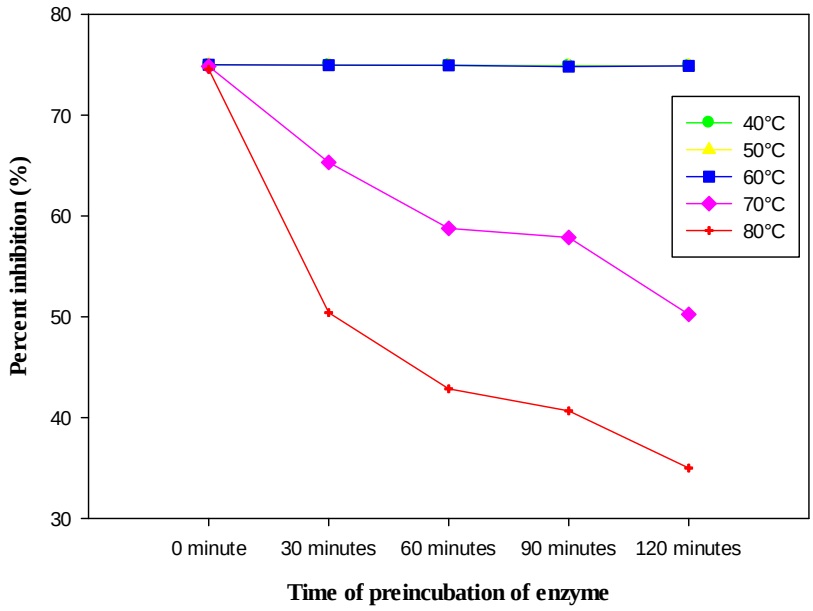

Figure 5: Thermostability profile of SOD from *Bacillus licheniformis* SPB-13

$R = +114.80 - 0.75 * A + 1.87 * B + 0.69 * C + 2.50 * D + 2.58 * AB - 1.60 * AC + 0.59 * AD + 2.72 * BC + 1.44 * BD + 4.37 * CD - 7.52 * A^2 - 7.71 * B^2 - 5.26 * C^2 - 3.64 * D^2$.

where, R is response (SOD activity, U/mL), A, B, C and D were coded readings for buffer molarity, buffer pH, reaction temperature and enzyme concentration respectively. After RSM application, SOD activity increased from 112 U/mL to 120 U/mL. The variance analysis for the given response surface quadratic model is shown in Table 4. The value of $p < 0.0006$ indicated that the linear, interactive and squared factors had a significant impact on SOD activity. The p-value for the lack of fit was 0.9695 that indicated that model has been properly adjusted into the data.

4.7. Validation of the model

The validation of response surface models of production and reaction parameters was done for predicted and actual responses. The results were compared with predicted responses and demonstrated. (Figures 7a and b).

Table 3: Central composite design (CCD) with predicted and actual responses

Std	Sr. No.	Tris-HCl concentration (mM)	Buffer pH	Reaction temperature (^{O}C)	Enzyme concentration (%)	Predicted response (U/mL)	Actual response (U/mL)
8	1	40	7.5	30	150	96.77	96.46
16	2	60	8	40	300	105.0	105.2
15	3	60	8	40	100	95.0	95.44
17	4	60	8	40	200	112.55	114.7
10	5	100	8	40	200	83.0	83.23
11	6	60	7	40	200	80.0	80.22
21	7	60	8	40	200	116.56	114.7
18	8	60	8	40	200	114.87	114.8
14	9	60	8	60	200	95.1	95.1
5	10	80	7.5	30	250	86.68	86.37
1	11	80	8.5	50	150	87.43	87.28
7	12	40	8.5	50	250	103.6	103.4
12	13	60	9	40	200	87.5	87.7
9	14	20	8	40	200	86.0	86.38
20	15	60	8	40	200	120.0	114.79
13	16	60	8	20	200	92.0	92.38
3	17	80	7.5	50	250	88.0	87.85
6	18	40	7.5	50	150	87.0	86.85
4	19	40	8.5	30	250	85.0	84.69
19	20	60	8	40	200	110.0	114.8
2	21	80	8.5	30	150	92.7	92.3

Table 4: Analysis of variance of response surface methodology (RSM) for production factors

Source	Sum of Squares	df	Mean Square	*F*-Value	*p*-Value	Prob. > *F*
Model	2953.49	14	210.96	21.40	0.0006	significant
A-Buffer molarity	4.50	1	4.50	0.46	0.5245	
B-Buffer pH	28.13	1	28.13	2.85	0.1422	
C-Reaction temperature	7.67	1	7.67	0.78	0.4116	
D-Enzyme concentration	50.00	1	50.00	5.07	0.0653	
AB	26.57	1	26.57	2.70	0.1517	

Table 4 (Cont.)

Source	Sum of Squares	df	Mean Square	*F*-Value	*p*-Value	Prob. > *F*
AC	20.42	1	20.42	2.07	0.2002	
AD	1.39	1	1.39	0.14	0.7200	
BC	59.30	1	59.30	6.01	0.0496	
BD	8.35	1	8.35	0.85	0.3929	
CD	152.78	1	152.78	15.50	0.0077	
A^2	1418.57	1	1418.57	143.88	< 0.0001	
B^2	1490.21	1	1490.21	151.15	< 0.0001	
C^2	693.23	1	693.23	70.31	0.0002	
D^2	333.10	1	333.10	33.79	0.0011	
Residual	59.15	6	9.86			
Lack of Fit	0.91	2	0.45	0.031	0.9695	Not significant
Pure Error	58.24	4	14.56			
Cor Total	3012.65	20				

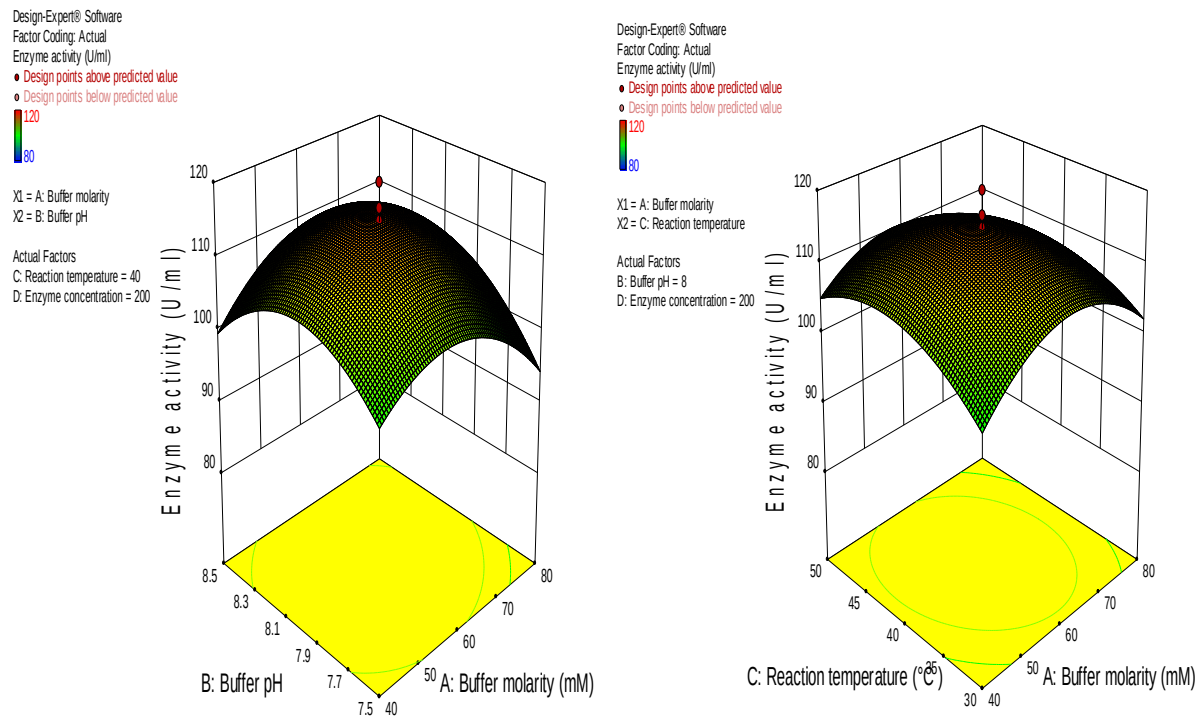

Figure 6: 3D response surface plots elucidating the effect of interactions of (a) **buffer pH and buffer molarity** (b) **reaction temperature and buffer molarity** (c) **enzyme conc. and buffer molarity** (d) **reaction temperature and buffer pH** (e) **enzyme conc. and buffer pH and** (f) **enzyme conc. and reaction temperature on superoxide dismutase activity of *B. licheniformis* SPB-13**

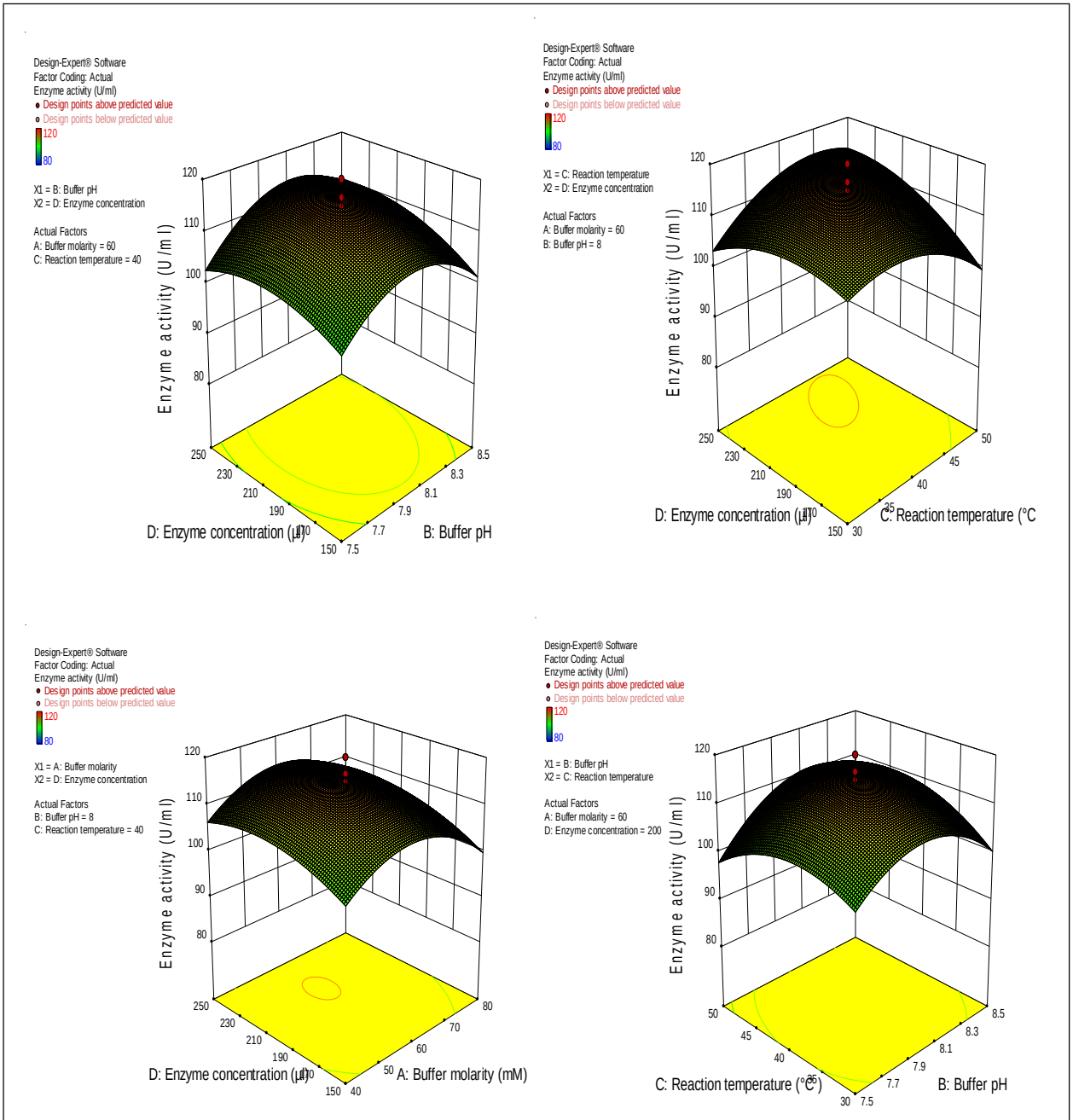

Figure 6 (Cont.)

4.8. Inhibition assay to study the type of SOD

The cells containing SOD were incubated with various types of inhibitors. After the SOD was incubated for 90 min in the presence of 10 mM H_2O_2, 80% of its activity was inhibited. On comparison, when the SOD was incubated with NaN3 at the same concentration, only 14% of the SOD activity was inhibited. EDTA as a metal sequestering agent did not inhibit the SOD activity at the concentration of 10 mM. This showed that this SOD belongs to Fe/Mn family of SOD (Figure 8).

5. Discussion

SOD enzyme is major antioxidant system present in all the organisms. To the best of our knowledge, SOD has been isolated and purified mostly from eukaryotic sources mainly from plants and animals (Guo *et al.*, 2008; Hadji *et al.*, 2007; Kumar *et al.*, 2012; Shrestha *et al.*, 2016; and Wang *et al.*, 2012). Attempts have been made for

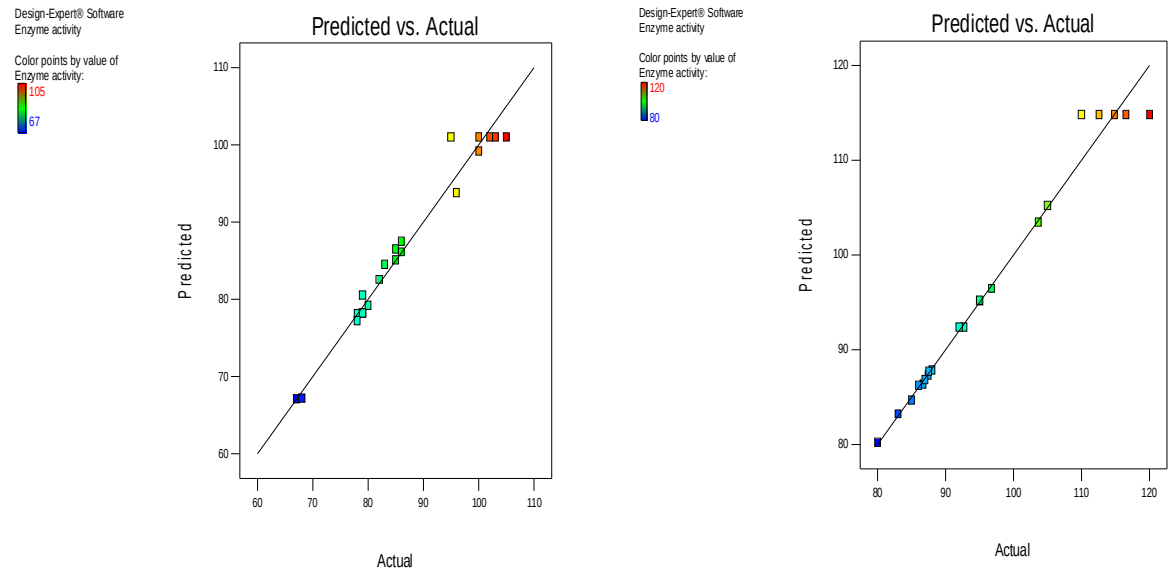

Figure 7: Parity plots showing distribution of predicted and actual values for (a.) production parameters and (b.) reaction parameters for SOD from *Bacillus licheniformis* SPB-13.

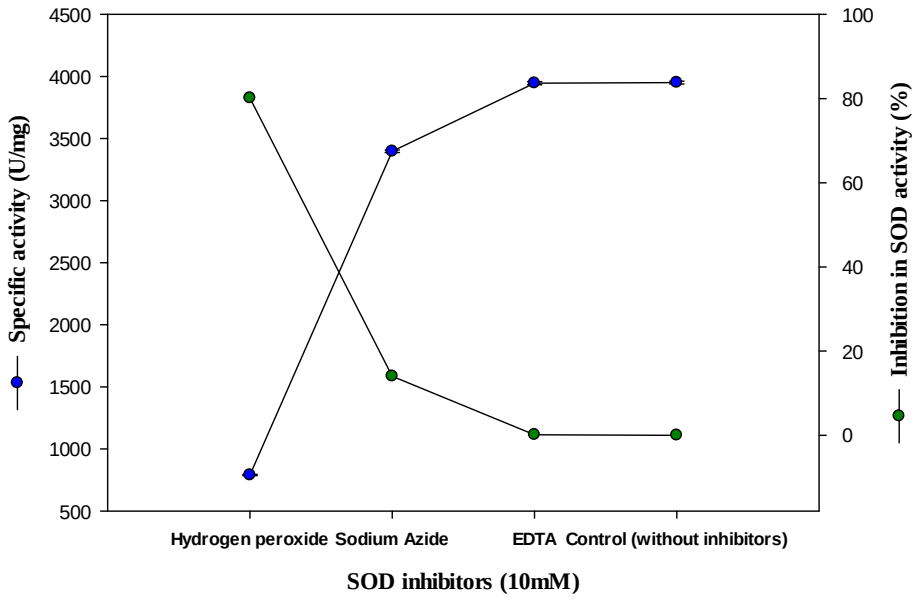

Figure 8: Inhibition assay of superoxide dismutase for SOD typing

SOD isolation from prokaryotic sources as bacterial sources are advantageous over plants and animals (Areekit *et al.*, 2011; Dhar *et al.*, 2013; Nam *et al.*, 2011; Santos *et al.*, 2000; Seatovic *et al.*, 2004). Among bacterial SODs, the present demand of medical, pharmaceutical and cosmetics industry is thermostable SODs as the production processes involve high temperature and stability is lost. Researchers have tried to explore thermostable SODs and some have achieved success (Boyadzhieva *et al.*, 2010) but no attempt has been made for production process optimization for bulk recovery of enzyme. In the present piece of work, natural thermal habitat, i.e., hot water springs of Himalayan region have been explored for thermostable SOD. *Bacillus licheniformis* SPB-13 was isolated from thermal springs of Himachal Pradesh and found to be hyperproducer of thermostable SOD. This strain was found to be very stable and retained 100% SOD activity at 80 °C for two hours and only 20%

activity loss was found at 90 °C. To scale up the SOD production process from *Bacillus licheniformis* SPB-13 for industrial purposes, all the production conditions and reaction conditions were optimized by conventional OVAT methodology followed by application of CCD component of RSM tool. A significant increase in SOD activity, i.e., 80 U/mL to 105 U/mL was obtained after classical and statistical optimization. Glucose, casein, yeast extract and NaCl concentrations had significant effect on production of SOD. As SOD is metalloenzyme, $FeCl_3$ had inducing effect on SOD production. For assay variables, Tris-HCl concentration and pH were vital factors along with reaction temperature and enzyme concentration for CCD analysis. After variables analysis, activity increased upto 120 U/mL. The antioxidative potential of this enzyme has also been explored at highest purity levels (Thakur *et al*., 2018). So, statistical tool application on *Bacillus licheniformis* SPB-13 enhanced the production and activity of thermostable SOD and this was done for the very first time for SOD from thermophilic bacterium. After isotyping analysis, the SOD was found to be of Fe/Mn family of SODs. This process can be exploited by industrial establishments for a thermostable SOD for topical ointments and other health products of antioxidant nature as industrial operating procedures for cosmetics production require a thermostable enzyme candidate (Zhang and Wu, 2011; and Cai *et al*., 2011).

6. Conclusion

The conclusion of present work is that *Bacillus licheniformis* SPB-13 produces a thermostable SOD with very good antioxidant activity. The production and reaction parameters have been optimized completely using classical and analytical methods to cut the production cost and to increase the yield of SOD. Finally, for the first time, basic production and reaction methodology optimization using RSM tool has been elucidated completely for SOD producing *Bacillus licheniformis* to make its production industry friendly.

Acknowledgment

The authors gratefully acknowledge Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla for providing the necessary facilities for the successful completion of this research work.

Conflict of interest

The authors declare that they have no conflict of interest in the publication.

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Cite this article as: Abhishek Thakur, Pradeep Kumar, Pankaj Kumari, Kavita Bhatia and Duni Chand (2020). Statistical augmentation of thermostable Superoxide dismutase (SOD) production from Bacillus licheniformis SPB-13 of Himalayan ranges. African Journal of Biological Sciences. 2 (2), 25-39.