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Enhancement and statistical optimization (Plackett-Burman and response surface methodology) of biosurfactant production by marine bacteria *Bacillus tequilensis* KM15 using Fish processing wastes

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Abstract---Biosurfactant (BS) production by *Bacillus tequilensis* KM15 is investigated using fish processing wastes as medium components. Bocha (B) and Rohu (RR) fish scales are selected as cost-effective substrates for BS production and optimized together with pH, the incubation period (IP), and inoculum size (IS) using the response surface method (RSM). RSM model has demonstrated the following optimum conditions for BS production: Bocha (B) and Rohu (RR) fish scales 4% each, and inoculum size 2%, at pH 8.0, 37 ℃ temperature

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in 48 h. A 2.8-fold increase in biosurfactant production was obtained (from 340 mg L^{-1} in basal medium to 944 mg L^{-1}) using the response surface methodology. The major portion of the production cost can be reduced by using the currently studied substrates.

Keywords---biosurfactant, response surface methodology, fish scales, production.

Introduction

Biosurfactants (BS) are surface-active secondary metabolites with an expansive range of applications in agriculture, industry, and medicinal goods (Muthusamy et al., 2008; Das and Chandran, 2011; Obayori et al., 2009; Kumar et al., 2015). BS has higher efficiency and stability than chemical surfactants, as well as lower toxicity and greater compatibility in extreme conditions, and can be produced from renewable raw materials (Romero et al., 2007). BS are the green surfactants that are produced by bacteria, yeasts, and fungi. Since they are less harmful and environmentally friendly, many forms of BS have the potential to be commercially manufactured for a range of applications in the pharmaceutical, cosmetics, petroleum, food, and agriculture sector (Zouari et al., 2014; Wang et al., 2015).

BS, despite having a wide range of applications pose a serious problem in meeting the demand to supply, with high production costs and a tedious purification process. Nowadays, to reduce production costs, more emphasis is being placed on the production of high-yielding strains, the use of low-cost medium ingredients, improved purification methods, and cost-effective engineering processes (Al-Bahry et al., 2013). The BS development by *Corynebacterium aquaticum* in submerged fermentation using fish residues and sugarcane bagasse was documented by Paola et al (2018). SOC was used as a carbon and nitrogen source during processing, according to Narender Kumar et al. (2017). Since the cost of the growth medium, which is mainly added by carbon and nitrogen sources, is estimated to account for 30–40% of the overall production cost when microbial products are produced for industrial purposes, the composition of the medium is one of the key parameters (Gnaneshwar et al, 2013; Kirk et al., 2002). Complex carbohydrates and crude proteins make up the majority of agro-industrial waste, both of which can provide nutrients for microbial development.

The fish processing industry generates solid biological waste (head, trimmings, skin, viscera, scale, bone, etc) based on the processing methods, which poses a serious effect on the environment. Fish waste is valorized by various treatment methods which are costly and generate effluents with a high organic load. As a consequence, designing potential value-added processes for these wastes is very appealing, as it becomes a waste management tool that is pleasant to the community. This study aims to see the fish wastes (scales) can be used as substrates for *Bacillus tequilensis* KM15 in submerged fermentation to produce BS, as well as to optimize key variables. Following an initial screening of various substrates to assess their effect on BS production, a select few preferred substrates were statistically screened with Plackett-Burman design and optimized by RSM to maximize BS production.

Materials and Methods

Microorganism and cultural conditions

In the present study, the *Bacillus tequilensis* KM15 strain developed in our lab was used. Inoculum of about 10^8 cells per mL was obtained by growing the culture in mineral salts medium (MSM) [$NaNO_3$ (0.5), $MgSO_4$ (0.5), KCl (0.1), $FeSO_4$ (0.01), K_2HPO_4 (0.5), KH_2PO_4 (0.5) g/L], pH 7 and temperature of 37 °C for 18 h at 200 rpm.

Biosurfactant production and extraction

Bacillus tequilensis KM15 was grown in 100 mL MSM in a 250 mL Erlenmeyer flask for 48 hours at 200 rpm, then centrifuged for 15 minutes at 10,000 rpm. The supernatant was precipitated by changing the pH to 2.0 with 6 N HCl (Dubey and Juwarkar, 2001) and deposited overnight at 4 ºC. The precipitate was obtained by centrifugation at 12,000 rpm for 20 minutes, followed by methanol extraction twice(Kim et al., 2004).

Substrates

In the present study, scales of the following fish species were tested for their potency as carbon and nitrogen sources.: Grass carp (GC), Kalbasu (KB), Roopchand (RC), Rohu/ravata (RR), Bocha (B), Murrel (M), Tilapia/Cichlid (T), Common carp (CC), Reba (R), Catla/ Indian carp (IC), Mrigal carp (MC), Tor tor (TT), Hilsa (H), Peddaperka (PP) and Pool barb (PB). All of these materials were gathered from local market yards and farms, washed, dried (50–60°C), and stored in a moisture-free container at room temperature. All the above varieties of fish scales were used as medium ingredients, directly without any pretreatment in fermentation media.

Screening by One substrate – One-time approach

Initially, 15 different fish waste (scales) of low or no value (Table1) were tested as substrates for BS production. In fermentation medium (MSM broth), these substrates were directly used at 1% level, inoculated with 1% of 18 h culture (10^8 cells mL^{-1}) at 37 °C, 7.0, and 200 rpm for 48 h.

Screening of most suitable substrates by Plackett-Burman method

Based on preliminary BS production results by *Bacillus tequilensis* KM15, 10 different substrates (Table-2) were chosen and further analyzed using the Plackett-Burman design to identify the most variables influencing BS production. A set of 16 experiments were conducted in 100mL sterile medium (MSM broth) in 250mL Erlenmeyer flasks with different concentrations of substrates as per the design (Table 2), inoculated with 1 % of 18 h culture (1 x 10^8 CFU mL-1) and incubated for 48 h at 200 rpm and 37 °C for screening the best substrate for BS production. In the results, the effects of variables on BS were investigated. Each row represents an experiment, while each column represents an independent variable in the experimental design. (Table 2). By comparing the average of

measurements taken at each factor's high (+1) and low level (-1) settings, the main effect was calculated. Plackett-experimental Burman's design was based on the first-order model experiments(Plackett and Burmann, 1946):

$$Y = \beta o + \sum \beta i Xi \qquad (1)$$

Where Y represents the response (BS productivity), βo represents the model intercept, and βi represents the variable estimates. The findings were analyzed using MINITAB-15 statistical tools and a Plackett–Burman architecture matrix (Table 2).

Optimization by response surface method

RSM was used to optimize substrate concentrations, pH, inoculum size, and incubation time for optimal BS production, after defining critical factors affecting BS production through PB design and other preliminary experiments. For optimization, five variables were chosen: Bocha (B), Rohu (RR), pH, inoculum size, and incubation duration. The central composite rotatable system (CCRD) (Montgomery, 1997)was used in a 32-experiment design with six replicates at the central stage (Table 8) with five coded levels (-2, -1, 0, +1, +2). The real experimental values of the variables were coded using the following equation(Neter et al., 1996)

$$x_i = (Xi - X0)/\Delta X \qquad (2)$$

Where xi is the variable's dimensionless coded level, Xi is the variable's actual value, X0 is the variable's average of maximum and minimum level values, and X is the variable's maximum value minus minimum value.

A second-order polynomial equation was used to measure BS production (mg L-1), and the data were fitted into the equation using a multiple regression process. The individual and interactive effects of test variables on the response are depicted in the 3-dimensional graphical representation of the model equation. The optimum levels of variables for maximum BS production were determined by solving the regression equation and analyzing response surface plots. The second-order polynomial equation defines the expected response (Y) in terms of the independent variables (X1, X2, X3, X4, and X5):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_{11} X^2_1 + \beta_{22} X^2_2 + \beta_{33} X^2_3 + \beta_{44} X^2_4 + \beta_{55} X^2_5 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{15} X_1 X_5 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{25} X_2 X_5 + \beta_{34} X_3 X_4 + \beta_{35} X_3 X_5 + \beta_{45} X_4 X_5 \qquad (3)$$

where β_0 is intercept term, β_1, β_2, β_3, β_4, β_5 linear coefficients, β_{11}, β_{22}, β_{33}, β_{44} quadratic coefficients and β_{12}, β_{13}, . . ., β_{45} interactive coefficient estimates. By running an experiment with the optimum values for variables determined by response optimization for validation of the predicted value, the optimum levels of variables (within the experimental range) for maximum BS yield were estimated, and the BS production was measured.

Reproducibility of results

The findings were based on the average of three different experiments that obtained consistent results, and all of the experiments were conducted in triplicate.

Results and Discussion

Screening by One substrate – One-time approach

BS production using various agro-wastes as substrates ranged from 104 to 326 mg/L after 48 hours of incubation (Table 1). For the second level of statistical screening, the top ten substrates (B, RC, KB, MC, M, IC, TT, GC, CC, and RR) with the highest BS output were chosen.

Table 1
Screening of fish wastes (scales) as substrates for BS production by *Bacillus tequilensis*KM15

Fish variety (Common name & Abbreviation used)	Scientific name	Biosurfactant production mg L^{-1}
Grass carp(GC)	Ctenopharyngodonidella	169 ±1.07
Kalbasu(KB)	Labeocalbasu	250 ± 1.09
Roopchand(RC)	Piaractusbrachypomus	317 ± 1.07
Mrigal carp(MC)	Cirrhinusmrigala	326 ± 1.04
Bocha(B)	Labeocatla	247 ± 0.99
Murrel(M)	Channastraitus	237 ± 1.1
Tilapia/Cichlid fish(T)	Oreochromis niloticus	121 ± 0.96
Common carp(CC)	Cyprinus carpio	145 ± 0.95
Reba(R)	Cirrhinusreba	119 ± 0.98
Catla/Indian carp (IC)	Catlacatla	137 ± 1.02
Rohu/Ravata(RR)	Labeorohita	222 ± 1.06
Tor Tor (TT)	Mahseer	197 ± 1.01
Hilsa(H)	Ilish shad	104 ± 1.01
Pedda parka(PP)	Osteochilichthysbrevidorsalis	134 ± 0.92
Pool barb(PB)	Puntius sophore	106 ± 1.03

Screening of most suitable substrates by Plackett-Burman method

After initial screening, 10 different agro-wastes were assessed as substrates (Table 2) for BS production using the Plackett-Burman statistical design. MINITAB-15 statistical software was used to measure the P & F values, and regression coefficient of the variables for BS production. The input data is analyzed, and the factors are ranked by magnitude of influence.

Table 2
The Plackett–Burman experimental design matrix: Screening agro-industrial wastes as substrates for BS production by *Bacillus tequilensis*KM15.

Run	B	RC	KB	MC	M	IC	TTGC	CC	RR	BS (mg L^{-1})
1	+	-	-	-	+	-	-	+	+	-
541										
2	+	+	-	-	-	+	-	-	+	+
658										
3	+	+	+	-	-	-	+	-	-	+
657										
4	+	+	+	+	-	-	-	+	-	-
644										
5	-	+	+	+	+	-	-	-	+	-
650										
6	+	-	+	+	+	+	-	-	-	+
705										
7	-	+	-	+	+	+	+	-	-	-
577										
8	+	-	+	-	+	+	+	+	-	-
546										
9	+	+	-	+	-	+	+	+	+	-
620										
10	-	+	+	-	+	-	+	+	+	+
626										
11	-	-	+	+	-	+	-	+	+	+
590										
12	+	-	-	+	+	-	+	-	+	+
630										
13	-	+	-	-	+	+	-	+	-	+
643										
14	-	-	+	-	-	+	+	-	+	-
496										
15	-	-	-	+	-	-	+	+	-	+
539										
16	-	-	-	-	-	-	-	-	-	-
442										

Number of runs - 16, variables - 10 and center points - 0

The varying influence of variables on BS production by *Bacillus tequilensis* KM15 has shown varied BS production from 442 mg/L to 705 mg/L in the 16 PB design experiments (Table-2). The regression coefficients of the ten variables revealed that B, RC, KB, MC, M, IC, CC, and RR had positive effects on BS output in the tested concentration range, while TT and GC had negative effects. Seven of the ten agro-wastes studied had a major effect on BS productivity, with p values less than 0.05, with B, RC, and RR being highly significant, KB, MC, M being significant, and TT being nearly significant (Table 3&4). All the above seven substrates favor impact on BS production, implying that they could be increased further to achieve higher output. With p values >0.05, the other variables, IC, GC, and CC, were

found to be negligible, implying that increasing their concentrations would have no impact on BS output.

Table 3
Estimated Effects and Coefficients for BS production (coded units): Plackett-Burmann design

Term	Effect	Coefficient	SE Coefficient	t	P	Significance
Constant		797.75	4.310	185.08	0.000	*
B	54.75	27.38	4.310	6.35	0.001	Significant
RC	73.25	36.63	4.310	8.50	0.000	Significant
KB	33.00	16.50	4.310	3.83	0.012	Significant
MC	43.25	21.63	4.310	5.02	0.004	Significant
M	34.00	17.00	4.310	3.94	0.011	Significant
IC	13.25	6.62	4.310	1.54	0.185	*
TT	-22.75	-11.38	4.310	-2.64	0.046	Significant
GC	-8.25	-4.13	4.310	-0.96	0.383	*
CC	7.25	3.62	4.310	0.84	0.439	*
RR	66.50	33.25	4.310	7.71	0.001	Significant

SE, Standard error; t, Student t value; p, Probability, *Insignificant.

Table 4
Analysis of Variance for BS production (coded units): Plackett-Burmann design

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	10	70859	70859	7085.9	23.84	0.001
Residual Error	5	1486	1486	297.2		
Total	15	72345				

DF, degrees of freedom; Seq.SS, Sum of squares; Adj. SS, the adjusted sum of squares; Adj. MS, the adjusted sum of squares; F, Variance ratio; p, Probability.

Optimization of various fermentation parameters by response surface method

RSM was used to increase BS output in *Bacillus tequilensis* KM15 submerged fermentation using B and RR as substrates. The effects of five variables, including B in combination with RR as a carbon and nitrogen source, pH, incubation time

(IP), and inoculum size (IS) on BS production were investigated at five levels using CCRD. Table 5 summarises the findings of 32 trials performed to decide the best settings for maximum BS production. The run with the highest BS output was run-16. Multiple regression analysis was used to describe the concurrent effect of variables and to adapt the response function to the experimental results, and from regression analysis, the following polynomial equation was derived:

$$Y=1893.38 + 286.85 X_1 + 405.76 X_2 + 131.40 X_3 - 46.10 X_4 + 280.43 X_5 + 41.58 X_1^2 + 19.53 X_2^2 + 88.24 X_3^2 + 11.78 X_4^2 + 12.58 X_5^2 - 20.40 X_1 X_2 + 5.44 X_1 X_3 + 46.06 X_1X_4 + 3.40 X_1X_5 + 46.69 X_2X_3 + 16.40 X_2X_4 + 180.40 X_2X_5 + 5.65 X_3X_4 + 64.81 X_3X_5 - 6.90 X_4X_5 \quad (4)$$

where Y was the yield of BS (predicted response), and X1, X2, X3, X4, and X5 were the coded values of the test variables B, RR, pH, IS and IP respectively.

Table 5
CCRD for optimization of five variables for BS production by Bacillus tequilensisKM15

Run	B	RR	pH	IS	IP	BS production (U/mL)	
						Exp.Value	Pred.Value
1	-1(2%)	-1(2%)	-1(6.0)	-1(1%)	+1(48h	367	380
2	+1(4%)	-1(2%)	-1(6.0)	-1(1%)	-1(24h)	486	456
3	-1(2%)	+1(4%)	-1(6.0)	-1(1%)	-1(24h)	486	456
4	+1(4%)	+1(4%)	-1(6.0)	-1(1%)	+1(48h)	797	785
5	-1(2%)	-1(2%)	+1(8.0)	-1(1%)	-1(24h)	367	364
6	+1(4%)	-1(2%)	+1(8.0)	-1(1%)	+1(48h)	578	597
7	-1(2%)	+1(4%)	+1(8.0)	-1(1%)	+1(48h)	818	792
8	+1(4%)	+1(4%)	+1(8.0)	-1(1%)	-1(24h)	650	629
9	-1(2%)	-1(2%)	-1(6.0)	+1(2%)	-1(24h)	309	302
10	+1(4%)	-1(2%)	-1(6.0)	+1(2%)	+1(48h)	485	502
11	-1(2%)	+1(4%)	-1(6.0)	+1(2%)	+1(48h)	646	622
12	+1(4%)	+1(4%)	-1(6.0)	+1(2%)	-1(24h)	603	578
13	-1(2%)	-1(2%)	+1(8.0)	+1(2%)	+1(48h)	386	395

14	+1(4%)	-1(2%)	+1(8.0)	+1(2%)	-1(24h)	501
502						
15	-1(2%)	+1(4%)	+1(8.0)	+1(2%)	-1(24h)	510
478						
16	+1(4%)	+1(4%)	+1(8.0)	+1(2%)	+1(48h)	944
922						
17	-2(1%)	0(3%)	0(7.0)	0(1.5%)	0(36h)	419
395						
18	+2(5%)	0(3%)	0(7.0)	0(1.5%)	0(36h)	800
700						
19	0(3%)	-2(1%)	0(7.0)	0(1.5%)	0(36h)	399
308						
20	0(3%)	+2(5%)	0(7.0)	0(1.5%)	0(36h)	771
740						
21	0(3%)	0(3%)	-2(5.0)	0(1.5%)	0(36h)	585
528						
22	0(3%)	0(3%)	+2(9.0)	0(1.5%)	0(36h)	738
667						
23	0(3%)	0(3%)	0(7.0)	-2(0.5%)	0(36h)	612
541						
24	0(3%)	0(3%)	0(7.0)	+2(2.5%)	0(36h)	541
491						
25	0(3%)	0(3%)	0(7.0)	0(1.5%)	-2(12h)	385
368						
26	0(3%)	0(3%)	0(7.0)	0(1.5%)	+2(60h)	769
666						
27	0(3%)	0(3%)	0(7.0)	0(1.5%)	0(36h)	515
503						
28	0(3%)	0(3%)	0(7.0)	0(1.5%)	0(36h)	522
503						
29	0(3%)	0(3%)	0(7.0)	0(1.5%)	0(36h)	515
	503					
30	0(3%)	0(3%)	0(7.0)	0(1.5%)	0(36h)	514
	503					
31	0(3%)	0(3%)	0(7.0)	0(1.5%)	0(36h)	518
	503					
32	0(3%)	0(3%)	0(7.0)	0(1.5%)	0(36h)	511
	503					

Table 6 shows the importance of each coefficient as calculated by the p-values and Student's t-test. A greater t-value and a smaller p-value indicate a higher degree of significance for the related coefficient. The constants, X1, X2, X3, X5, X32, and X2X5 were found to be important (P 0.05) based on the p-value of each model expression. Positive coefficients of X1, X2, X3, X5, X12, X22, X32, X42, X52, X1X3, X1X4, X1X5, X2X3, X2X4, X2X5, X3X4, and X3X5 had a direct effect on the response Y, while negative coefficients suggested an inverse effect on BS production. To perform ANOVA for BS production, MINITAB-15 was used. The ANOVA results of the second-order response surface model fitting are shown in Table 7. According to ANOVA, the regression and linear effects are very significant for BS production (Table-7). The model equation's fit was evaluated using the

regression-based determination coefficient R^2. The model will better explain the difference between experimental and expected values if the R^2 values are close to 1 (Dhanya, et al., 2008). The model, which had a high determination coefficient (R^2 = 0.96) and explained 96 percent of the variability in the answer, could only explain 4% of the total variations. The adjusted determination coefficient was also very high (Adj R^2 = 0.886), suggesting that the observed and expected responses were well-matched. According to the current analytical results, the response equation provided a suitable model for process optimization of BS production.

Table 6
Coefficients and t-values for BS production by Bacillus tequilensis KM15 using central composite rotatable design (CCRD)

Variable	Coefficients	Standard error coefficient	t-Value	Probability
Constant	1893.38	74.93	25.269	0.000
B	286.85	38.35	7.480	0.000
RR	405.76	38.35	10.581	0.000
pH	131.40	38.35	3.427	0.006
IS	-46.10	38.35	-1.202	0.255
IP	280.43	38.35	7.313	0.000
B * B	41.58	34.69	1.199	0.256
RR *RR	19.53	34.69	0.563	0.585
pH * Ph	88.24	34.69	2.544	0.027
IS*IS	11.78	34.69	0.340	0.740
IP*IP	12.58	34.69	0.363	0.724
B * RR	-20.40	46.97	-0.434	0.672
B * pH	5.44	46.97	0.116	0.910
B *IS	46.06	46.97	0.981	0.348
B *IP	3.40	46.97	0.072	0.944
RR * pH	46.69	46.97	0.994	0.342
RR *IS	16.40	46.97	0.349	0.734

RR *IP		180.40	46.97	3.841
	0.003			
pH *IS		5.65	46.97	0.120
	0.906			
pH *IP		64.81	46.97	1.380
	0.195			
IS*IP		-6.90	46.97	-0.147
	0.886			

S = 187.9 R-Sq = 96.0% R-Sq(adj) = 88.6%

Table 7
Analysis of variance for FFase production by Bacillus tequilensis KM15 using CCRD

Source		DF	Seq SS	Adj SS	Adj MS	F	P
Regression		20	9214460	9214460	460723		
	13.05	0.000					
Linear		5	8279006	8279006	1655801		
	46.92	0.000					
Square		5	265852	265852	53170		
	1.51	0.265					
Interaction		10	669602	669602	66960		
	1.900	0.154					
Residual Error		11	388206	388206	35291		
Lack-of-Fit		6	387374	387374	64562		
	387.81	0.000					
Pure Error		5	832	832	166		
Total		31	9602667				

DF, degrees of freedom; Seq.SS, sum of squares; Adj. SS, the adjusted sum of squares; Adj. MS, the adjusted sum of squares; F, Variance ratio; p, Probability.

A graphic representation of the response surface is used to visualize the effect of B, RR, and pH on BS production, as shown in Fig. 1&2. It was discovered that as the variables were increased, the BS yield increased. At the "+1" stage of B (4%), RR (4%), and pH (4%), a high BS output was observed (8.0). The response surface plot (Fig.3) depicts the interaction of concentration and incubation time, with the form of the response surface suggesting a positive interaction between the two variables.

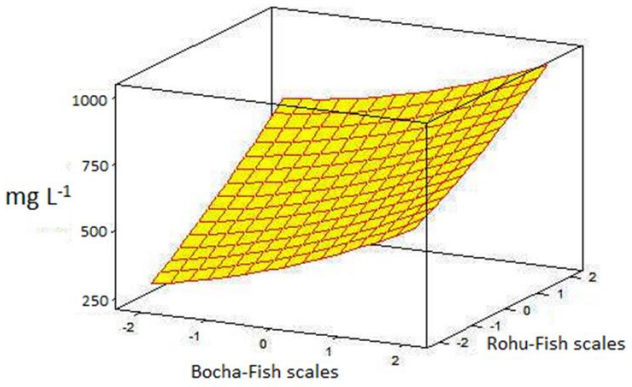

Fig. 1. Response surface plot showing the effect of B and RR concentrations on BS production

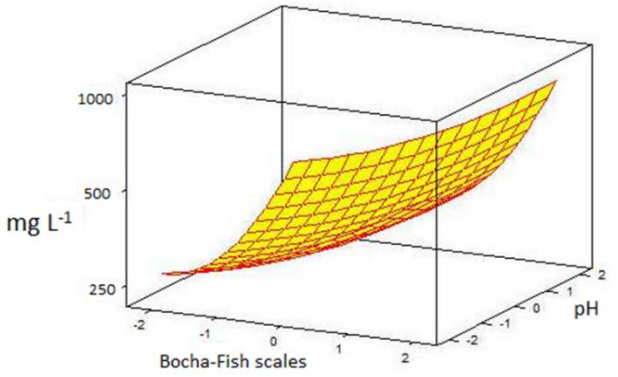

Fig. 2. Response surface plot showing the effect of B and pH on BS production

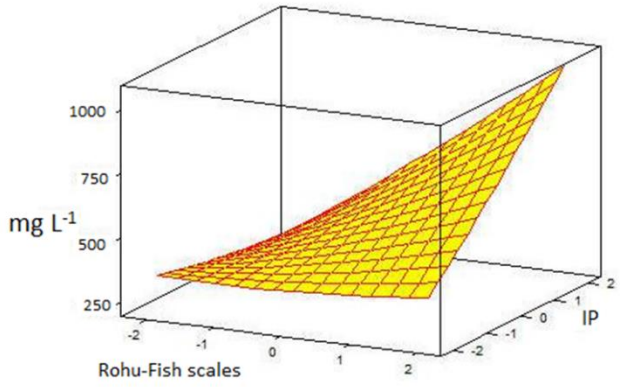

Fig. 3. Response surface plot showing the effect of RR concentration and IP on BS production

In Fig. 4, the response surface depicts the effect of incubation time and inoculum size on BS development. The characteristics and rate of growth of the culture

decide the duration of the incubation period. Increased IP resulted in an improvement in BS production up to 48 hours, but further incubation did not boost BS production. There was a large increase in BS production when the IS was set to 2%; but, as the IS was increased, the BS production steadily decreased. This may be due to bacterial metabolism depleting basic nutrients and accumulating toxic secondary metabolites in the fermentation medium. Bacillus spp. produced the most BS after 48 hours of incubation, according to Narendra Kumar et al. (2017).

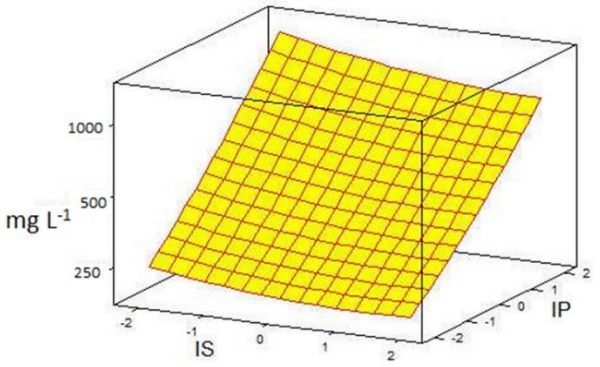

Fig. 4. Response surface plot showing the effect of IS and IP on BS production

Under ideal conditions, the maximum BS yield was 944 mg L-1, according to data. The exact reason for increased BS production by B and RR is unknown; however, free amino acids and soluble protein content released from the substrates during fermentation can play a role. In this analysis, the substrates B and RR were found to be extremely important in the production of BS. Fish waste is a potentially valuable resource from which high-value products can be obtained. As a locally produced waste, fish scales represent a cost-effective and sustainable biomaterial compared to conventional polymer materials. The recycling and reuse of this material can also reduce the need for landfill areas and environmental pollution, reducing the cost of processing fish scale waste. Expanding the application of fish scale waste as a medium ingredient for biosurfactant production is an effective means to achieve significant economic and environmental benefits. As a result, this study aimed to use locally available, low-cost fish wastes as substrates for BS production using *Bacillus tequilensis* KM15 in submerged fermentation, and to optimize various parameters for improved BS production. Therefore the present strain *Bacillus tequilensis*KM15 strain may be used for commercial production because it has the potential to generate large amounts of BS in a limited amount of time without polluting the environment with fish wastes.

Conclusion

In the present research, efforts were made to establish a low-cost medium formulation using agro-wastes as substrates for BS production. Plackett–Burman architecture was used to pick better ingredients for statistical screening, and the optimization of substrate concentrations was done by RSM. The use of fish scales

(Bocha and Rohu) instead of commercial sucrose, peptone, yeast extract, and malt extract resulted in a 2.8-fold increase in BS production (944 mg L-1). This is the first study to look at the use of Bocha and rohu fish scales in tandem for BS production by *Bacillus tequilensis* KM15.

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Conflict of interest

The authors declare that they have no conflict of interest in this study.

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