

## Response surface optimization in biosurfactant production by using a renewable growth substrate

Zulfiqar Ali Raza<sup>1\*</sup>, Naseer Ahmad<sup>1</sup>, Zafar M Khalid<sup>2</sup> and Niaz Ahmad<sup>1</sup>

<sup>1</sup>Chemistry Research Laboratory, National Textile University, Faisalabad 37610, Pakistan

<sup>2</sup>Department of Bioinformatics and Biotechnology, International Islamic University, Islamabad 44000, Pakistan

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The present study investigated the use of blackstrap molasses as renewable carbon source by a *Pseudomonas aeruginosa* strain to produce rhamnolipid surfactant under shake flask conditions. The process factors considered were total sugar concentration, carbon to nitrogen ratio and incubation period, whereas the responses were utilized total sugar, dry cell biomass, rhamnolipid yield, surface tension and certain kinetic parameters. This is the first report on response surface optimization in biosurfactant production by *P. aeruginosa* strain grown on molasses. Statistical modeling for all the considered responses was done through desirability, which expressed that the percentage of prediction error was much low. This explains that the prediction performance of the models is quite adequate. The highest dry cell biomass (1.63 g/L) and rhamnolipid (1.46 g/L) yields were observed at 5 d of incubation on 2% total sugars-based molasses amended with sodium nitrate (at C:N, 20:1). The surface tension of this culture medium dropped to 28.0 from 50.0 mN/m.

**Keywords:** Biosurfactant, optimization, rhamnolipid, response surface methodology, surfactant

### Introduction

Surfactants and detergents have the ability to reduce superficial and interfacial tension between multiple phases. Most of these surface-active compounds are synthetic, while few of them are of biological origin, hence termed as biosurfactants<sup>1,2</sup>. Interest in potential applications of biosurfactants has significantly been increased recently, especially due to their reduced toxicity, eco-friendly nature and sustainability as compared to their synthetic counterpart<sup>1-9</sup>. However, from a financial perspective, biosurfactants have not been commercialized extensively.

Different ways can be adopted to lessen the production expenses through enhanced product yields of biosurfactants. Use of inexpensive renewable substrates, optimal process conditions and adoption of high-production microbial strains could reduce the cost on biosurfactant production process. Molasses, a byproduct of the sugar industry, is a major raw material for the production of diverse organic and biochemical compounds. It is comparatively low in price as compared to other polysaccharides and is rich in various nutrients besides sucrose. These include

minerals, organic compounds and vitamins, which are valuable for the microbial process<sup>10</sup>.

The conventional method for medium optimization involves changing one variable at a time, while keeping factors fixed at a specific set of conditions. However, these methods might lead to unpredictable results and misleading conclusions. Moreover, performing experiments with every possible combination of the factors is impractical, because this results in large number of trials<sup>2</sup>. On the other hand, the response surface methodology (RSM) is a collection of mathematical and statistical techniques for designing experiments, building models and evaluating the factors' effects. The main function of RSM is to convert objective and constraint functions into polynomials, which are simple and smooth functioning<sup>11</sup>.

In the present study, rhamnolipid (RL) production was optimized through RSM by growing *Pseudomonas aeruginosa* gamma ray-induced mutant strain in the minimal medium provided with blackstrap molasses adjusted at specific C/N ratios under shake flask conditions of 100 rpm and 37°C. The biosurfactant production process was followed up by measuring and utilizing various process parameters including total sugars, dry cell biomass, rhamnolipid yield, surface tension and some kinetic parameters.

\*Author for correspondence:

Tel: +92-41-9230081, Fax: +92-41-9230098  
zarazapk@yahoo.com

## Materials and Methods

### Growth Substrate

The molasses, rich in various nutrients and one of the major byproducts of sugar production industry, was evaluated as the cheapest substrates for value-addition production. Blackstrap molasses was clarified as per described elsewhere<sup>12</sup>.

### Bacterial Strain

*P. aeruginosa* EBN-8 mutant<sup>13</sup> was separately enriched in the minimal medium containing five successively increasing concentrations of clarified molasses in Erlenmeyer flasks and incubating on the orbital shaker at 37°C and 100 rpm. The strain was then streaked on an agar plate amended with the clarified molasses and incubated at 37°C for 24 h. The inoculum of EBN-8 was prepared as reported earlier<sup>13</sup>.

### Shake Flask Experiments

The study was conducted in Erlenmeyer flasks encompassing sterilized aqueous medium supplied with the clarified molasses as per RSM design (Table 1). The carbon contents in the medium were set on the basis of total organic carbon contents. The factors and levels of the present study have

been chosen on the basis of pilot experimentation using one factor at a time approach method. The input parameters were total sugars (TS), C:N ratio and incubation time (Time or T). The 3-level of input parameters were used to develop the models for RL yield and to optimize the parameters for fermentation process.

Sodium nitrate was added to respective concentrations of molasses to adjust the C/N ratio (10, 20 or 30) of the media and pH value of the media was set at 7, following sterilization. An ampoule of 1% (v/v) inoculum was added to the minimal medium and incubated at 37°C and 100 rpm. For analysis, samples were taken at time-defined intervals and subjected to examine various process traits. The chemicals were of analytical grade and used as solutions after filter-sterilization, when applicable.

### Analytical Methods

TS in clarified molasses were determined by the standard dinitrosalicylic acid (DNS) method<sup>14</sup>. The dry cell biomass (DCBM) in culture medium was determined after harvesting the cells by centrifugation (10,000 rpm for 15 min, 4°C). The cell pellet was desiccated at 60°C to a constant mass. Alongside, the cell-free culture broth (CFCB) obtained was used to

Table 1—Experimental design matrix and results

	Input parameters						Responses							
	Coded			Actual			Process parameters				Kinetic parameters			
	A	B	C	TS (%w/v)	C:N ratio	Time (d)	UTS (%w/v)	DCBM (g/L)	RL (g/L)	ST (mN/m)	$Y_{P/S}$ (g/g)	$Y_{P/X}$ (g/g)	$Y_{X/S}$ (g/g)	$P_V$ (g/L/h)
1	-1	-1	-1	1	10	3	24	0.65	0.80	32	3.33	1.23	2.71	0.0111
2	1	-1	-1	3	10	3	8	0.90	0.80	31	1.00	0.09	11.25	0.0011
3	-1	1	-1	1	30	3	25	1.10	0.86	31	3.44	0.78	4.40	0.0119
4	1	1	-1	3	30	3	9	1.00	0.98	30	10.89	0.98	11.11	0.0136
5	-1	-1	1	1	10	7	46	0.95	0.80	32	1.74	0.84	2.07	0.0048
6	1	-1	1	3	10	7	19	0.85	0.95	31	5.00	1.12	4.47	0.0057
7	-1	1	1	1	30	7	47	1.10	0.88	31	1.87	0.80	2.34	0.0052
8	1	1	1	3	30	7	20	0.90	1.04	30	5.20	1.16	4.50	0.0062
9	-1	0	0	1	20	5	39	1.11	0.90	31	2.31	0.81	2.85	0.0075
10	1	0	0	3	20	5	16	1.23	1.41	29.5	8.81	1.15	7.69	0.0118
11	0	-1	0	2	10	5	19	1.30	1.00	30	5.26	0.77	6.84	0.0083
12	0	1	0	2	30	5	20	1.40	1.20	29	6.00	0.86	7.00	0.0100
13	0	0	-1	2	20	3	14	1.51	1.20	28.5	8.57	0.79	10.79	0.0167
14	0	0	1	2	20	7	26	1.5	1.45	28	5.58	0.97	5.77	0.0086
15	0	0	0	2	20	5	21	1.62	1.45	28	6.90	0.90	7.71	0.0121
16	0	0	0	2	20	5	20	1.61	1.44	28	7.20	0.89	8.05	0.0120
17	0	0	0	2	20	5	21	1.63	1.46	28	6.95	0.90	7.76	0.0122
18	0	0	0	2	20	5	20	1.62	1.45	28	7.25	0.90	8.10	0.0121
19	0	0	0	2	20	5	20	1.61	1.44	28	7.20	0.89	8.05	0.0120
20	0	0	0	2	20	5	21	1.62	1.45	28	6.90	0.90	7.71	0.0121

determine other responses. The rhamnase equivalents of the CFCB were determined by the standard orcinol method<sup>15</sup>. The RL concentrations were calculated from a standard curve prepared with L-rhamnase and expressed as rhamnase equivalents (mg/mL). The RLs were calculated as 3.4 times the rhamnase contents. The equilibrated surface tension of the CFCB was measured by the ring method using a de Noüy digital tensiometer (Krüss K10T, Hamburg, Germany). The kinetics of fermentation experiments was studied in terms of the product yields related to substrate consumption ( $Y_{P/S}$ , g/g) and to biomass ( $Y_{P/X}$ , g/g), biomass yield related to substrate consumption ( $Y_{X/S}$ , g/g), and volumetric productivity ( $P_V$ , g/L/h) of the culture media, following the standard methods of Aiba *et al*<sup>15</sup>.

**Statistical Analysis**

The Microsoft Excel 2010 (Microsoft, Corp., Redmont, WA) and Design Expert 7.0.0 softwares were used to optimize the composition of the culture media and to plot the experimental data and models. The statistical significance was assessed by ANOVA. The level of significance was defined at  $p < 0.05$ . All the experiments were conducted in triplicates and the results reported are the means of three concordant readings.

**Response Surface Modeling of Biosurfactant Production**

The RSM has been applied for modeling and analysis of process and kinetic parameters in the biosurfactant production process in order to obtain the relationship to the TS, C:N ratio and Time. In the RSM, the quantitative form of relationship between desired response and independent input variables is represented as follows:

$$Y = f(\text{TS, C:N, Time}) \quad \dots (1)$$

Where, Y is the desired response and f is the response function (or response surface). For the purpose of analysis, the approximation of Y was proposed using the fitted second-order polynomial regression model, which is called as the quadratic model. The quadratic model of Y is written as follows:

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i=1}^k \sum_{\substack{j=1 \\ i < j}}^k \beta_{ij} X_i X_j \dots (2)$$

Where, Y is the desired response and the xi (1, 2, k) are the independent of k quantitative process variables. The  $\beta_0$  is constant and  $\beta_i$ ,  $\beta_{ii}$  and  $\beta_{ij}$  are the coefficients of linear, quadratic and cross product terms, respectively.

**Multi-response Optimization through Desirability**

One of the useful approaches to optimize the multiple responses is the use of simultaneous optimization technique. This approach includes the concept of desirability functions. The general approach is to first convert each response ( $y_i$ ) into an individual desirability function ( $d_i$ ) and varied over the range  $0 \leq d_i \leq 1$ . Where, if the response  $y_i$  is at its goal or target, then  $d_i=1$ . The response is outside an acceptable region ( $d_i=0$ ). Finally, the individual desirability functions are combined to provide a measure of the overall desirability of the multi-response system. This measure of composite desirability is the weighted geometric mean of the individual desirability for the responses. The optimal operating conditions were determined by maximizing the composite desirability<sup>17</sup>.

$$D = (d_1 \times d_2 \times d_3 \times \dots \times d_n)^{1/n} = (\prod_{i=1}^n d_i)^{1/n} \quad \dots (3)$$

Where, n is the number of responses in the measure. If any of the responses or factors falls outside the desirability range, the overall function becomes zero. It can be extended to reflect the possible difference in the importance of different responses, where the weight  $w_i$  satisfies and  $0 < w_i < 1$  and  $w_1 + w_2 + \dots + w_n = 1$ .

$$D = (d_1^{w_1} \times d_2^{w_2} \times d_3^{w_3} \times \dots \times d_n^{w_n})^{1/n} \quad \dots (4)$$

In the present investigation, various process and kinetic response parameters were chosen to maximize the overall desirability. The factor settings with maximum total desirability were considered to be the optimal parameter conditions.

**Results and Discussion**

**Determination of Main Effects on UTS**

Based on the proposed third-order polynomial model, the effect of the process variable on the utilized total sugars (UTS) has been determined by computing the values using Design Expert software and the relevant data from Table 1. The mathematical relationship for correlating the UTS and the considered process variables is obtained as follows:

$$\text{UTS} = 4.62955 - 7.70682 \text{ TS} + 0.63182 \text{ C:N} + 12.63636 \text{ Time} - 5.87500 \text{ TS} * \text{Time} + 0.92045 \text{ TS}^2 - 0.014545 \text{ C:N}^2 - 0.23864 \text{ Time}^2 + 1.12500 \text{ TS}^2 * \text{Time} \quad \dots (5)$$

It has been concluded from Eq. 5 that there are two factor interactions, *i.e.*, between TS and Time, and  $\text{TS}^2$  and Time. When the values of probability (P values) are less than 0.05, it means that the factor is

significant. The estimated regression coefficients and ANOVA (after backward elimination process) for UTS using 95% of confidence interval (CI) are shown in Table 2. The other essential coefficient  $R^2$ , which is called determination coefficient in the subsequent ANOVA table, is defined as the ratio of the explained variation to the total variation and is a measure of the degree of fit. When  $R^2$  approaches unity, the response model fits better to the actual data and shows less difference between the predicted and actual data. The obtained values predicted  $R^2$  of 0.9902, which is in reasonable agreement with the adjusted  $R^2$  of 0.9974.

Based on Fig. 1a, the UTS were mainly affected by TS and time of incubation. The UTS decreased dramatically from 36.2 to 14.4% as TS was increased by 1 to 3%. As for incubation time, UTS increased from 16.0 to 31.6% when incubation time was increased from 3 to 7 d. Since TS and time of incubation showed the higher percentage contribution as compared to the C:N ratio, they could be considered most significant to the UTS. Thus for an economical process, minimum carbon source should be utilized at minimum incubation time and optimum C:N ratio. For that reason, the minimum UTS was achieved when the factors were set at TS = 3%, C:N = 20 and Time = 3 d. Residuals plots in Fig. 1b also satisfy the developed model. It was observed that errors are normally distributed that fall on a straight line.

#### Determination of Interaction Effects for UTS

Based on Table 2, there are two factor interactions between TS and Time, and  $TS^2$  and Time. Fig. 1c shows the significant interactions of the parameters for the UTS. The P value of  $TS \times T$  and  $TS^2 \times T$  interactions are <0.0001 and 0.0002, respectively. Therefore, we consider the interaction between

Table 2—The analysis of variance for UTS

Source	SS	DOF	MS	F value	P > F	Remarks
Model	2010.67	8	251.33	897.09	< 0.0001	significant
A	1188.10	1	1188.10	4240.71	< 0.0001	significant
B	2.50	1	2.50	8.92	0.0124	significant
C	72.00	1	72.00	256.99	< 0.0001	significant
AC	60.50	1	60.50	215.94	< 0.0001	significant
A <sup>2</sup>	117.82	1	117.82	420.53	< 0.0001	significant
B <sup>2</sup>	5.82	1	5.82	20.77	0.0008	significant
C <sup>2</sup>	2.51	1	2.51	8.94	0.0123	significant
A <sup>2</sup> C	8.10	1	8.10	28.91	0.0002	significant

$R^2 = 0.9985$   
Adjusted  $R^2 = 0.9974$   
Predicted  $R^2 = 0.9902$

incubation time and TS concentration at fixed C:N ratio of 20. When TS concentration was set at 3% and incubation time was varied from 3 to 7 d, UTS increased from 9.87 to 20.87%. The maximum utilization of total sugars was 26.17% at 1%TS and 3 d of incubation. It was observed that a decrease in the TS concentration led to the increase of the UTS at full incubation tenure. The interaction of incubation time with C:N ratio at fixed TS concentration of 2% showed that, at C:N ratio of 10, on increasing incubation time from 3 to 7 d, the UTS increased from 11.77 to 23.77%. Whereas the interaction of C:N ratio with TS at fixed incubation time of 5 d showed the maximum UTS of 36.17% at 1%TS and C:N ratio of 10.

#### Determination of Main Effects on DCBM

The mathematical relationship for correlating the DCBM and the considered process variables is obtained as follows:

$$\text{DCBM} = -1.75037 + 1.92762 \text{ TS} + 0.11550 \text{ C:N} + 0.063250 \text{ Time} - 5.62500 \text{ E-003 TS} \times \text{C:N} - 0.028125 \text{ TS} \times \text{Time} - 0.41938 \text{ TS}^2 - 2.39375 \text{ E-003 C:N}^2 \dots (6)$$

It has been concluded from Eq. 6 that there are two factor interactions between TS and C:N, and TS and Time. The estimated regression coefficients and ANOVA for DCBM are shown in Table 3. The obtained values are predicted  $R^2$  of 0.8527, which is in reasonable agreement with the adjusted  $R^2$  of 0.9541.

Based on Fig. 2a, the DCBM concentration was affected by all the factors of TS, C:N ratio and time of incubation. Since DCBM being a side product, it must be minimum during biosurfactant production, but not below a critical limit as biosurfactants are produced once the cellular growth reach the stationary phase. This suggests that, at 3% TS and 7 d of incubation, minimum of carbon source might be used in limited biomass formation making the process more efficient. Hence, the minimum DCBM was achieved when the factors were set at TS = 3%, C:N = 30 and Time = 7 d. Residuals plots in Fig. 2b also satisfy the developed model and errors are normally distributed that fall on a straight line.

#### Determination of Interaction Effects for DCBM

Based on Table 3, there are two factor interactions, *i.e.*, between TS and C:N, and TS and Time. Fig. 2c shows the significant interactions of the parameters for the DCBM. The P value of  $TS \times C:N$  and  $TS \times \text{Time}$

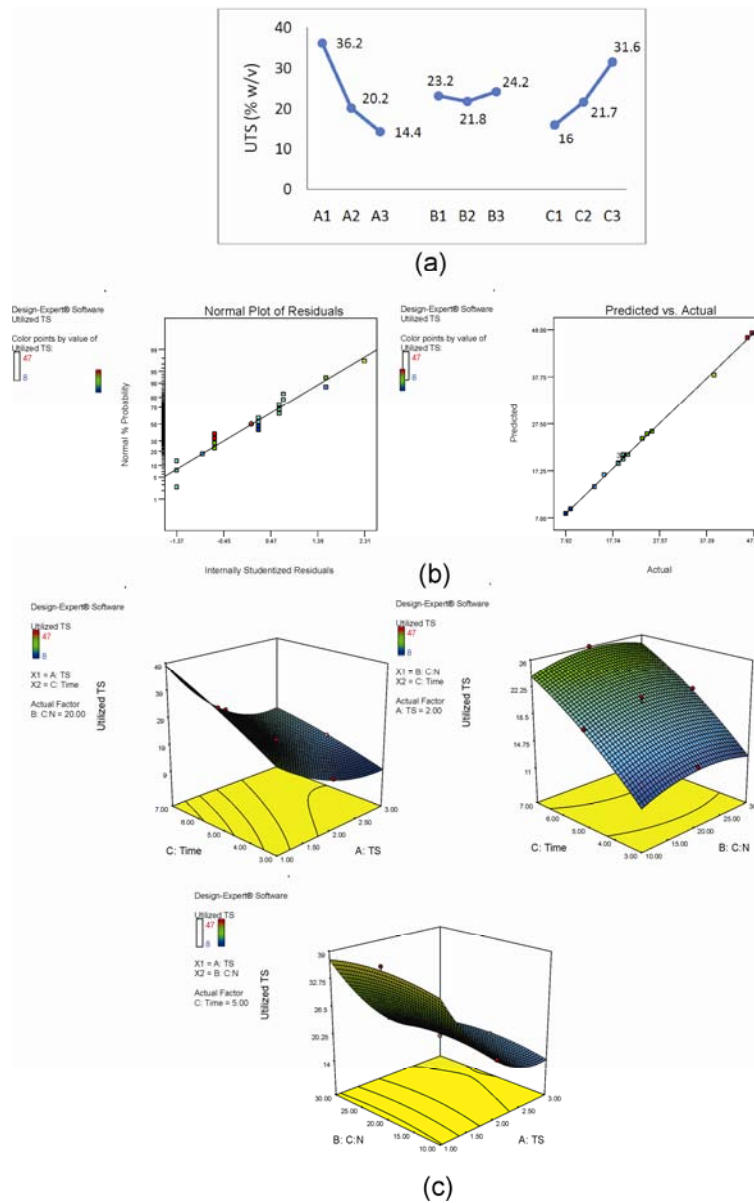


Fig. 1 (a-c)—Main effect of residuals and interaction plots for UTS: (a) Process parameters effect, (b) Residuals plots, & (c) Interaction plots between T×TS, T×C:N, & C:N×TS.

Table 3— The analysis of variance for DCBM

Source	SS	DOF	MS	F value	P > F	Remarks
Model	1.8931	7	0.2704	57.38	< 0.0001	significant
A	0.0001	1	0.0001	0.02	0.8924	
B	0.0722	1	0.0722	15.33	0.0021	significant
C	0.0020	1	0.0020	0.42	0.5312	
AB	0.0253	1	0.0253	5.37	0.0390	significant
AC	0.0253	1	0.0253	5.37	0.0390	significant
A <sup>2</sup>	0.5628	1	0.5628	119.40	< 0.0001	significant
B <sup>2</sup>	0.1834	1	0.1834	38.90	< 0.0001	significant

R<sup>2</sup>=0.9710  
 Adjusted R<sup>2</sup> = 0.9541  
 Predicted R<sup>2</sup> = 0.8527

interactions are 0.0390 each. The interaction of C:N ratio with TS concentration at fixed incubation time of 5 d expresses that minimum DCBM of 0.79 g/L was observed at C:N ratio of 10 and TS concentration of 1%. The interaction of incubation time with TS concentration at fixed C:N ratio of 20 exhibits that minimum DCBM of 1.10 g/L was observed at TS concentration of 1 % and incubation time of 3 d. Whereas, the interaction between incubation time and C:N ratio at fixed TS of 2% shows that minimum DCBM of 1.25 g/L was observed at C:N ratio of 10 and incubation time of 3 d.

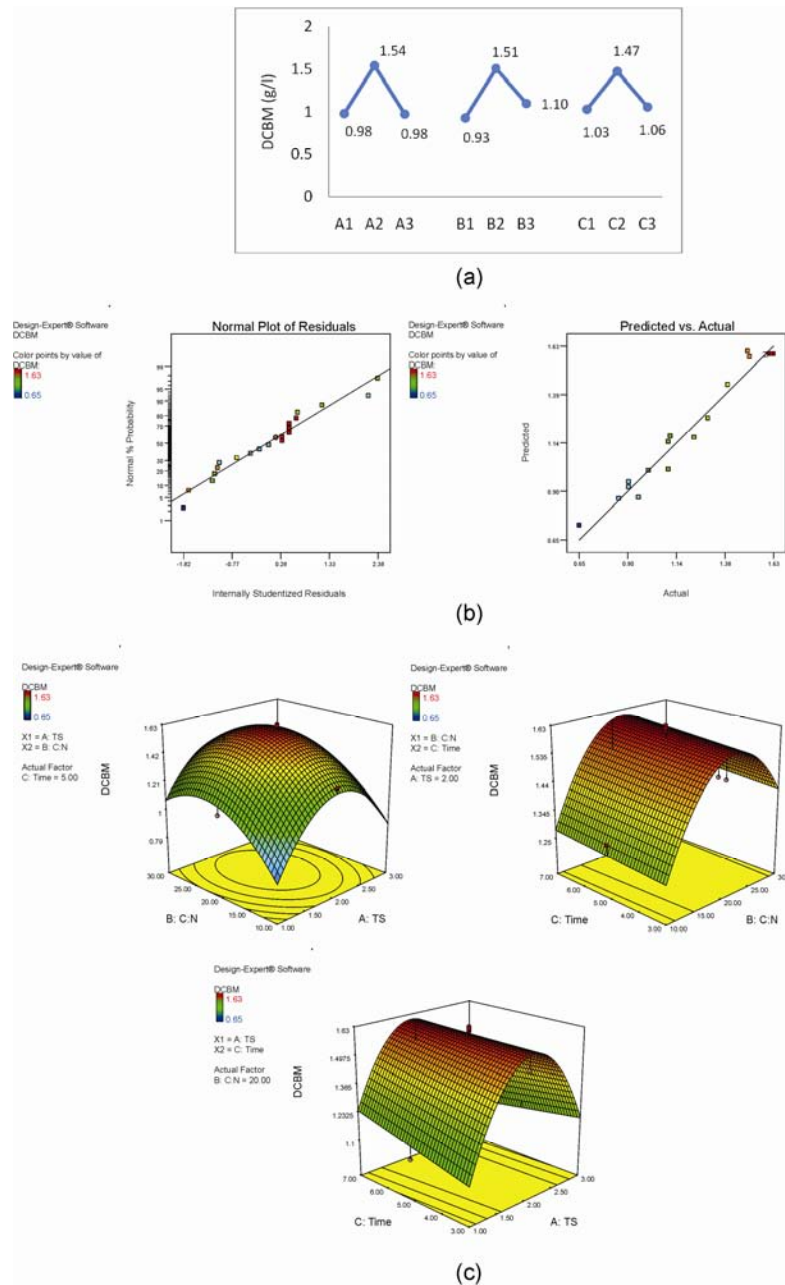


Fig. 2 (a-c)—Main effect of residuals and interaction plots for DCBM: (a) Process parameters effect, (b) Residuals plots, & (c) Interaction plots between C:N×TS, T×TS, and T×C:N.

**Determination of Main Effects on RL**

The mathematical relationship for correlating the RL and the considered process variables is obtained as follows:

$$\begin{aligned}
 RL = & 1.48361 - 0.92017 \text{ TS} - 0.14559 \text{ C:N} + 0.040909 \\
 & \text{Time} + 0.15306 \text{ TS} * \text{C:N} + 0.16063 \text{ TS} * \text{Time} + 5.43750 \\
 & \text{E} - 003 \text{ C:N} * \text{Time} - 0.25136 \text{ TS}^2 + 2.76136 \text{ E} - 003 \\
 & \text{C:N}^2 - 0.020341 \text{ Time}^2 - 5.18750 \text{ E} - 003 \text{ TS} * \text{C:N} * \text{Time} \\
 & - 2.91250 \text{ E} - 003 \text{ TS} * \text{C:N}^2 \dots (7)
 \end{aligned}$$

It has been concluded from Eq. 7 and Table 4 that there are four factor interactions, *i.e.*, between TS and C:N, TS and Time, C:N and Time, and between TS, C:N and Time. The quadratic functions of TS, C:N and Time have significant effects on RL and can be used to predict RL within limits of control factors. The estimated regression coefficients and ANOVA for RL are shown in Table 4. As per ANOVA, the predicted R<sup>2</sup> of 0.8327 is in reasonable agreement with the adjusted R<sup>2</sup> of 0.9911.

Table 4— The analysis of variance for RL

Source	SS	DOF	MS	F value	P > F	Remarks
Model	2.37	11	0.22	194.25	< 0.0001	significant
A	0.13	1	0.13	117.15	< 0.0001	significant
B	0.18	1	0.18	159.34	< 0.0001	significant
C	0.14	1	0.14	129.71	< 0.0001	significant
AB	0.09	1	0.09	81.35	< 0.0001	significant
AC	0.10	1	0.10	93.24	< 0.0001	significant
BC	0.08	1	0.08	70.27	< 0.0001	significant
A <sup>2</sup>	0.17	1	0.17	156.52	< 0.0001	significant
B <sup>2</sup>	0.26	1	0.26	232.50	< 0.0001	significant
C <sup>2</sup>	0.02	1	0.02	16.40	0.0037	significant
ABC	0.09	1	0.09	77.57	< 0.0001	significant
AB <sup>2</sup>	0.14	1	0.14	122.26	< 0.0001	significant

R<sup>2</sup> = 0.9963  
 Adjusted R<sup>2</sup> = 0.9911  
 Predicted R<sup>2</sup> = 0.8327

Based on Fig. 3a, the RL yield was affected by all the factors. The RL yield was observed maximum at TS = 2%, C:N = 20 and T= 5 d. The residuals plots in Fig. 3b also satisfy the developed model and the errors are observed as normally distributed that fall on a straight line.

**Determination of Interaction Effects for RL**

Based on Table 4, the P value of TS×C:N, TS× Time, C:N× Time, and TS×C:N×Time interactions are <0.0001 each. Fig. 3c shows the significant interactions of the parameters for the RL yield. The interaction of C:N ratio with TS concentration at fixed incubation time of 5 d shows that maximum RL yield of 1.41 g/L was observed at C:N ratio of 25 and TS

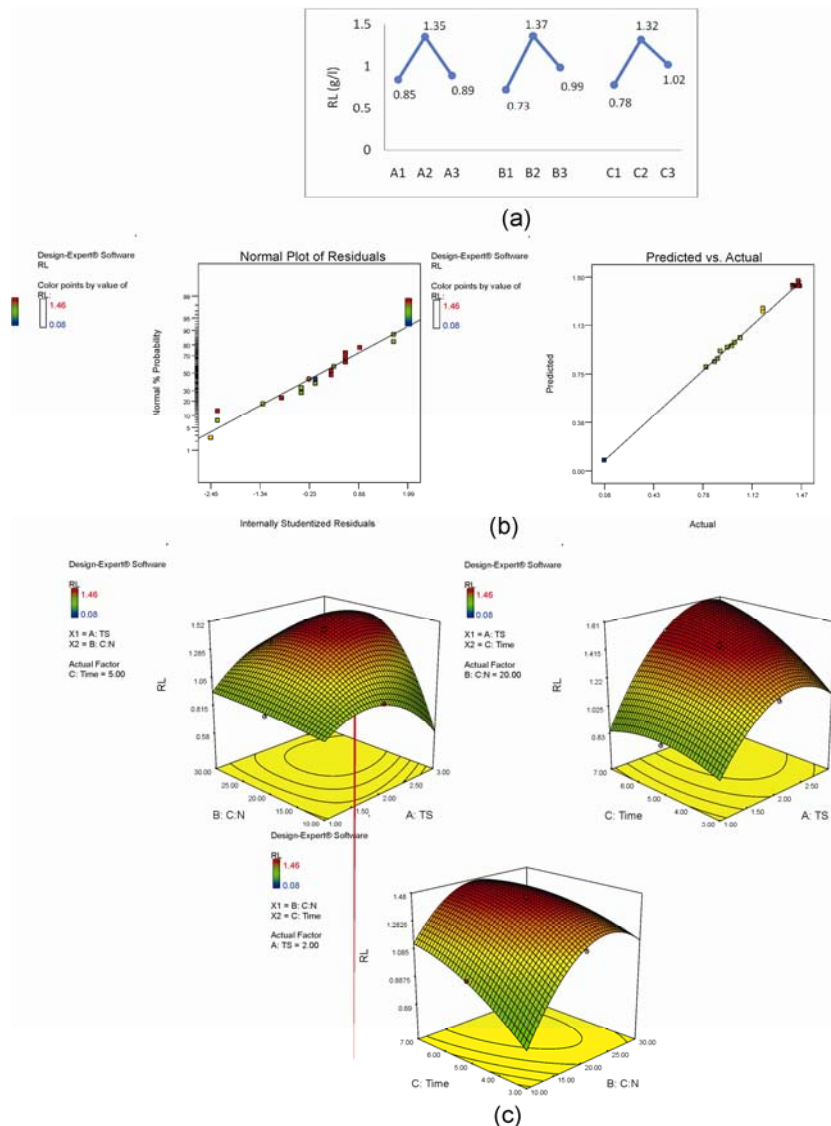


Fig. 3 (a-c)—Main effect of residuals and interaction plots for RL: (a) Process parameters effect, (b) Residuals plots, & (c) Interaction plots between C:N×TS, T×TS, and T×C:N.

concentration of 3%. The interaction of incubation time with TS concentration at fixed C:N ratio of 20 exhibits that maximum RL yield of 1.59 g/L was observed at TS concentration of 3% and incubation time of 7 d. Whereas, the interaction between incubation time and C:N ratio at fixed TS of 2% shows that maximum RL yield of 1.47 g/L was observed at C:N ratio of 20 and incubation time of 7 d.

**Determination of Main Effects on Surface Tension (ST)**

The effectiveness of a biosurfactant is estimated by its ability to lower the ST of the medium. The presence of biosurfactant reduces workload to bring a molecule from the bulk of a medium to its surface, hence the ST of that medium decreases<sup>18</sup>. The mathematical relationship for correlating the ST and the considered process variables is obtained as follows:

$$ST = +41.98125 - 7.92500 TS - 0.48750 C:N + 1.84375 TS^2 + 0.010938 C:N^2 \dots (8)$$

It has been concluded from Eq. 8 and Table 5 that no factor interactions were observed between TS, C:N and Time. The estimated regression coefficients and ANOVA for surface tension are shown in Table 5. As per ANOVA, the predicted R<sup>2</sup> of 0.9634 is in reasonable agreement with the adjusted R<sup>2</sup> of 0.9786.

Based on Fig. 4a, the ST was affected by all the considered factors. The minimum surface

Table 5—The analysis of variance for ST

Source	SS	DOF	MS	F value	P > F	Remarks
Model	40.60	4	10.15	218.49	< 0.0001	significant
A	3.03	1	3.03	65.11	< 0.0001	significant
B	2.50	1	2.50	53.81	< 0.0001	significant
A <sup>2</sup>	10.88	1	10.88	234.15	< 0.0001	significant
B <sup>2</sup>	3.83	1	3.83	82.40	< 0.0001	significant

R<sup>2</sup> = 0.9831  
 Adjusted R<sup>2</sup> = 0.9786  
 Predicted R<sup>2</sup> = 0.9634

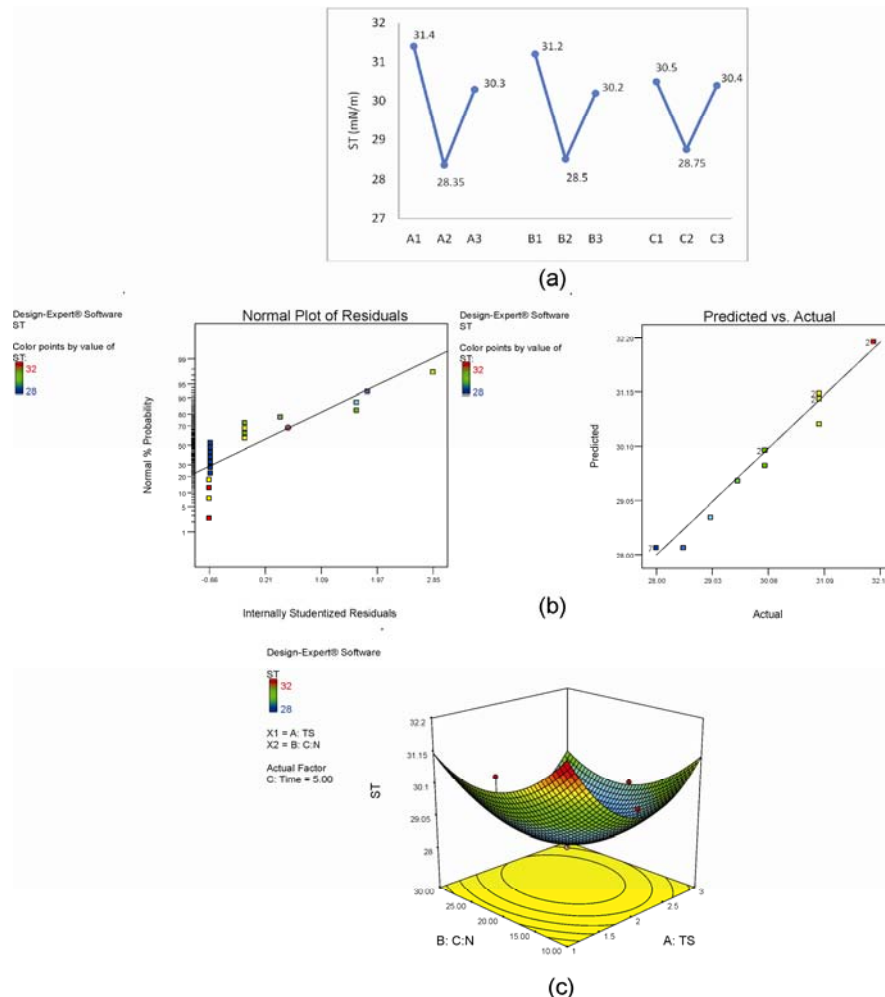


Fig. 4 (a-c)—Main effect of residuals and interaction plots for ST: (a) Process parameters effect, (b) Residuals plots, & (c) Interaction plot between C:N×TS.



tension of ~28.5 mN/m was observed at central points of the parameters continuum, *i.e.*, TS = 2%, C:N = 20 and Time= 5 d, which coincides the maximum RL yield conditions as described above. The residuals plots in Fig. 4b also satisfy the developed model. It was observed that errors are normally distributed that fall on a straight line. Fig. 4c shows interaction of C:N ratio with TS at fixed incubation time of 5 d. Here, the minimum ST of 28 mN/m was attained at 2%TS and C:N ratio of 20.

**Determination of Main Effects on  $Y_{P/S}$**

The mathematical relationship for correlating the  $Y_{P/S}$  and the considered process variables is obtained as follows:

$$Y_{P/S} = +1.50250 + 9.53187 \text{ TS} - 0.98644 \text{ C:N} - 0.37250 \text{ Time} + 0.77256 \text{ TS} * \text{C:N} - 0.47188 \text{ TS} * \text{Time} + 0.060938 \text{ C:N} * \text{Time} - 5.60438 \text{ TS}^2 + 0.021025 \text{ C:N}^2 - 0.060688 \text{ TS} * \text{C:N} * \text{Time} + 0.092125 \text{ TS}^2 * \text{C:N} + 0.44438 \text{ TS}^2 * \text{Time} - 0.017862 \text{ TS} * \text{C:N}^2 \dots (9)$$

It has been concluded from Eq. 9 that there are three factor interactions, *i.e.*, between TS and C:N, TS and Time, and C:N and Time. The quadratic functions of TS, C:N and Time have significant effects on  $Y_{P/S}$  and can be used to predict  $Y_{P/S}$  within limits of control factors.

Based on Fig. 5a, the  $Y_{P/S}$  was affected by all the factors of TS concentration, C:N ratio and time of incubation. Again, maximum  $Y_{P/S}$  was observed at central levels of considered factors, *i.e.*, the optimum set for maximum  $Y_{P/S}$  was observed to be TS = 2%, C:N = 20 and Time = 5 d.

**Determination of Interaction Effects on  $Y_{P/S}$**

The P value of TS×C:N, TS×Time and C:N×Time interactions are <0.0001, 0.0081 and <0.0001, respectively. The interaction of C:N ratio with TS concentration at fixed incubation time of 5 d shows that maximum  $Y_{P/S}$  yield of 8.96 g/g was observed at C:N ratio of 27 and TS concentration of 3%. The interaction of incubation time with TS concentration at fixed C:N ratio of 20 exhibits that maximum  $Y_{P/S}$  yield of 9.21 g/g was observed at TS concentration of 3% and incubation time of 3 d. Whereas, the interaction between incubation time and C:N ratio at fixed TS of 2% shows that maximum  $Y_{P/S}$  yield of 8.68 g/g was observed at C:N ratio of 30 and incubation time of 3 d.

**Determination of Main Effects on  $Y_{P/X}$**

The mathematical relationship for correlating the  $Y_{P/X}$  and the considered process variables is obtained as follows:

$$Y_{P/X} = +4.53809 - 2.38989 \text{ TS} - 0.22436 \text{ C:N} - 0.33477 \text{ Time} + 0.13613 \text{ TS} * \text{C:N} + 0.22625 \text{ TS} * \text{Time} + 0.013000 \text{ C:N} * \text{Time} + 0.023409 \text{ TS}^2 + 3.30909 \text{ E} - 003 \text{ C:N}^2 - 4.77273 \text{ E} - 003 \text{ Time}^2 - 7.87500 \text{ E} - 003 \text{ TS} * \text{C:N} * \text{Time} + 1.00000 \text{ E} - 003 \text{ TS}^2 * \text{C:N} + 7.50000 \text{ E} - 003 \text{ TS}^2 * \text{Time} - 2.07500 \text{ E} - 003 \text{ TS} * \text{C:N}^2 \dots (10)$$

It has been concluded from Eq. 10 that there are three factor interactions, *i.e.*, between TS and C:N, TS and Time, and C:N and Time. The quadratic functions of TS, C:N and Time have significant

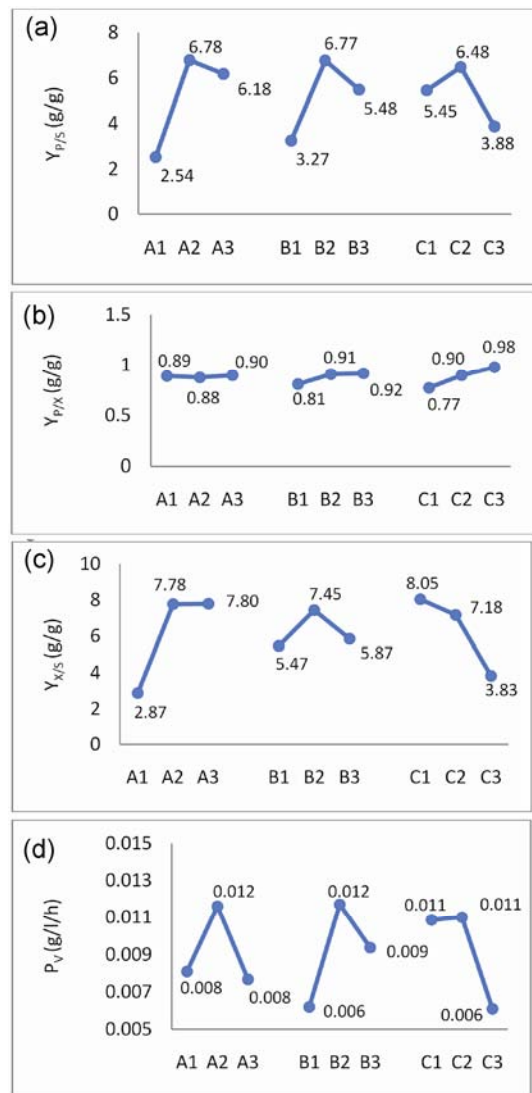


Fig. 5 (a-d)—Main process parameters on: (a)  $Y_{P/S}$ , (b),  $Y_{P/X}$ , (c)  $Y_{X/S}$  and (d)  $P_V$ .

effects on  $Y_{P/X}$  and can be used to predict  $Y_{P/S}$  within limits of control factors.

Based on Fig. 5b, the  $Y_{P/X}$  was slightly affected by C:N ratio and time of incubation, with maximum  $Y_{P/X}$  was observed at central levels of respective factors, *i.e.*, the optimum set for maximum  $Y_{P/S}$  was observed to be C:N = 30 and Time = 7 d.

#### Determination of Interaction Effects on $Y_{P/X}$

The P value of TS×C:N, TS×Time and C:N×Time interactions are <0.0001 each. The interaction of C:N ratio with TS concentration at fixed incubation time of 5 d shows that maximum  $Y_{P/X}$  yield of 1.19 g/g was observed at C:N ratio of 25 and TS concentration of 3%. The interaction of incubation time with TS concentration at fixed C:N ratio of 20 exhibits that maximum  $Y_{P/X}$  yield of 1.43 g/g was observed at TS concentration of 3% and incubation time of 7 d. Whereas, the interaction between incubation time and C:N ratio at fixed TS of 2% shows that maximum  $Y_{P/X}$  yield of 0.94 g/g was observed at C:N ratio of 25 and incubation time of 7 d.

#### Determination of Main Effects on $Y_{X/S}$

The mathematical relationship for correlating the  $Y_{X/S}$  and the considered process variables is obtained as follows:

$$Y_{X/S} = -7.43395 + 15.32381 \text{ TS} + 0.31192 \text{ C:N} - 1.29539 \text{ Time} - 0.66812 \text{ TS*Time} - 2.37955 \text{ TS}^2 - 7.29545 \text{ E-003 C:N}^2 + 0.15761 \text{ Time}^2 \quad \dots (11)$$

It has been concluded from Eq. 11 that there is one factor interaction between TS and Time.

Based on Fig. 5c, the  $Y_{X/S}$  was affected by all the factors of TS concentration, C:N ratio and time of incubation. The minimum  $Y_{X/S}$  was observed at central levels of considered factors, *i.e.*, the optimum set for maximum  $Y_{P/S}$  was observed at TS = 3%, C:N = 10 and Time = 7 d.

#### Determination of Interaction Effects on $Y_{X/S}$

The P value of TS×T interaction is <0.0001. The interaction of C:N ratio with TS concentration at fixed incubation time of 5 d shows that minimum  $Y_{X/S}$  yield of 2.02 g/g was observed at C:N ratio of 10 and TS concentration of 1%. The interaction of incubation time with TS concentration at fixed C:N ratio of 20 exhibits that minimum  $Y_{X/S}$  yield of 4.36 g/g was observed at TS concentration of 1% and incubation time of 3 d.

#### Determination of Main Effects on $P_V$

The mathematical relationship for correlating the  $P_V$  and the considered process variables is obtained as follows:

$$P_V = +0.013598 + 0.014603 \text{ TS} - 1.38841 \text{ E-003 C:N} - 2.06818 \text{ E-003 Time} + 1.32000 \text{ E-003 TS*C:N} - 2.06250 \text{ E-003 TS*Time} + 6.75000 \text{ E-005 C:N*Time} - 9.41023 \text{ E-003 TS}^2 + 3.02727 \text{ E-005 C:N}^2 + 1.56818 \text{ E-004 Time}^2 - 7.25000 \text{ E-005 TS*C:N*Time} + 9.25000 \text{ E-005 TS}^2 * \text{C:N} + 1.03750 \text{ E-003 TS}^2 * \text{Time} - 2.95000 \text{ E-005 TS}^2 * \text{C:N}^2 \quad \dots (12)$$

It has been concluded from Eq. 12 that there are three factor interactions, *i.e.*, between TS and C:N, TS and Time, and C:N and Time. The quadratic functions of TS, C:N and Time have significant effects on  $P_V$  and can be used to predict  $P_V$  within limits of control factors.

Based on Fig. 5d, the  $P_V$  was affected by all the factors of TS concentration, C:N ratio and time of incubation. Here, the maximum  $P_V$  was observed at central levels of considered factors, *i.e.*, the optimum set for maximum  $P_V$  was observed to be TS = 3%, C:N = 30 and Time = 7 d.

#### Determination of Interaction Effects on $P_V$

The P value of TS×C:N, TS×T and C:N×T interactions are <0.0001 each. The interaction of C:N ratio with TS concentration at fixed incubation time of 5 d shows that maximum  $P_V$  of 0.0093 g/L/h was observed at C:N ratio of 30 and TS concentration of 3%. The interaction of incubation time with TS concentration at fixed C:N ratio of 20 exhibits that maximum  $P_V$  of 0.0118 g/L/h was observed at TS concentration of 3% and incubation time of 7 d. Whereas, the interaction between incubation time and C:N ratio at fixed TS of 2% shows that the highest  $P_V$  of 0.0163 g/L/h was observed at C:N ratio of 30 and incubation time of 3 d.

#### Modeling for All Responses through Desirability

In the present study, Design Expert has been used to optimize the response variables. The objective was to maximize RL yield,  $Y_{P/S}$ ,  $Y_{P/X}$  and  $P_V$  and minimize UTS, DCBM, ST and  $Y_{X/S}$ . This is obtained by applying the multi-objective optimization technique. Hence, the lower limit for the RL yield is set at 0.08 g/L with a target of 1.46 g/L,  $Y_{P/S}$  with a target of 10.89 g/g,  $Y_{P/X}$  with a target of 1.23 g/g and  $P_V$  with a target of 0.1067 g/L/h. Whereas, the upper limit for the UTS is set at 47% with a target of 8%, DCBM

with a target of 0.65 g/L, surface tension with a target of 28 mN/m and  $Y_{X/S}$  with a target of 2.07 g/g. The ranges and targets of input parameters, *viz.*, ST concentration, C:N ratio and incubation time are given in Table 6. The values of upper wt and lower wt are identical since all the process and kinetic parameters are equally important in this study. The objective was to choose an optimal setting to maximize the desirability function, whereas the objective of optimization was to determine the optimum conditions. The RSM model has been used to predict the 15 optimal desirability solutions for the considered response parameters, as shown in Table 7.

Fig. 6a shows all of these 15 responses corresponding to those input parametric combinations, and Fig. 6b and 6c show the multi objective optimization through bar graph and three dimensional surface desirability plot, respectively. The prediction error has been defined as a follows.

Prediction error (%) =

$$\left[ \text{Experimental results} - \frac{\text{Predicted results}}{\text{Experimental results}} \right] \times 100$$

The main effect plot for predicted and experimental values is shown in Fig. 7. It has been determined that the percentage of prediction errors is much less and

Table 6—Desirability analysis for all the considered responses

Parameters	Target	Lower limit	Upper limit	Lower weight	Upper weight	Importance
TS		1	3	1	1	3
C:N		10	30	1	1	3
Time		3	7	1	1	3
UTS	minimize	8	47	1	1	3
DCBM	minimize	0.65	1.63	1	1	3
RL	maximize	0.08	1.46	1	1	3
ST	minimize	28	32	1	1	3
$Y_{P/S}$	maximize	1	10.89	1	1	3
$Y_{P/X}$	maximize	0.09	1.23	1	1	3
$Y_{X/S}$	minimize	2.07	11.25	1	1	3
$P_V$	maximize	0.0011	0.0167	1	1	3

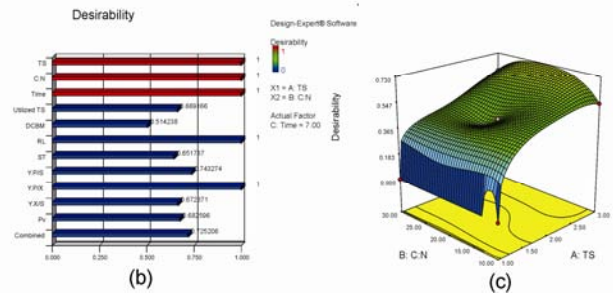
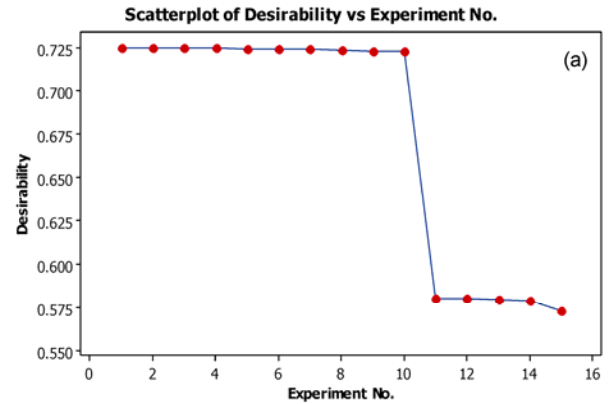


Fig. 6 (a-c)—Prediction of process characteristic by desirability plots.

Table 7—Optimal solution for all the considered responses

Run	Factors			Response variables								
	UTS (% w/v)	C:N ratio	Time (d)	Utilized TS (% w/v)	DCBM (g/L)	RL (g/L)	ST (mN/m)	$Y_{P/S}$ (g/g)	$Y_{P/X}$ (g/g)	$Y_{X/S}$ (g/g)	$P_V$ (g/L/h)	Desirability
1	3.00	20.77	7.00	20.9025	1.1260	1.5869	29.3931	8.3510	1.4318	5.0776	0.0117	0.72521
2	3.00	20.84	7.00	20.9045	1.1260	1.5864	29.3906	8.3477	1.4316	5.0783	0.0117	0.72520
3	3.00	20.48	7.00	20.8933	1.1261	1.5879	29.4034	8.3598	1.4322	5.0746	0.0118	0.72514
4	3.00	21.08	7.00	20.9052	1.1266	1.5847	29.3802	8.3328	1.4301	5.0838	0.0117	0.72498
5	3.00	20.22	7.00	20.8766	1.1272	1.5883	29.4097	8.3597	1.4314	5.0763	0.0118	0.72475
6	3.00	20.38	7.00	20.8798	1.1282	1.5884	29.3999	8.3562	1.4309	5.0820	0.0118	0.72473
7	3.00	19.76	7.00	20.8600	1.1244	1.5864	29.4374	8.3583	1.4313	5.0614	0.0118	0.72447
8	2.99	19.82	7.00	20.8344	1.1313	1.5877	29.4110	8.3442	1.4274	5.0897	0.0117	0.72351
9	3.00	21.65	6.82	20.5767	1.1274	1.5732	29.3722	8.3661	1.4060	5.2866	0.0116	0.72300
10	3.00	22.57	7.00	20.9049	1.1168	1.5576	29.3687	8.1733	1.4178	5.0702	0.0114	0.72294
11	1.50	30.00	3.00	17.8862	1.3194	1.0636	29.4541	6.4543	0.7735	7.5329	0.0150	0.57985
12	1.52	29.96	3.00	17.7198	1.3265	1.0687	29.4144	6.5303	0.7740	7.6245	0.0150	0.57967
13	1.50	29.81	3.00	17.9887	1.3241	1.0657	29.4343	6.4401	0.7739	7.5271	0.0150	0.57920
14	1.56	30.00	3.07	17.5311	1.3426	1.0863	29.3262	6.6711	0.7771	7.7762	0.0150	0.57867
15	1.38	13.98	3.00	19.6088	1.2225	0.9392	29.8796	5.3510	0.9098	6.8796	0.0135	0.57271

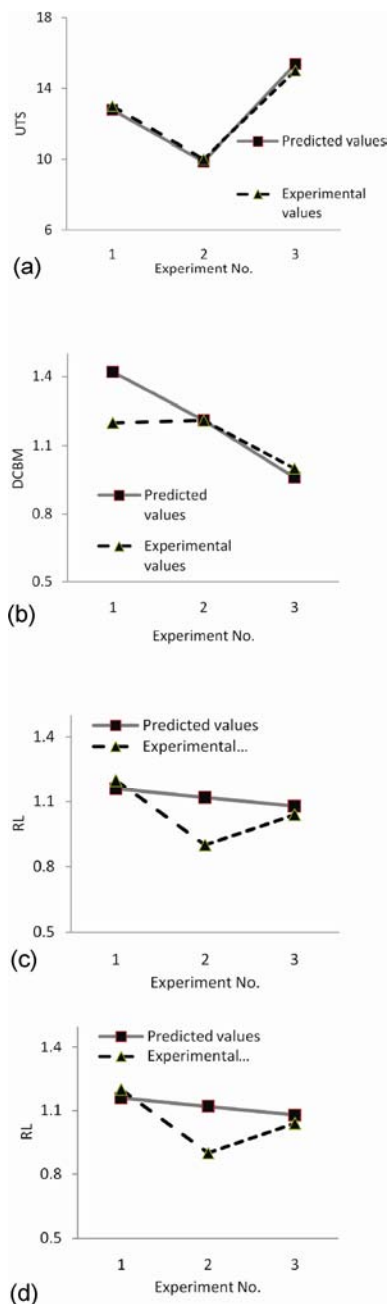


Fig. 7 (a-d)—Plots for predicted and experimental values: (a) UTS, (b) DCBM, (c) RL, & (d) ST.

hence the prediction performance of the models is quite satisfactory.

### Conclusion

In the present study, some statistical models have been proposed for different process and kinetic parameters to correlate the dominant fermentation process parameters like total sugar concentration, C:N ratio and incubation time. The analytical evaluation in

the biosurfactant production process has been done according to the developed mathematical models to obtain the conclusion that an efficient biosurfactant production could have been done on blackstrap molasses by a *P. aeruginosa* strain.

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