

Application of Response Surface Methodology in Medium Optimization for Protease Production by the New Strain of *Serratia marcescens* SB08

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Abstract

For production of protease by a new strain, *Serratia marcescens* SB08, optimization of the fermentation medium and environmental conditions, were carried out by applying factorial design and response surface methodology. The results of factorial design showed that pH, agitation, incubation time and yeast extract were the key factors affecting protease production. The optimal cultural conditions for protease production obtained with response surface methodology were pH 6.0, agitation 100 rpm, incubation time 51.0 h and yeast extract 3.0 g/l. This model was also validated by repeating the experiments under the optimized conditions, which resulted in the maximum protease production of 281.23 U/ml (Predicted response 275.66 U/ml), thus proving the validity of the model. Unexplored *Serratia marcescens* SB08 strain isolated from enteric gut of sulphur butterfly (*Kricogonia lyside*) was taken up for this study. This study demonstrates the ability of the new strain, *Serratia marcescens* SB08, for protease production and also that smaller and less time consuming statistical experimental designs are adequate for the optimization of fermentation processes for maximum protease production.

Key words: *Serratia marcescens* SB08, central composite design (CCD), protease production

Introduction

The role of enzymes in industries and medicines has been known for a long time. Existence of secondary metabolites in microbes has thrown new possibilities for the emergence of different high ending industrial processes. Enzymes which have been associated mainly from microbes, have found a number of commercial applications (Demirijan *et al.*, 2001). Advances in this area have been possible with the isolation of a large number of beneficial microorganisms from different ecological zones and consequent extraction of useful enzymes from them (Burrows, 1973; Antranikian *et al.*, 1987; Groboillot, 1994; Bharat and Hoondal, 1998; Bauer *et al.*, 1999; Kohilu *et al.*, 2001). This research was one such attempt to isolate a beneficial microorganism from a distinct ecological zone, *i.e.* enteric gut associated microflora of insects, possessing industrially important enzymes and also to optimize the fermentation conditions for maximum enzyme production from the isolated strain.

Proteases constitute one of the most important groups of industrial enzymes and have applications in different industries *viz.*, detergent, food, pharmaceutical, leather, silk and recovery of silver from used x-ray films (Masse and Tilburg, 1983; Anisworth, 1994; Outtrup *et al.*, 1995; Wolff *et al.*, 1993; Inhs *et al.*, 1999). Probably the largest application of proteases is in laundry detergents, where they help removing protein based stains from clothing (Banerjee *et al.*, 1999). Proteases are produced by wide range of microorganisms including bacteria, moulds and yeast.

Recent research efforts have focused on process development (optimization) and scale up of enzyme production. The medium factors in optimum ratio play a vital role in enhancing the enzyme production. In general, medium optimization by the traditional 'one factor at a time' technique was used (Gokhade, 1991) in such investigations. Single variable optimization methods are not only tedious, but also can lead to misinterpretation of results, especially because the interaction effects between different factors are overlooked

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(Wenster-Botz, 2000). This method is not only laborious and time consuming but also often leads to an incomplete understanding of the system behaviour, resulting in confusion and lack of predictive ability.

Limitations and drawbacks of the single factor optimization can be eliminated by employing response surface methodology (RSM) which is used to explain the combined effects of all the factors in a fermentation process (Elibol, 2004). Response surface methodology not only deals with experimental strategies but also deals with mathematical methods and statistical inference for constructing and exploring an approximate functional relationship between a response variable and a set of design variables.

Statistical methods have been applied for optimization of enzyme production (Dey *et al.*, 2001; Francis *et al.*, 2002; Ahuja *et al.*, 2004; Kunamneni *et al.*, 2005). No defined medium has been established for the optimum production of enzymes from different microbial sources. Each organism has its own special conditions for maximum enzyme production. The use of a good reliable statistical model is essential to develop better strategies for the optimization of the fermentation process (Ghaly *et al.*, 2005). In the present work, a new strain *Serratia marcescens* SB08 (GenBank Accession Number: AB061685) has been subjected to special conditions using response surface methodology for enhancing the production of protease with optimum medium factors and conditions.

Experimental

Materials and Methods

Microorganisms and media. The new strain *Serratia marcescens* SB08 was isolated from enteric gut of sulphur butterfly and the culture was maintained at 4°C and subcultured every two weeks. Nutrient broth medium containing (g/l): Beef extract: 3.0 g; Yeast extract: 3.0 g; NaCl: 5.0 g; Peptone: 5.0 g was prepared for the production of protease from *Serratia marcescens* SB08. The pH of the medium was adjusted to 7.0 with 1N NaOH or 1 N HCl and was autoclaved at 121°C for 15 minutes.

Production of protease. 100 ml of nutrient broth was inoculated with 1 ml of the inoculum (containing 10⁶ cells/ml) and was incubated at 30°C for 18 hours. After incubation the crude enzyme was obtained by centrifugation of the culture broth at 10000×g for 10 minutes at 30°C. The cell free supernatant which contains the enzyme was assayed for protease activity.

Enzyme assay. Protease production was assayed in terms of protease activity exhibited by the culture supernatant in the enzyme assay. Protease assay was done by a modification of the casein digestion method

of Kunitz (1947). To 3 ml of 0.6% casein in a phosphate buffer (100 mM) of pH 7, 0.5 ml of crude enzyme was added and incubated for 30 minutes at 37°C after which 3 ml of 5% trichloroacetic acid was added to stop the reaction and allowed to stand for 15 minutes at room temperature. The resultant mixture was filtered through Whatman No 1 filter paper. The absorbance of this filtrate was measured at 280 nm in a UV – Visible spectrophotometer. A suitable control was run simultaneously, in which TCA was added prior to the addition of enzyme solution. One unit of proteolytic activity was defined as that amount of enzyme, which liberated 1 µg of tyrosine (Sigma-Aldrich) per ml per minute under the specific conditions of assay. The absorbance at 280 nm (test-control) indicated the tyrosine content of the filtrate, which has been released by the hydrolysis of the protein substrate by the enzyme. The tyrosine content of the sample was read from the standard calibration curve prepared with pure tyrosine.

Experimental design. This study was done by Plackett-Burman design for screening medium components with respect to their main effects and not their interaction effects (Plackett and Burman, 1946) on enzyme production by *Serratia marcescens* SB08. The medium components were screened for eleven variables at two levels, maximum (+) and minimum (–). According to the Plackett-Burman design, the number of positive signs (+) is equal to (N+1)/2 and the number of negative signs (–) is equal to (N–1)/2 in a row. A column should contain equal number of positive and negative signs. The first row contains (N+1)/2 positive signs and (N–1)/2 negative signs and the choice of placing the signs is arbitrary. The next (N–1) rows are generated by shifting cyclically one place (N–1) times and the last row contains all negative signs. The medium was formulated as per the design and the flask culture experiments for protease were assayed as described earlier. Response was calculated at the rate of enzyme production and expressed as U/ml. All experiments were performed in triplicate and the average of the rate of enzyme production was considered as the response.

The effect of each variable was calculated using the following equation

$$E = (\sum M_+ - \sum M_-) / N$$

Where E is the effect of tested variable, M₊ and M_– are responses (enzyme activities) of trials at which the parameter was at its higher and lower levels respectively and N is the number of experiments carried out.

The standard error (SE) of the variables was the square root of variance and the significance level (p-value) of each variables calculated by using Student's t-test.

$$t = E_{xi} / SE$$

Where E_{x_i} is the effect of tested variable. The variables with higher confidence levels were considered to influence the response or output variable.

Response surface methodology. Response surface methodology is an empirical statistical modeling technique employed for multiple regression analysis using quantitative data obtained from factorial design to solve multivariable equations simultaneously (Rao *et al.*, 2000). The screened medium components affecting enzyme production were optimized using central composite design (CCD) (Box and Wilson, 1951; Box and Hunter, 1957).

According to this design, the total number of treatment combinations is $2^k + 2k + n0$ where 'k' is the number of independent variables and n0 the number of repetitions of the experiments at the center point. For statistical calculation, the variables X_i have been coded as x_i according to the following transformation:

$$x_i = X_i - X_0 / \delta X$$

Where x_i is dimensionless coded value of the variable X_i , X_0 the value of the X_i at the center point, and δX is the step change. A 2^k -factorial design with eight axial points and six replicates at the center point with a total number of 30 experiments was employed for optimizing the medium components.

The behavior of the system is explained by the following quadratic equation:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j$$

Where Y is the predicted response, β_0 the intercept term, β_i the linear effect, β_{ii} the squared effect, and β_{ij} is the interaction effect. The regression equation was optimized for maximum value to obtain the optimum conditions using Design Expert Version 7.1.5 (State Ease, Minneapolis, MN).

Validation of the experimental model. The statistical model was validated for protease production

under the conditions predicted by the model in shake flask conditions. Samples were withdrawn at the desired intervals and protease assay was determined as described above.

Results

The influence of eleven medium factors and conditions namely pH, temperature, agitation, inoculum concentration, incubation time, sucrose, peptone, KH_2PO_4 , yeast extract, NaCl and CaCl_2 in the production of protease was investigated in 12 runs using Plackett-Burman design. Table I represents the Plackett-Burman design for 11 selected variables and the corresponding response for protease production. Variations ranging from 41.23 to 271.31 U/ml in the production of protease in the 12 trials were observed by Plackett-Burman design.

Among the variables screened, the most effective factors with high significance level were in the order of yeast extract, incubation time, pH and agitation. They were identified as most significant variables in protease production and selected for further optimization.

Statistical analysis of the Plackett-Burman design demonstrates that the model F value of 0.94 is significant. The values of $p < 0.05$ indicate model terms are significant (Table II). The R^2 value (multiple correlation coefficient) closer to 1 denotes better correlation between the experimental and predicted responses. In this case the value of R^2 (0.81) indicates good correlation between the experimental and predicted values. The coefficient of variation (CV) indicates degree of precision with which the experiments are compared. The lower reliability of the experiment is usually indicated by high value of CV. In the present case, a low

Table I
Plackett-Burman experimental design for evaluating factors influencing protease by *Serratia marcescens* SB08

Run	A: pH	B:Temp (°C)	C:Agit (rpm)	D:Ino conc (%)	E:Incu time (h)	F: Sucrose (g/l)	G: Peptone (g/l)	H: KH_2PO_4 (g/l)	J:Yeast extract (g/l)	K:NaCl (g/l)	L:CaCl ₂ (g/l)	Protease (U/ml)
1	4	40	200	1	96	20	6	2	2	0.1	0.2	65.09
2	4	40	200	5	6	5	3	6	2	0.5	0.2	41.23
3	4	40	0	5	96	5	6	6	4	0.1	0.05	72.81
4	4	20	0	1	6	5	3	2	2	0.1	0.05	151.92
5	8	20	200	5	96	5	3	2	4	0.1	0.2	271.31
6	8	20	0	1	96	5	6	6	2	0.5	0.2	242.03
7	4	20	200	1	96	20	3	6	4	0.5	0.05	175.83
8	8	40	0	1	6	20	3	6	4	0.1	0.2	97.02
9	4	20	0	5	6	20	6	2	4	0.5	0.2	73.99
10	8	40	200	1	6	5	6	2	4	0.5	0.05	135.13
11	8	40	0	5	96	20	3	2	2	0.5	0.05	153.56
12	8	20	200	5	6	20	6	6	2	0.1	0.05	45.65

Table II
Analysis of variance for protease production by *Serratia marcescens* SB08

Source	Sum of square	Degree of freedom	Mean square	F-Value	p-Value
Model	27794.3	5	5558.87	0.942	0.0061
A-pH	147	1	147	0.025	0.0098
C-Agitation	85.3333	1	85.3333	0.014	0.0082
E-Incubation time	9976.33	1	9976.33	1.69	0.0013
J-Yeast extract	17025.3	1	17025.3	2.884	0.0404
Residual	35416.3	6	5902.72		
Cor Total	62650.3	10			

CV – 4.50; R² – 0.81

CV (4.50) denotes that the experiments performed are highly reliable. The *p* values denotes the significance of coefficients and also important in understanding the pattern of mutual interactions between the variables.

Regression analysis was performed on the results and first order polynomial equation was derived representing protease production as a function of the independent variables.

$Y = 167.33 + 3.50 A + 2.67 C + 37.67 E + 6.83 J$
Where Y is the predicted protease yield and A, C, E and J is the coded values of pH, agitation, incubation time and yeast extract respectively. The magnitude of the effects indicates the level of the significance of the variable on protease production. Consequently, based on the results from this experiment, statistically significant variables *i.e.* yeast extract, incubation time, pH

Table III
Experimental plan for optimization of protease production using central composite design

Run	pH	Agitation rpm	Incubation time h	Yeast extract g/l	Protease (U/ml)	
					Experimental	Predicted
1	4	0	6	2	36.34	36.375
2	6	0	6	2	31.15	39.625
3	4	200	6	2	65.98	68.291667
4	6	200	6	2	69.87	71.541667
5	4	0	96	2	34.2	47.291667
6	6	0	96	2	161.11	171.04167
7	4	200	96	2	111.26	112.70833
8	6	200	96	2	232.09	236.45833
9	4	0	6	4	57.15	43.958333
10	6	0	6	4	35.35	45.208333
11	4	200	6	4	71.45	72.875
12	6	200	6	4	72.45	74.125
13	4	0	96	4	51.12	52.375
14	6	0	96	4	162.46	174.125
15	4	200	96	4	98.5	114.79167
16	6	200	96	4	212.05	236.54167
17	3	100	51	3	0	0.8333333
18	7	100	051	3	214	225.83333
19	5	100	51	3	0	0
20	5	300	51	3	76	91.5
21	5	100	39	3	0	0
22	5	100	141	3	187.93	170
23	5	100	51	1	212.64	204.5
24	5	100	51	5	220.64	212.16667
25	5	100	51	3	269.52	275.66667
26	5	100	51	3	280.82	275.66667
27	5	100	51	3	265.18	275.66667
28	5	100	51	3	280.05	275.66667
29	5	100	51	3	281.23	275.66667
30	5	100	51	3	279.92	275.66667

and agitation with positive effect were further investigated with central composite design to find the optimal range of these variables.

Based on the Plackett-Burman design yeast extract, incubation time, pH and agitation were selected for further optimization by response surface methodology. To examine the combined effect of these medium components on protease production, a central composite design was employed. The central composite design for 4 variables and the corresponding experimental data are shown in Table III.

The results obtained were subjected to analysis of variance on Stat-Ease package, with the regression model given as

$$Y = 275.67 + 31.25 A + 23.58 B + 43.33 C + 1.92 D + 0.000 AB + 30.13 AC - 0.50 AD + 8.38 BC - 0.75 BD - 0.62 CD - 53.08 A^2 - 57.83 B^2 - 48.08 C^2 - 16.83 D^2$$

Where Y is the response value (protease production) and A, B, C and D is the coded levels of pH, agitation, incubation time and yeast extract respectively.

The adequacy of the model was checked using analysis of variance and the results are presented in Table IV. The analysis of variance of the quadratic regression model suggested that the model is very significant as was evident from the Fisher's F-test. The model F value of 7.53 implies the model is significant. The value of R^2 (0.87) indicates good correlation between the experimental and predicted values. The low CV (3.95) denotes the experiments performed are

highly reliable. The p values denotes the significance of coefficients and also important in understanding the pattern of mutual interactions between the variables.

The interaction effects and optimal levels of the variables were determined by plotting the response surface curves. The fitted response and contour for the above regression model was plotted in Figure 1. 3D response surface curves were plotted to understand the interactions of medium components and their effect on protease production. Graphs highlight the roles played by various factors and also to emphasize the roles played by the physical constraints.

Figure 1A shows the response surface plot obtained as function of pH vs agitation, while all other variables are maintained at zero level. An increase in protease yield was observed at pH 6.0 and agitation of 120 rpm. Figure 1B shows the response surface plot obtained as function of pH vs incubation time, while all other variables are maintained at zero level. An increase in protease yield was observed at pH 6.0 and 51 h. Figure 1C shows the response surface plot obtained as function of pH vs yeast extract, while all other variables are maintained at zero level. An increase in protease yield was observed at pH 6.0 and yeast extract 3 (g/l). Figure 1 D shows the response surface plot obtained as function of agitation vs incubation time, while all other variables are maintained at zero level. An increase in protease yield was observed at agitation 100 rpm and incubation time 51 h.

The maximum experimental response for protease production was 281.23 U/ml whereas the predicted

Table IV
ANOVA for the experimental results of the central composite design (quadratic model)

Source	Sum of square	Degree of freedom	Mean square	p -Value	F-Value
Model	278920	14	19922.8	7.53883122	0.0002
A-pH	23438	1	23437.5	8.86878922	0.0094
B-Agitation	13348	1	13348.2	5.0509686	0.0401
C-Incubation time	45067	1	45066.7	17.0533021	0.0009
D-Yeast extract	88.167	1	88.1667	0.03336241	0.8575
AB	0	1	0	0	1.0000
AC	14520	1	14520.3	5.4944869	0.0333
AD	4	1	4	0.00151361	0.9695
BC	1122.3	1	1122.25	0.42466128	0.5245
BD	9	1	9	0.00340562	0.9542
CD	6.25	1	6.25	0.00236501	0.9619
A ²	77289	1	77289.3	29.2464131	< 0.0001
B ²	91740	1	91740.2	34.7146416	< 0.0001
C ²	63415	1	63415	23.9963601	0.0002
D ²	7772.2	1	7772.19	2.94100988	0.1069
Residual	39640	15	2642.69		
Lack of Fit	39405	10	3940.51	83.7218484	< 0.0001

CV – 3.95; R^2 – 0.87

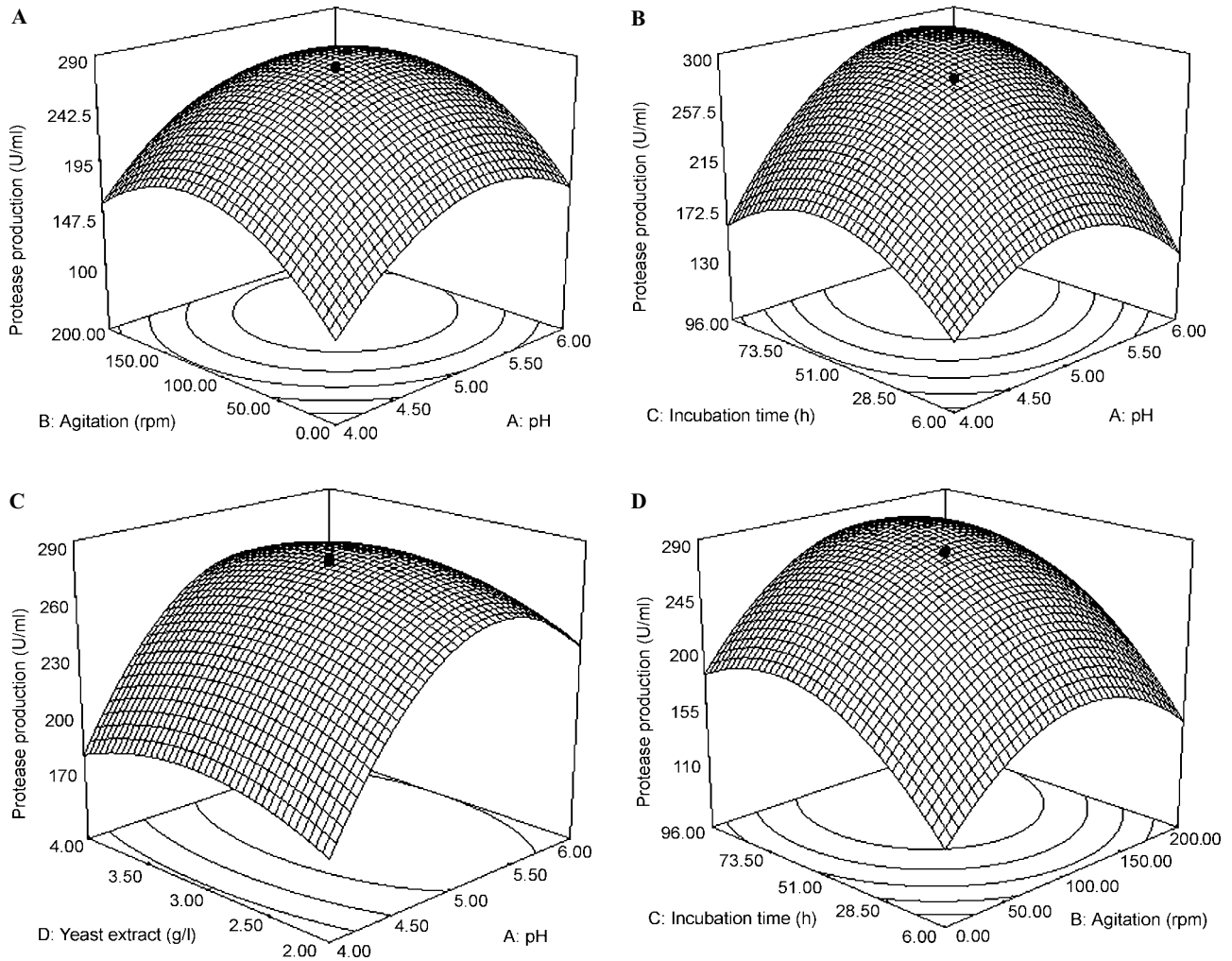


Fig. 1. Response surface graph showing interaction between pH and agitation on protease production by *Serratia marcescens* SB08.

value was 275.66 U/ml indicating a strong agreement between them. The optimum values of the tested variables are pH 6.0, agitation 100 rpm, incubation time 51.0 h and yeast extract 3.0 g/l. The model was also validated by repeating the experiments under the optimized conditions, which resulted in the protease production of 279.05 U/ml (Predicted response 275.66 U/ml), thus proving the validity of the model.

To optimize industrial conditions for protease production, scale-up study was carried out in a jar fermentor by using medium under optimum conditions. The maximum production of 284.94 U/ml protease was achieved comparing to the shake flask condition yield of 281.23 U/ml. The results are encouraging for optimization under pilot scale or industrial scale conditions.

Discussion

Numerous studies to optimize the production of various microbial secondary metabolites by applying response surface methodology have already been car-

ried out by the scientific community. The response surface methodology, a smaller and less time consuming experimental design, could generally satisfy the optimization of many microbial processes. Central composite design, a response surface methodology maximizes the amount of information that can be obtained, while considering the interaction of independent variables and limiting the numbers of individual experiments required. (Chauhan and Gupta, 2004; Elibol, 2004; Abdel-Fattah *et al.*, 2005). This study is an attempt that has demonstrated the application of a multifactorial statistical approach for determining the fermentation conditions that lead to the maximum yield of protease production from *Serratia marcescens* SB08, a newly isolated strain.

By applying response surface methodology, in protease production by *Serratia marcescens* SB08, optimum amount of pH, agitation, incubation time and yeast extract are found as positive factors playing significant role. Extended period of incubation might lead to the decomposition of enzyme due to interaction with other components in the media (Ramesh

and Lonsane, 1987). There was a decline in enzyme production (31.15 U/ml) under static conditions, which could be due to the reduction in dissolved oxygen and inadequate mixing (Uma Maheshwar Rao and Satanarayana, 2003).

In this work, maximum protease production of 281.23 U/ml was observed when yeast extract (3 g/l) was supplemented at optimum levels. Yeast extract contains complex nutrients such as vitamin, nucleic acid, lipid and other substances which might be necessary for growth and production of secondary metabolites from microbes. Yeast extract is the key nutrient material which controls the biosynthesis of this enzyme. This fact has also been suggested previously during other enzyme production experiments on nitrogen repression effects (Cruegar and Cruegar, 1984; Frankena *et al.*, 1986; Kole *et al.*, 1988; Giesecke *et al.*, 1991).

This study also demonstrates the potentiality of the new strain *Serratia marcescens* SB08 for the production of protease. Response surface curves are very helpful in visualizing the main effects and interaction of factors. The optimum culture medium obtained in this experiment gives a basis for further study with batch or fed-batch cultivation in a bioreactor for large scale production of target secondary metabolites from *Serratia marcescens* SB08.

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