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Optimization of Exopolysaccharide Production from Lactobacillus acidophilus MTCC 10307 by Response Surface Methodology

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Abstract: With the rising awareness of microbial exopolysaccharides (EPSs) application in various fields, microorganisms which produce EPSs have received broad attention. The study has utilized central composite design and response surface methodology to derive a statistical model for optimizing the conditions for maximum production of EPS. The experimental design was used with four independent variables i.e., temp, pH, time and conc. of carbon source for responses as EPS (mg/L), optical density, total plate count and dry cell weight. The results showed that EPS production is dependent on the selected variables. The conditions optimized for EPS production were sucrose concentration of 15%, pH 6, temperature of 32.5°C and time duration of 72 hours.

Keywords: exopolysaccharides, Lactobacillus, RSM, optimization.

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Introduction

Food industries are continuously in search of value-added compounds or additives of natural origin having increased functionality and bioactivity. Moreover, in the present time, an increasing trend may be observed in the consumer toward healthier foodstuffs. Bacterial exopolysaccharides (EPSs) play an important role in the improvement of the rheological and sensory characteristics of food products by positively influencing the food texture and organoleptic properties. Additionally, EPSs have also gained potential research interest for pharmacological and nutraceutical applications due to their biocompatibility, non-toxicity, and biodegradability (Prete *et al*. 2021).

Lactic acid bacteria are already well known for their techno-functional food applications in the dairy industry (Korcz *et al*. 2021).

Exopolysaccharides (EPS) are high-molecular-weight polymers that consist of sugar residues which widely vary in structure and function. The accumulation of exopolysaccharides (EPS) produced by microorganisms occurs in the presence of excess substrate and limiting conditions of elements that are essential to growth, such as nitrogen, phosphorus, sulfur, and magnesium. The EPS matrix consists not only of polysaccharides, proteins (the major component in waste water and environmental biofilms) and nucleic acids. Major proportion of the EPS is strongly hydrated, however, hydrophobic EPS also occurs; one such example is cellulose which is produced by a range of microorganisms. The EPS matrix encases the microbial cells within it and allows communication among them by biochemical signals as well as gene exchange. It traps extracellular enzymes and keeps them in close proximity to the cells, and hence presents an external digestion system that allows stable synergistic micro consortia of different species (Wingender and Flemming, 2010). There is a vast scope of implication of EPS of microorganisms in the food, pharmaceutical and biotechnology field due to their structural diversity, physical and rheological properties.

EPS produced by microorganisms mainly lactobacillus species possess Generally Regarded As Safe (GRAS) status and are allowed to be incorporated in food without labeling. EPS imparts highly desirable rheological properties to the food matrix like increased viscosity, improved texture and reduced syneresis. EPS may improve the rheology (viscosity and elasticity) of a final product by acting as a texturiser as well as physical stabilizers by binding hydration water and interacting with other milk constituents (ions and proteins) to avoid syneresis. The physical and rheological properties are based on the chemical composition, molecular size, charge, presence of side chains, rigidity of the molecules and 3D-structures of the EPS polymers. It also acts as a gelling agent in gum and candies and can be used as a crystallization inhibitor in ice-creams (Singh and Saini, 2017). It provides viscosity and mouthfeel to the pudding mixes. EPS has already been widely used as bioflocculants, biosorbents, encapsulating materials, heavy metal removing agents, drug delivery agents, ion exchange resins, and a natural immunomodulator (Chatterjee and De, 2017). In addition to the distinct biophysicochemical properties of bacterial EPS, it has also proven its importance in the food industry as viscosifying, stabilizing, emulsifying, antioxidant and antibiofilm agents.

Response surface methodology (RSM) is one of the most used optimization technologies in food technology. It provides superb statistical tools for design

and analysis of experiments aimed at process optimization. At the final stage of process development, RSM illuminates the sweet spot where high yield of in-specification products can be achieved at lowest possible cost. It produces statistically validated predictive model and, with the aid of specialized software, response surface maps that point the way to pinnacles of process performance. The study aimed to isolate the maximum amount of EPS from the growth of selected strain. This method was used to investigate the influence of variable pH, temperature and time conditions on the optimization of EPS content of reference culture Lactobacillus acidophilus MTCC 10307.

2. Materials and Methods

2.1. Optimization of EPS production

Experimental design selected for optimization purposed was Central Composite Rotatable Design (CCRD), for 4 independent variables at five levels. The experiments conducted in the first phase of investigation revealed that temperature of incubation, pH of growth medium, concentration of carbon sources and time of incubation were the most critical factors to produce EPS. Response surface methodology (RSM) was used to reduce the number of experiments without affecting the accuracy of results and determine the interactive effect of variables on the response (Meyers, 1976; Box and Hunter, 1978). The Box and Behnken design were used to determine the combination of variable levels in each experiment. The variable factors considered for RSM studies were temperature of incubation (25 to 40 °C), pH (5.5 to 7.0), time of incubation (24 to 72 hours) and sucrose concentration (5% to 20%). The variable factors are given in Table 1. The data were analyzed using Design-Expert software (6.0.10 version) and generalized second degree polynomial equation (Equation 3.1) using the method of least squares (Snedecor and Cochran, 1968).

$$Y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_{11} x_1^2 + b_{22} x_2^2 + b_{33} x_3^2 + b_{12} x_1 x_2 + b_{13} x_1 x_3 + b_{23} x_2 x_3 + C \quad \text{(Eq. 1)}$$

The coefficients of the polynomial models were represented by b_0 (constant term), b_1, b_2, b_3, b_4 (linear terms), b_{11}, b_{22}, b_{33}, b_{44} (quadratic terms); and b_{12}, b_{13}, b_{14}, b_{23}, b_{24}, b_{34} (interactive terms). Adequacy of model was evaluated using F ratio and coefficient of determination (R^2). The lack of fit was also calculated. Model was considered adequate when F-calculated was more than table F-value and R^2 was more than 70 per cent (Henika, 1982). The effect of variables at linear, quadratic and interactive level on individual response was described using significance at

1, 5 and 10 per cent level of confidence. The magnitude and sign of coefficients described the extent of dependency of variation on increasing or decreasing the response depending on positive or negative sign of coefficient terms. Response surface plots were also developed using second order polynomial models for all responses keeping the two processing variables at centre point. Response surface was used to determine the interaction between two variables on responses.

Table 1: Experimental variable for EPS production, their coded and uncoded values

Variables	*Code*	*Coded level*				
		-α	*-1*	*0*	*+1*	*+α*
Sucrose conc.	X_1	5	10	15	20	25
pH	X_2	5	5.5	6	6.5	7
Temp.	X_3	25	28.75	32.5	36.25	40
Time	X_4	24	36	48	60	72

2.2. Growth Measurement

The growth of the culture in MRS broth medium was measured as optical density (O.D) at 600 nm using a spectrophotometer and by plating appropriate dilutions of bacterial growth on MRS indicator agar.

2.3. Dry cell Weight

Cells were harvested by centrifugation (10,000 rpm, for 20 min) of the fermented culture broth. The cell pellet was dried in an oven at 105ºC to a constant weight.

2.4. Isolation and Purification of EPS

Released EPS was extracted from planktonic culture of Lactobacillus spp. using an optimized procedure (Kavita *et al*. 2014). Briefly, MRS broth (500 mL) was inoculated with 2% (v/v) overnight culture (OD 600nm= 0.6) and incubated for 16 hours at 37ºC on a rotatory shaker (180 rpm). The bacterial culture was centrifuged at 10,000 rpm for 20 min at 25°C. Now discard the harvested cell and collect supernatant and add an equal amount of cold ethanol and retain it for precipitation at 4°C for 16 hours. Again, centrifuge the supernatant at 8,000 rpm at 5°C for 25 min. After centrifugation, the obtained pellets dry in a hot air oven and weight is noted.

2.5. Total Viable Count

The total viable counts in burfi samples were determined according to IS 5402 (2012) procedure. Appropriate dilutions of the sample (1ml) were

transferred aseptically to sterile petri plates in duplicate and mixed well with 10-15 ml of presterilized molten plate count agar at 45°C. After solidification plates were incubated at 37°C for 24-48 hours. The average count multiplied by dilution factor was expressed as colony forming unit (cfu) per gram of sample.

3. Results and Discussion

Central composite design (CCD) with four factors was used for fitting a second order response surface. The responses were analyzed by multiple regression through the least squares method to fit equation 2

$$\text{Where, } Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j \qquad \text{(Eq 2)}$$

measured response variable; β_0, β_i, β_{ii} and β_{ij} are constant, linear, quadratic and cross product regression coefficients of the model, respectively; and X_i and X_j represent the independent variables (pH, time, temp, and glucose conc.) in coded values.

The coefficient of the response function, their statistical significance and process conditions for maximum EPS content were evaluated by using a statistical software "Design Expert, Trial version 7.1.3" (Statease Inc., Minneapolis, USA) to determine the individual and compromise optima of the response and level of different variables.

Thirty combinations of the independent variables were selected as per experimental design for three parameters as shown in Table 2. The data for optical density, dry cell weight, EPS, and total plate count (TPC) under variable experimental runs are recorded in Table 2. The independent and dependent variables were fitted to the second order model equation and evaluated for the goodness of fit. The analysis of variance was performed to determine the lack of fit and the significance of the linear, quadratic and interaction effects of the independent variables on the dependent variables. The lack of fit is a measure of the failure of a model to represent data in the experimental dominant which parts were not included in the regression (Varnalis *et al*. 2004). The result revealed that the models for all the response variables were highly adequate because they obtained satisfactory levels of R^2 and there is no significant lack of fit in all the response variables (Table 3).

3.1. Effect of Independent Variables on Optical Density

The effect of carbon concentration, variable pH, temperature and time conditions on optical density is shown in Fig 1. The optical density is the

Table 2: Experimental design of EPS production from reference culture (Lactobacillus acidophilus)

Expt. No.	Coded form and level				Responses			
	X_1	X_2	X_3	X_4	OD	EPS	DCW	TPC
1	(-1) 10	(-1) 5.50	(-1) 28.75	(-1) 36	1.74	282	379	463
2	(+1) 20	(-1) 5.50	(-1) 28.75	(-1) 36	1.73	281	378	464
3	(-1) 10	(+1) 6.50	(-1) 28.75	(-1) 36	1.76	285	378	464
4	(+1) 20	(+1) 6.50	(-1) 28.75	(-1) 36	1.74	286	376	468
5	(-1) 10	(-1) 5.50	(+1) 36.25	(-1) 36	1.79	288	377	463
6	(+1) 20	(-1) 5.50	(+1) 36.25	(-1) 36	1.75	284	378	462
7	(-1) 10	(+1) 6.50	(+1) 36.25	(-1) 36	1.76	283	379	464
8	(+1) 20	(+1) 6.50	(+1) 36.25	(-1) 36	1.75	285	382	466
9	(-1) 10	(-1) 5.50	(-1) 28.75	(+1) 60	1.79	284	383	467
10	(+1) 20	(-1) 5.50	(-1) 28.75	(+1) 60	1.8	283	384	466
11	(-1) 10	(+1) 6.50	(-1) 28.75	(+1) 60	1.78	286	382	468
12	(+1) 20	(+1) 6.50	(-1) 28.75	(+1) 60	1.79	289	382	469
13	(-1) 10	(-1) 5.50	(+1) 36.25	(+1) 60	1.75	285	385	467
14	(+1) 20	(-1) 5.50	(+1) 36.25	(+1) 60	1.76	287	384	468
15	(-1) 10	(+1)6.50	(+1) 36.25	(+1)60	1.78	288	383	469
16	(+1) 20	(+1) 6.50	(+1) 36.25	(+1) 60	1.8	289	384	468
17	(-α) 5	(0) 6	(0) 32.50	(0)48	1.73	285	379	463
18	(+α) 25	(0) 6	(0) 32.50	(0) 48	1.77	286	380	469
19	(0) 15	(-α) 5	(0) 32.50	(0) 48	1.77	286	384	462
20	(0) 15	(+α) 7	(0) 32.50	(0) 48	1.76	285	379	464
21	(0) 15	(0) 6	(-α) 25	(0) 48	1.76	284	378	465
22	(0) 15	(0) 6	(+α) 40	(0) 48	1.75	285	380	466
23	(0) 15	(0) 6	(0) 32.50	(-α) 24	1.74	284	379	467
24	(0) 15	(0) 6	(0) 32.50	(+α) 72	1.81	290	385	470
25	(0)15	(0) 6	(0) 32.50	(0) 48	1.79	289	384	465
26	(0) 15	(0) 6	(0) 32.50	(0) 48	1.78	287	385	464
27	(0) 15	(0) 6	(0) 32.50	(0) 48	1.79	288	383	468
28	(0) 15	(0) 6	(0) 32.50	(0) 48	1.77	286	384	467
29	(0) 15	(0) 6	(0) 32.50	(0) 48	1.78	287	382	466
30	(0) 15	(0) 6	(0) 32.50	(0) 48	1.76	285	383	465

Table 3: Analysis of variance (ANOVA)

	O.D	EPS	DCW	TPC
Mean±SD	1.77±0.015	285.73±1.52	381.30±1.38	465.90±1.43
Model	Quadratic	Quadratic	Quadratic	Quadratic
F-value	3.06	3.18	7.06	4.10
p-value prob<F	0.0196	0.0167	0.0003	0.0052
Lack of fit	0.00285	24.75	23.25	20.00
R^2	0.7410	0.7479	0.8683	0.7926
Adj R^2	0.4992	0.5127	0.7454	0.5991
CV%	0.87	0.53	0.36	0.31

measure of growth of biomass as well as EPS production in the broth. Equation 4.1 was fitted into the response variable, optical density (OD) using least square regression analysis. The ANOVA is given in Table 3. F value suggests that the model was significant at $p<0.0196$. Coefficient of determination, R^2 was 0.7410 suggesting 74.10% of the variability in the data was explained by the model. Therefore, model was adequate to predict the response and interpret the effect variables on the response.

The optical density (O.D) value was found in the range of 1.730-1.810. The highest value obtained at sucrose conc. of 15%, pH 6, Temp. 32.5° C, and time 72 hours. Ahmed *et al*. (2006) have also reported that the growth of L. lactis and L. acidophilus was found to be maximum between 30-35ºC. The model was obtained as follows:

Y=0.010+0.0001042* sucrose concentration +0.0000375 *pH +0.000004167* temperature +0.00570* time +0.0000562 * sucrose concentration * pH +0.00000625 * sucrose concentration * temperature +0.001056* sucrose concentration *time +0.0000562* pH * temperature +0.000156 * pH * time+0.0001406 *time*temperature +0.00103 * sucrose concentration^2 +0.000157 *pH^2+ 0.000657 *temperature^2 + 0.000000297 time^2

3.2. *Effect of Independent Variables on Exopolysaccharide Content*

The model of EPS yield was very significant, as is evident from the model F-value and a very low probability value ((P model>F) = 0.0167). The value of determinant coefficient R^2 = 0.7479 suggested that the total variation of 75% for EPS was attributed to the independent variables and about 25% of the total variation cannot be explained by the model. The Exopolysaccharide (EPS) content was found in the range of 281-290 mg/l. The highest value obtained at sucrose conc. of 15%, pH 6, Temp. 32.50°C, time 72 hours. Kemavongse *et al*. (2014) have also studied the optimization of exopolysaccharides production by oral Lactobacillus fermentum, and reported that the optimum values of tested variables for EPS production were 25% w/v sucrose, pH 6.78 and 54.29 h for cultivation time. This culture condition enhanced EPS production which increased EPS about 9.25 fold compared with original culture conditions from 1.9±0.17 to 17.57±0.61 g/l.

Y=103.12+1.04 * sucrose concentration +9.37 *pH +9.37 *temperature +35.04 *time +7.56 * sucrose concentration * pH +0.062 * sucrose concentration *temperature +3.06 * sucrose concentration * time +14.06 * pH*temperature +5.06 * pH*time +0.063 *temperature* time +5* sucrose concentration^2+5*pH^2+12.57 *temperature^2 +0.074 *time^2

3.3. Effect of Independent Variables on Dry Cell Weight

The cell density can be quantified as grams of dry weight per liter of sample; it is the measure of number of viable/dead cells per ml. The model for effect of carbon concentration, variable pH, temperature and time conditions on dry cell weight is found to be significant with very low P value at $p<0.0003$. Coefficient of determination, R^2 was 0.8683 suggesting 86.83% of the variability in the data was explained by the model. The dry cell weight (DCW) value was found in the range of 376-385 mg/l. The highest value obtained at sucrose conc. of 15%, pH 6, Temp. 32.50°C, time 72 hours.

Y=189.55+.67 * sucrose concentration +6 * pH +8.17 * temperature+112.67 * time+0.25 * sucrose concentration *pH+2.25 * sucrose concentration * temperature+0.00 * sucrose concentration* time+6.25 * pH* temperature+4 *pH*time+0.00 temperature* time+24.11 * sucrose concentration ^{2}+5.25* pH^2+30.96 * temperature ^{2}+2.68 * time^2

3.5. Effect of Independent Variables on Total Plate Count

The total plate count is the enumeration of aerobic, mesophillic organisms that grow in aerobic conditions under moderate temperatures of 20-45°C. This includes all aerobic bacteria, yeast, molds and fungi that grow in the specific agar. The model of TPC was very significant, as is evident from the model F-value and a very low probability value ((P model>F) = 0.0052). The value of determinant coefficient R^2 = 0.7926 suggested that the total variation of 79% for TPC was attributed to the independent variables and about 21% of the total variation cannot be explained by the model. The TPC value was found in the range of 376-385 mg/l. The highest value obtained at sucrose conc. of 15%, pH 6, Temp. 32.50°C, time 72 hours.

Y=117.87+13.50 * sucrose concentration +16.67 * pH+0.00 *temperature +48.17 *time +2.25* sucrose concentration *pH+1 * sucrose concentration *temperature+2.25 *sucrose concentration *time+0.25 *pH *temperature+1*pH*time+2.25 *temperature *time +0.19 *sucrose concentration^2+12.19 * pH^2+0.048 *temperature^2+13.76 *time^2

Sharma *et al*. (2017) also reported potential probiotic lactic acid bacteria Pediococcus acidilactici KM0 (accession number KX671557) isolated from milk cream, capable of excellent production of EPS. The five important parameters (incubation time, temperature, pH, carbon concentration and nitrogen concentration) had significant positive effects on the EPS production. The optimum values of these five variables were optimized by RSM by using Design of Experiments (DOE) and interactive effects of the process parameters

were evaluated. Maximum EPS production of 32.64 mg/ml was observed with 65.81% increase after 24 h incubation at 35ºC using 1.50 % and 3% of carbon and nitrogen concentration respectively, at pH 6.5.

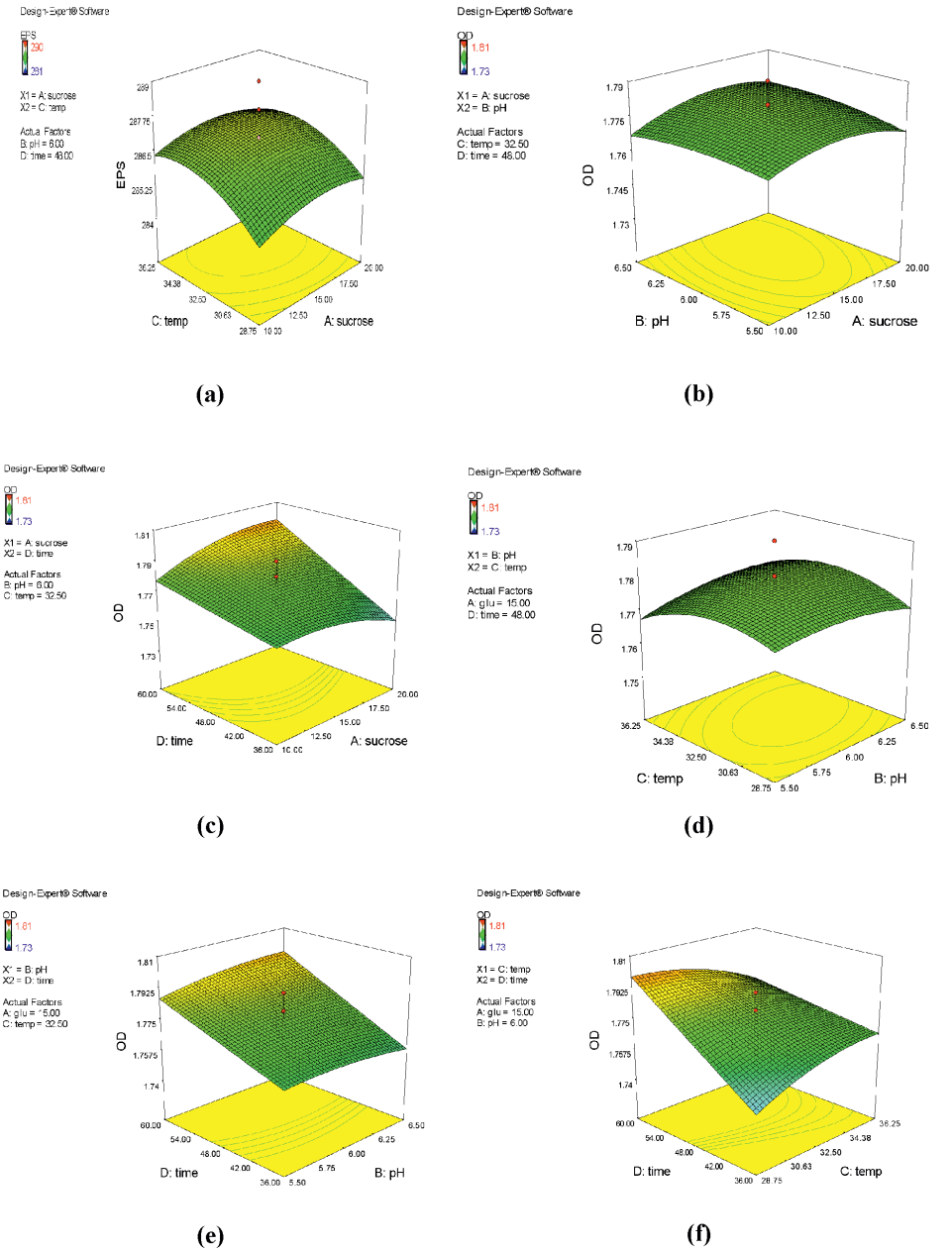
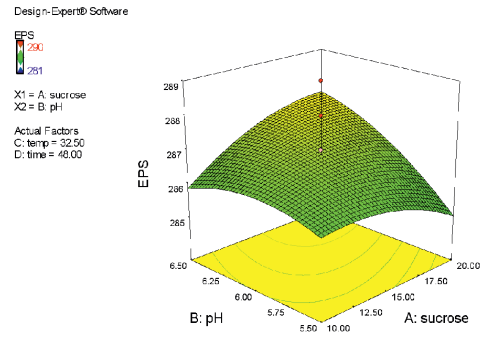
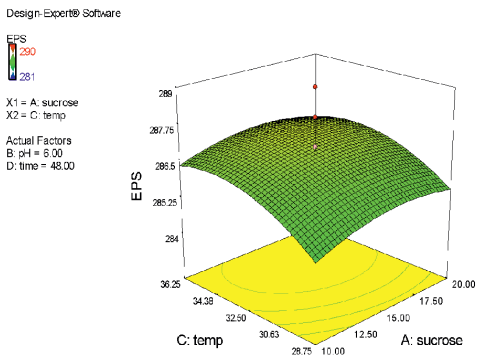


Figure 1: (a-f): Interactive effect of variables on optical density



(a)



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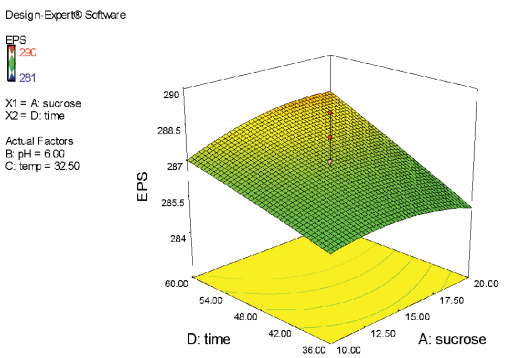

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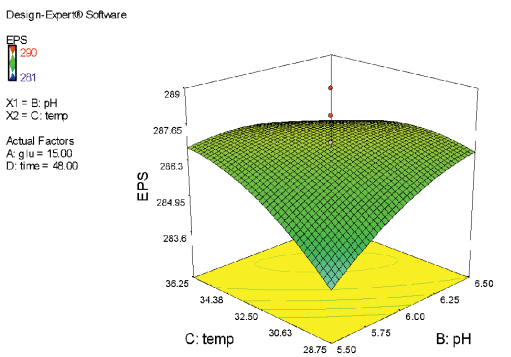

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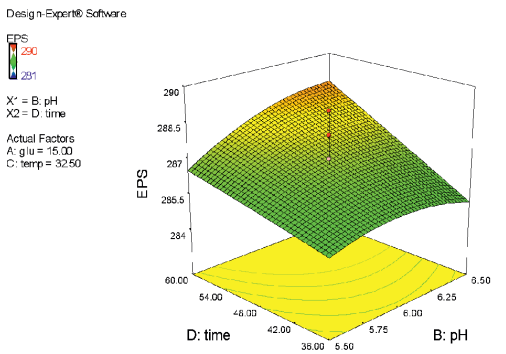

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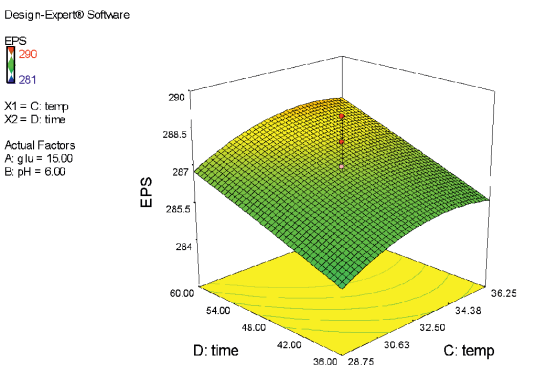

(f)

Figure 2: (a-f) : Interactive effect of variables on EPS

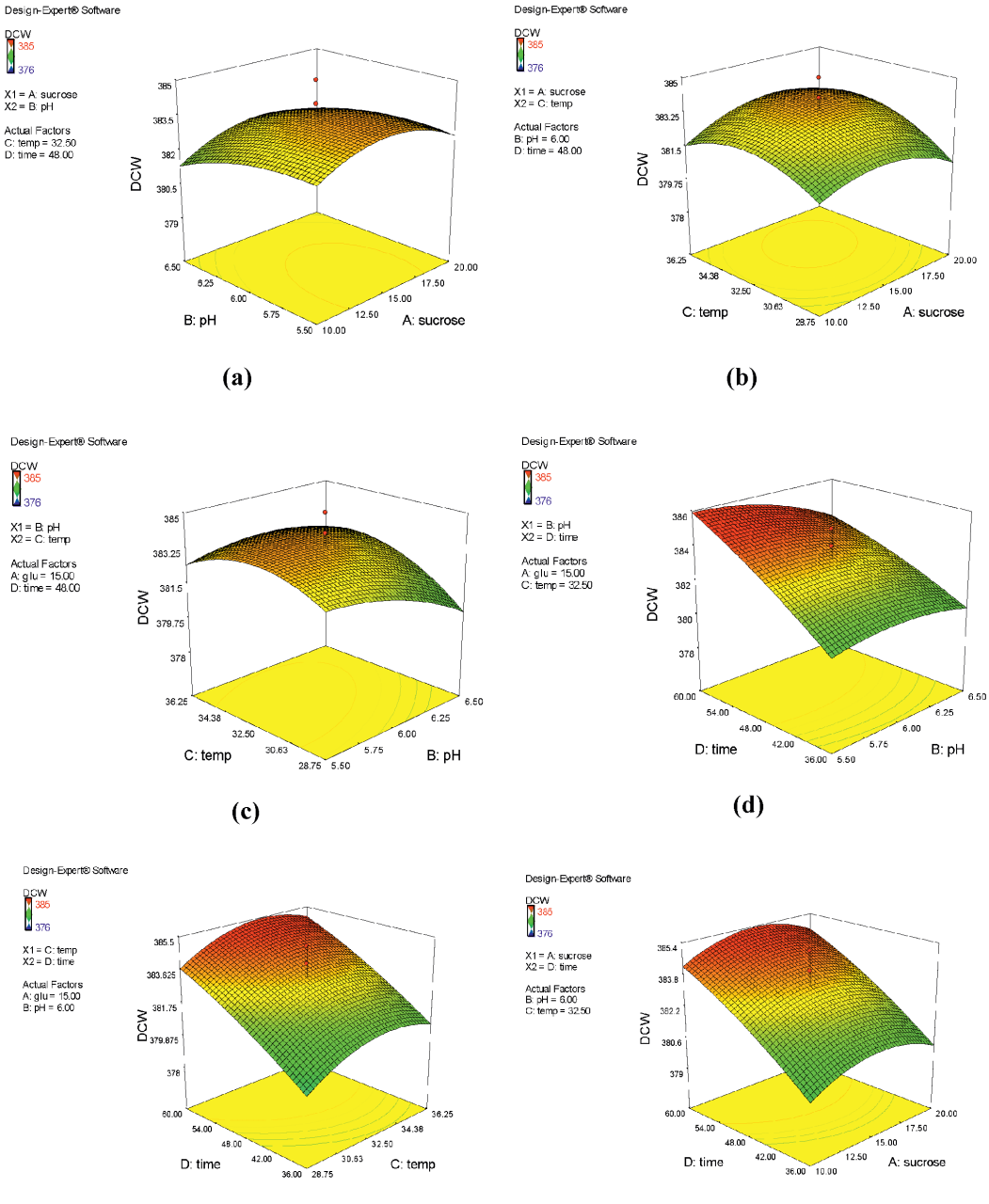

Figure 3: (a-f): Interactive effect of variables on dry cell weight

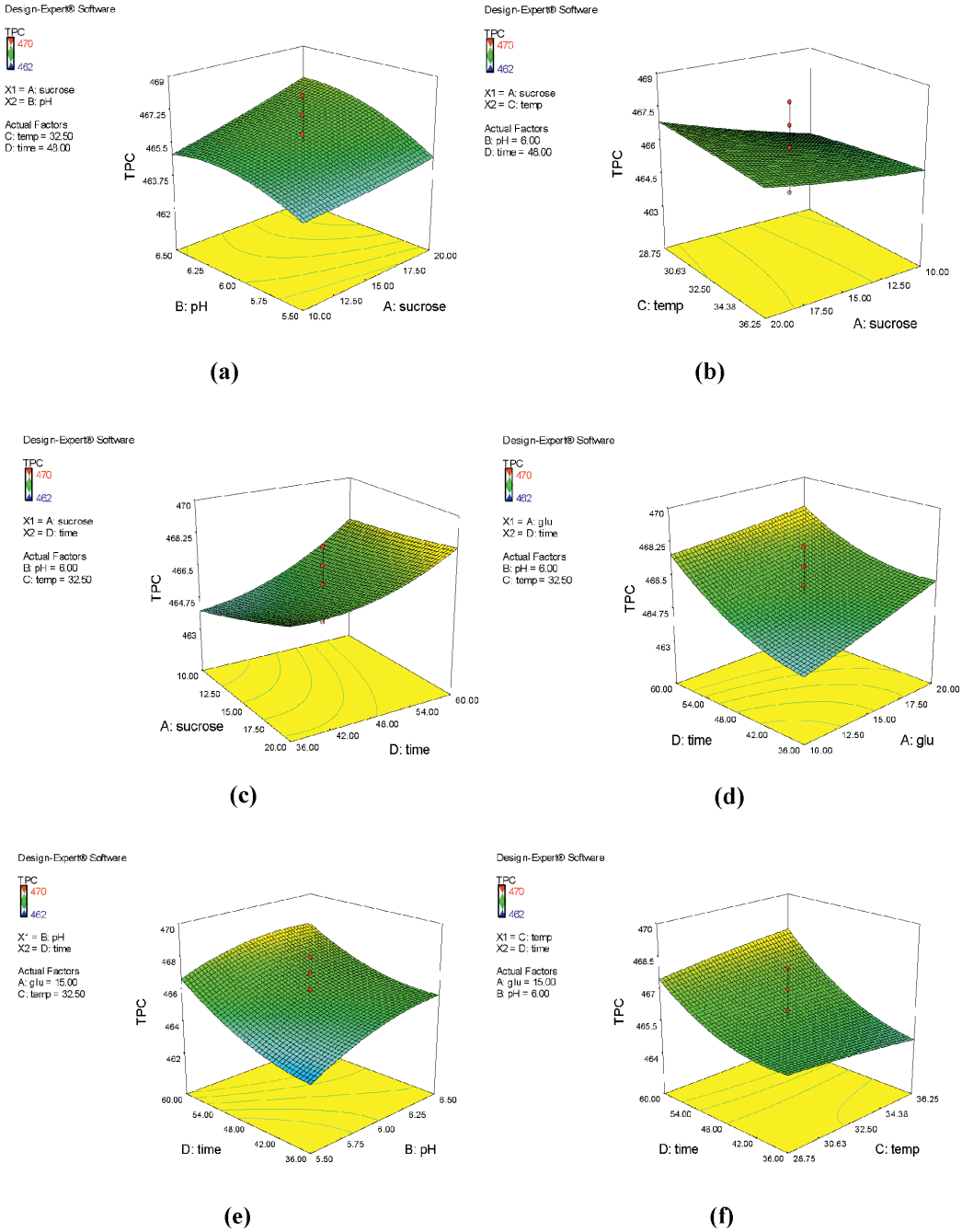

Figure 4: (a-f): Interactive effect of variables on total plate count

4. Conclusion

The present study was conducted with the objective of optimizing conditions for maximum production of exopolysaccharides. For optimization of EPS from Lactobacillus strains a Central Composite Rotatable Design (CCRD), experimental design was used with four independent variables (temp, pH, time and conc. of carbon source) at five levels. The responses were taken as EPS (mg/L), optical density, total plate count and dry cell weight. The optimization of EPS was done at sucrose conc. of 15%, pH 6, temp. 32.5°C and time duration of 72 hours. It was found that increasing concentration of sucrose, increased the EPS production but after a certain extent the EPS production decreased. Temperature and pH conditions were found to be favorable at 32.50°C and pH 6, respectively showing that the EPS production is dependent on the amount of cell growth, which is maximum at the above-mentioned conditions.

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