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Optimization study for enhanced production of hyaluronic acid from *Streptococcus equisimilus* MK156140

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Abstract−Hyaluronic acid finds its complete application in areas such as therapeutics, cosmetics, and as a health supplement. In the present investigation, standardization for the production of hyaluronic acid by *Streptococcus equisimilus* MK156140 in complex media was performed. Some of the selected physicochemical parameters such as pH, temperature, speed, incubation time, sucrose, yeast extract, and beef extract were screened using Plackett-Burman foldover design. Further, the screened parameters interaction was investigated using central composite design (CCD) and closely compared with OVAT studies. At a pH of 7.38, with beef extract, 12.15%, and yeast extract 7.64%, the observed yield was 7.16 g/L, which was in close line with the predicted value of 7.21 g/L.

Keywords: Response Surface Methodology, OVAT, Plackett Burman Design, Central Composite Design

INTRODUCTION

Hyaluronic acid is a linear mucopolysaccharide composed of repeating disaccharide units of β(1,4)-glucoronic acid (GlcUA) and β(1,3)-N-acetylglucosamine (GlcNAC) [1].

Hyaluronic acid, also called hyaluronan, is an anionic, nonsulfated glycosaminoglycan distributed widely throughout connective, epithelial, and neural tissues. Being nonsulfated, it is unique among glycosaminoglycan, which forms an integral part of the plasma membrane, and it has a very high molecular weight, often reaching to millions [2]. A person weighing around 70 kg (154 lb) has roughly 15 grams of hyaluronic acid in the body, one-third of which is turned over (degraded and synthesized) every day.

Hyaluronic acid in the living organism is produced by hyaluronan synthase enzyme, which can synthesize large, linear with other significant groups of glycol polymers of repeating disaccharides β(1,4)-GlcA-β(1,3)-GlcNAc by an alternate addition of monomers FlcUA and GlcNAc to the growing chain using their associated nucleotide sugars as substrates [3,4]. Hyaluronic acid is a member of the glycosaminoglycan family, including chondroitin sulfate, domain sulfate, and heparin sulfate. It differs from the other significant groups of the family due to the absence of the sulfate group [5]. Each repeating disaccharide unit consists of one acetamido group, four hydroxyl groups, and one carboxylate group. Hyaluronic acid also exists in random coil configuration, which is polyanionic at physiological pH. When it reaches high molecular weights, these random coils become entangled and form a viscoelastic gel [6]. Hyaluronic acid holds a unique set of characteristics: its solution demonstrates very unusual rheological properties, hydrophilic, and is lubricious [7].

Streptococcus equisimilus (MK156140) is identified as a potent strain of hyaluronic acid producer. In the present study, an attempt was made to optimize the environmental parameters such as pH, temperature, incubation period, speed, sucrose, beef extract, and yeast extract for enhanced production of hyaluronic acid by *Streptococcus equisimilus*, which was isolated by us from horse nasal swab.

Low resolution Placket-Burman design was used to screen out significant independent variables based on their input. Further, high resolution central composite design (CCD) provided the optimal interaction of independent variables for higher productivity of hyaluronic acid. The experimental design facilitates overcoming the systematic or randomized errors associated with conventional methods.

MATERIALS AND METHODS

1. Strains and Culture Conditions

Streptococcus equisimilus (MK156140) strain was isolated from a horse nasal swab and maintained in our laboratory. It was subcultured and maintained in nutrient media. The culture was incubated at 32 °C for 24 h. Glycerol stock (50%) of the culture was maintained in a deep freezer (–20 °C) for future experimental studies.

2. Inoculum Preparation

The inoculum was prepared in 250 ml Erlenmeyer flasks containing 50 ml of LB media of pH 7.0. The media was autoclaved at 121 °C (15 lbs) for 20 min and inoculated with *Streptococcus equisimilus* MK1561140. The inoculated flasks were kept on an orbital shaker (Scigenics Biotech) at 150 rpm for 24 h, and the grown culture was used as inoculum. The inoculum size of 10^{-8} was used throughout the study for subsequent inoculation unless otherwise specified [8].

3. Selection of Basal Media for the Optimization Process

Before the optimization study by OVAT (One-variable-at-a-time), the following four different media were tested (Table 1) for the production of hyaluronic acid, such as heart infusion broth, nutrient broth, Luria Bertani broth, and tryptic soy broth.

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Table 1. Hyaluronic acid production using different media

SN	Media	The concentration of HA g/L
1	Nutrient broth	0.6
2	Luria Bertani broth	0.91
3	Tryptic soy broth	0.77
4	Heart infusion broth	0.42

The basal media used for Plackett-Burman design contains sodium chloride 10 g/L, sucrose 20 g/L, tryptone 10 g/L.

4. Extraction of Hyaluronic Acid from *Streptococcus equisimilus*

To extract the hyaluronic acid, the fermented broth was initially blended with a 10% volume of 5% (w/v) SDS for 10 min and centrifuged at 5,000 xg/3 min. Hyaluronic acid was precipitated by mixing the supernatant with three volumes of ethanol and then centrifuged at 5,000 xg/10 min. The sediment was redissolved with one volume of 1.5 M NaCl and three volumes of ethanol and centrifuged again at 5,000 xg/10 min. Finally, the sediment was resuspended in distilled water to estimate hyaluronic acid [3].

5. Estimation of Hyaluronic Acid by CTAB Method

To estimate the hyaluronic acid, the fermented broth sample was diluted with equal volume of 0.1% of SDS and incubated for 10 minutes at room temperature to free the capsular bound hyaluronic acid. The mixture was then filtered through a 0.45 μm syringe filter and used in a turbidimetric HA quantification assay. One ml of filtrate was taken in a clean test tube, to this 1 ml of 0.1 M acetic acid and 2 ml of CTAB reagent (2.5% of CTAB dissolved in 0.5 M NaOH) was added and incubated for 20 minutes at room temperature. Optical density was recorded at 600 nm using a UV-vis spectrophotometer. A standard curve was drawn using 200 mg/L hyaluronic acid stock solution prepared from hyaluronic acid obtained from *S. zooepidemicus* HA (Sigma, India) [10].

6. Selection of Parameters for Process Optimization

The conventional OVAT method was used for the selection of optimization of physical and nutritional factors for hyaluronic acid production, which was carried out by shake flask studies. The nutritional media components required for the production of hyaluronic acid by *Streptococcus equisimilus* MK156140 were carried out in 100 ml Erlenmeyer flasks containing 10 ml basal medium. The optimized parameter obtained was based on the highest production of hyaluronic acid. The physical parameter and media components that significantly affected the hyaluronic acid production were further studied by statistical analysis using Placket-Burman design and central composite design [8].

6-1. One Variable at A Time Method

The one variable at a time method (OVAT) is to design the experiments which involve the testing of factors one at a time instead of testing multiple factors. Luria Bertani (LB) broth was used as a production medium. To enhance the production, six different parameters (Table 2) were selected based on the literature survey.

6-2. Statistical Methodology: Plackett-Burman Design (P-B Foldover Design)

Screening of the most significant processing parameters affecting hyaluronic acid production was studied by P-B foldover design, which is regarded as a high-resolution category IV design matrix,

Table 2. Different parameters selected for the OVAT method

Sl. No	Parameter	Range			
1	pH	5	6	7	8
2	Temperature (°C)	25	30	35	38
3	Speed (RPM)	100	120	150	180
4	Sucrose (%)	1	2	3	4
5	Beef extract (%)	2	4	6	8
6	Incubation time (hrs)	16	20	24	28

Table 3. Coded factors for Plackett-Burman foldover design

Factors	Level		
	−1	+1	Unit
F1: pH	4	7	
F2: Temperature	25	38	°C
F3: Sucrose	2	6	g/100 ml
F4: Error 1	**	**	**
F5: Yeast extract	2	6	g/100 ml
F6: Beef extract	2	6	g/100 ml
F7: Error 2	**	**	**

The two factors: F4 and F7, designated as "dummy variables".

used for screening statistically significant independent variables. Totally, seven (n) variables, including three nutritional (sucrose, yeast extract, and beef extract), two physical (temperature and pH), and two dummy variables designated as F4 and F7 were studied in 16 experimental runs, [n+1] (+foldover) experiments. The identification of coded factors value and their concentrations are shown in Table 3. Each variable is represented at two levels, high and low, denoted by +1 and −1 signs, respectively. The difference between the two values was taken large enough to ensure that the peak area for the highest hyaluronic acid production [10].

The number of positive and negative signs per experiment or trial is (n+1)/2 and (n−1)/2, respectively, with each column having an equal number of positive and negative signs. The design matrix applied to this study is shown in Table 4. The effect of each variable or factor is the difference between the average of the measurement made at the high level of the factor and the average of the measurements made at the low level of that factor. The significant level (P-value) of the effect of each concentration and the square root of the variance of an effect, i.e., standard error (S.E) was determined by Student's t-test [8].

6-3. Central Composite Design

Response surface methodology was used to develop an empirical model of the process and to get a more precise estimate of optimum operating conditions obtained by the interaction of factors involved. In 16 factorial central composite designs, the influence of all significant experimental variables and their interaction effects on the response were investigated. Three significant variables (short-listed from PB design) were set as factors for CCD design. Each variable in the design was studied at five different levels, with four cube points, six replicates, and four-star points for one factor, an axial distance to the center of $\pm\alpha$, whereas the other factor was at level 0, a total of 16 experiments was employed. Hyaluronic acid

Table 4. Plackett-Burman foldover design (Resolution IV) experimental design

Standard run	F1 (pH)	F2 (temperature)	F3 (sucrose)	F4 (error 1)	F5 (yeast extract)	F6 (beef extract)	F7 (error 2)	Observed value	Predicted value
1	−1	−1	−1	1	1	1	−1	3.5	3.521
2	1	−1	−1	−1	−1	1	1	4.6	4.525
3	−1	1	−1	−1	1	−1	1	0.8	1.215
4	1	1	−1	1	−1	−1	−1	0.4	0.315
5	−1	−1	1	1	−1	−1	1	0.3	0.212
6	1	−1	1	−1	1	−1	−1	5.0	4.838
7	−1	1	1	−1	−1	1	−1	0.9	0.883
8	1	1	1	1	1	1	1	6.4	6.2
9	1	1	1	−1	−1	−1	1	1.2	1.212
10	−1	1	1	1	1	−1	−1	0.9	0.912
11	1	−1	1	1	−1	1	−1	0.3	1.121
12	−1	−1	1	−1	1	1	1	3.9	4.323
13	1	1	−1	−1	1	1	−1	6.3	5.232
14	−1	1	−1	1	−1	1	1	0.1	0.938
15	1	−1	−1	1	1	−1	1	5.1	4.8
16	−1	−1	−1	−1	−1	−1	−1	0.4	0.344

production was taken as a response (Y); the observed experimental values and predicted values were tabulated. Multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response measured to the independent variable.

The analysis of experimental results was performed based on the first-order polynomial model to calculate the coefficient value of each selected constituents using the following Eq. (1).

$$Y=\beta_0+\beta_1A+\beta_2B+\beta_3C+\beta_4D+\beta_{11}A_2+\beta_{22}B_2+\beta_{33}C_2+\beta_{44}D_2 +\beta_1\beta_2AB+\beta_1\beta_3AC+\beta_1\beta_4AD+\beta_2\beta_3BC+\beta_2\beta_4BD+\beta_3\beta_4CD \quad (1)$$

where Y is the hyaluronic acid production rate, β_0 is intercept, β_1, β_2, β_3, β_4 are linear coefficients β_{11}, β_{22}, β_{33}, β_{44} are squared coefficients $\beta_1\beta_2$, $\beta_1\beta_4$, $\beta_2\beta_3$, $\beta_2\beta_4$, $\beta_3\beta_4$ are interaction coefficients and A, B, C, D, A_2, B_2, C_2, D_2, AB, AC, AD, BC, BD, and CD are independent variables [8].

6-4. Software and Data Analysis

Experimental design matrix was generated by using the statistical software package, Stat Soft®, STATISTICA version 6.0. Statistical analysis of experimental design was also performed by using this software. The coefficient of determination (R^2 value) and its significance was validated by using F-test to express the quality of the first-order polynomial model. The 3D interaction contour surface plot of a significant variable was generated. The critical experimental values and highest predicted response for the model were also obtained by this software [11].

RESULTS

Among four different media (Table 1), Luria Bertani broth was selected for further study, as the production of HA was significantly higher than that of the other three media used.

1. One Variable at a Time Method

1-1. Production of Hyaluronic Acid at Different pH

The production of hyaluronic acid at different pH is given in Fig. 1. At acidic pH 4.0, the production was found to be 0.43±0.06 g/L. However, the production linearly increased with increased pH, and the maximum yield was observed (1.11±0.06) at neutral pH 7.0. According to Aroskar et al. [3], various pH ranges (5.5, 5.8, 6.2, 6.4, 6.6) were tested for determining the optimum pH

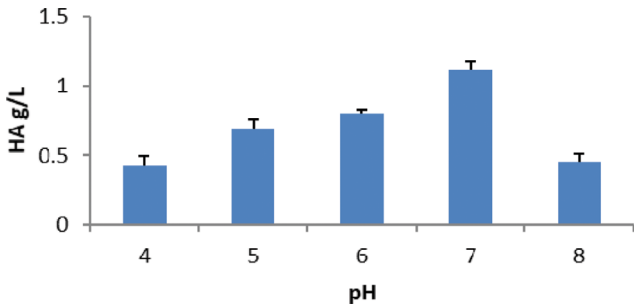

Fig. 1. Effect of different pH on the hyaluronic acid production from *Streptococcus equisimilus* MK156140.

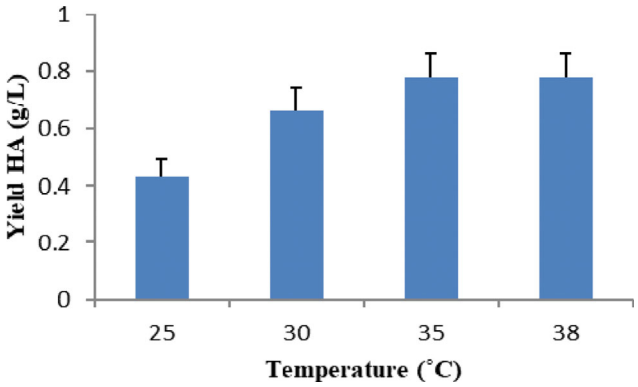

Fig. 2. Influence of different temperature on the yield of on hyaluronic acid from *Streptococcus equisimilus* MK156140.

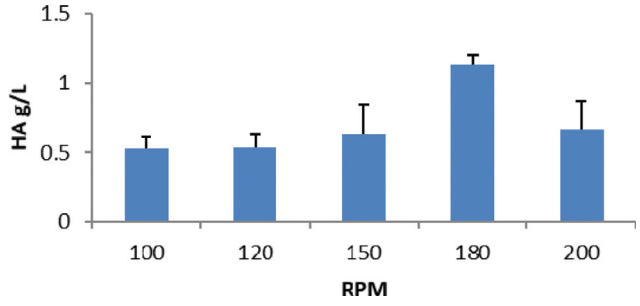

Fig. 3. Effect of speed at shaken condition (as measured by rpm) the yield of hyaluronic acid form *Streptococcus equisimilus* MK156140.

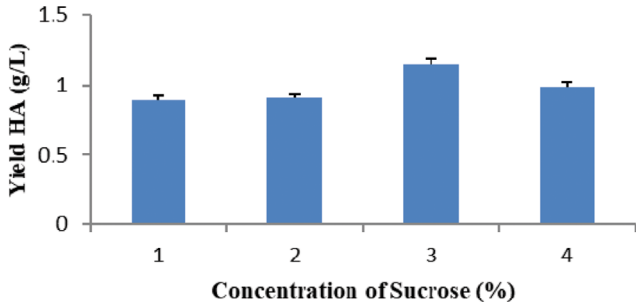

Fig. 4. Effect of concentration of sucrose (%) on the yield of hyaluronic in *Streptococcus equisimilus* MK156140.

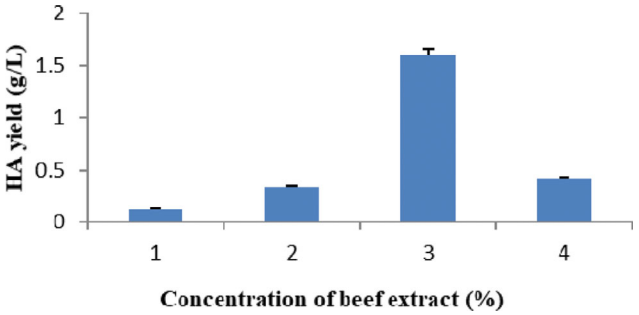

Fig. 5. Effect of different concentration of beef extract (%) on the yield of hyaluronic acid from *Streptococcus equisimilus* MK156140.

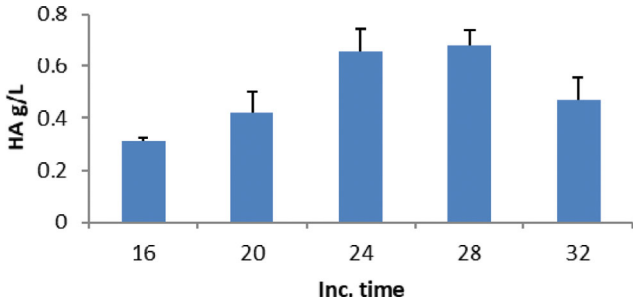

Fig. 6. Effect of incubation time on the recovery of hyaluronic acid from *Streptococcus equisimilus* MK156140.

for the production of hyaluronic acid. The fermentation media inoculated with the seed at pH 6.4 gave a maximum yield of 0.70 g/L.

1-2. Production of Hyaluronic Acid at Different Temperature

The effect of temperature on the production of hyaluronic acid demonstrated significant variations in the yield (Fig. 2). The yield was about 0.43±0.06 at 25 °C, the lowest temperature selected in the study. Maximum yield was recorded at 35° and 38 °C with the yield of 0.78±0.08 g/L. According to Aroskar et al. [3], the maximum yield of HA was 0.70±0.03 at 37 °C in *S. equi* Subsp. *zooepidemicus* ATCC 39920.

1-3. Production of Hyaluronic Acid at Different Speed (rpm)

Similar to pH and temperature, the yield of the hyaluronic acid significantly varied with respect to increase in the shaking speed (rpm) during the growth period (Fig. 3). At 100 rpm, the minimum speed we selected, the yield was about 0.53±0.08485 g/L. However, the yield increased with increase in speed (rpm) with the maximum yield of 1.135±0.06 g/L at 180 rpm. On the contrary, according to Aroskar et al. [3], 70-90 rpm speed was found to be more significant for the production of hyaluronic acid with maximum yield of 0.72±0.03 g/L in *S. equi* Subsp. *zooepidemicus* ATCC 39920.

1-4. Effect of Carbon Source Concentration on Production of HA

The concentration of sucrose towards the growth and HA yield *Streptococcus equisimilus* resulted in significant variations (Fig. 4). The yield of hyaluronic acid was very low (0.895±0.12 g/L) at 1% sucrose concentration, but constantly increased with increase in sucrose concentration with the maximum yield (1.15±0.084 g/L) at 3% concentration of sucrose.

1-5. Effect of Concentration of Beef Extract on HA Production

The beef extract (HIMEDIA - RM002) used at various concentrations also showed variations in the yield of hyaluronic acid. At low concentration (1%), the yield was observed to be 0.125±0.007 g/L. Beef extract showed maximum yield (1.61±0.04 g/L) at 3% concentration (Fig. 5). At higher concentration, i.e., 4%, however, the yield was found to be very low: 0.415±0.007 g/L.

1-6. Influence of Incubation Time on HA Production

The effect of incubation time for the growth to reach maximum production was also monitored. At the initial incubation period (16 h), the yield was about 0.31±0.01 g/L. However, the production of HA gradually increased with increase in incubation period, showing maximum yield, 0.68±0.056 g/L, at 28 h (Fig. 6).

2. Analysis of Plackett-Burman Design for Hyaluronic Acid Production

In the present study, out of five variables, which were expected to play a very important role in enhancing the production of hyaluronic acid, three factors such as pH (F1), Yeast Extract (F5) and Beef Extract (F6) were significantly affecting the production of hyaluronic acid. The two dummy variables, F4 and F7, which were included in the design showed no change on the production of hyaluronic acid, which overall makes the design significant in screening the independent variables.

To identify the factors that are significant in enhancing the production of hyaluronic acid the pareto chart (Fig. 7) of standardized effect was plotted based on the experimental data. The effect of the selected factors is ranked in order to their level of significance (P=0.05). The standardized effects of dummy variables or

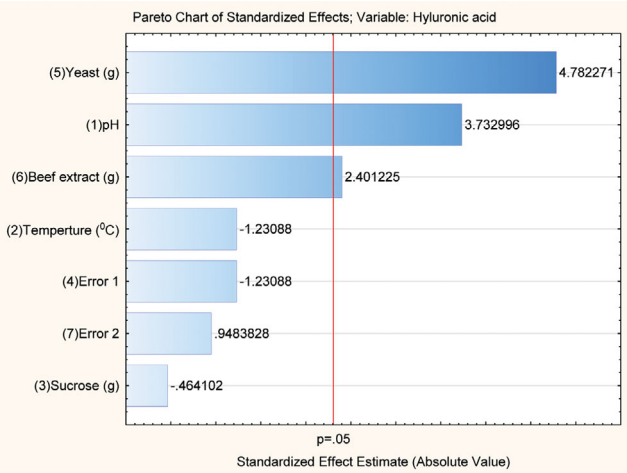

Fig. 7. Pareto chart of standardized effects of independent variables of HA yield by *Streptococcus equisimilus*.

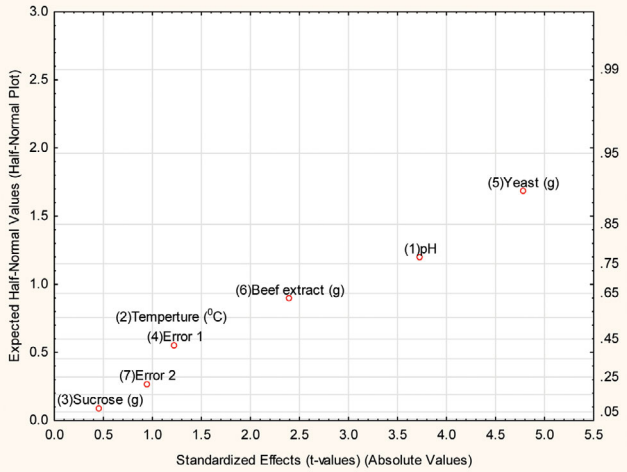

Fig. 8. The Half-normal probability plot of effects on hyaluronic acid production (g/L) by *Streptococcus equisimilus*.

error were neglected by using "ignore" option of "Statistica-6", software. It is noticeable in the pareto chart that the most significant variables are F1 (pH), F5 (Yeast extract) and F6 (Beef extract) having standardized effects (absolute value) 3.73, 4.78 and 2.40, respectively. These results were further confirmed by ANOVA. The observed highest F-value (22.87) and lowest p-value (0.0013), that is, "F>P value", was observed with yeast extract (F5), followed by F1 (pH) and F6 (beef extract) as shown in Table 5.

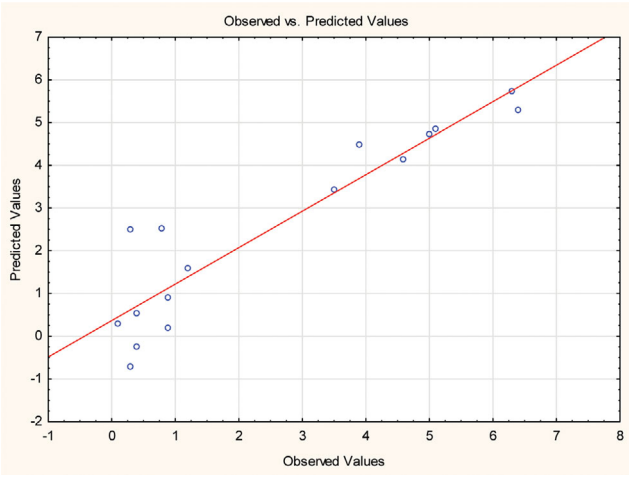

Fig. 9. Plot of observed v/s. predicted hyaluronic acid concentration g/L values for *Streptococcus equisimilus*. The HA concentration is response variable of interest. The expected HA concentration values are determined by the model equations determined for P-B Foldover design.

The Half-Normal probability plot of effects (Fig. 8) is very useful for separating random noise from 'real effects' based on their distribution on the plot as shown in the figure. It is evident from that among the selected factors Yeast extract (F5), pH (F1) and beef extract (F6) were positioned outliers with better factor confidence levels [11].

The observed and predicted values show that 8th and 13th experimental runs (Table 4) depict the highest yield rate in terms of production of hyaluronic acid; the observed yield was 6.4 g/L and 6.3, respectively. Fig. 9 represents the relationship between the predicted and observed hyaluronic acid concentration for P-B foldover design. Most of the points are nearby the line adjustment, which means that the values determined experimentally are similar to those determined by the model. Thus, the P-B Foldover design

Table 5. ANOVA results of P-B (foldover design)

Factor	SS	Df	MS	F	P
(1) pH	21.39063	1	21.39063	13.93526	0.005763
(2) Temperture (°C)	2.32563	1	2.32563	1.51507	0.253326
(3) Sucrose (g)	0.33063	1	0.33063	0.21539	0.654942
(4) Error 1	2.32562	1	2.32562	1.51507	0.253326
(5) Yeast (g)	35.10563	1	35.10563	22.87011	0.001387
(6) Beef extract (g)	8.85063	1	8.85063	5.76588	0.043094
(7) Error 2	1.38063	1	1.38063	0.89943	0.370699
Error	12.28000	8	1.53500		
Total SS	83.98938	15			

Note: SS; sum of square, Df; Degree of freedom, MS; mean square, F; Fisher's test, P; probability.

signified the importance of independent variables, such as pH, yeast extract and beef extract.

The experiments conducted with several trials based on the predicted values resulted in variations in the yield of the hyaluronic acid obtained from the observed values. Few observed values showed decreased yield compared to the predicted values.

According to Yu et al. [12], peptone (2%), meat extract (2%), manganese sulphate (0.1%), temperature (40 °C), di potassium phosphate, (0.04%), yeast extract (1%), glucose (0.4%), ammonium citrate (0.4%), sodium acetate (1%) gave the highest yield in *S. zooepidermicus*. The maximum yield of hyaluronic acid produced by *S. zooepidermicus* (ATCC 43079) was 42.38 mg/L. Whereas, the PBD performed by us gave the yield around 6.4 g/L in *S. equisimilus* MK156140.

3. Optimization of Screened Variables and Their Interaction Analysis: Central Composite Design

The optimum level of significant factors such as pH, yeast extract and beef extract and the effect of their interaction on the production of hyaluronic acid were determined by factorial central composite design (CCD). The observed and predicted concentration of hyaluronic acid for factorial CCD design showed positive correlation since most of the values are falling in the same line of adjustment (Table 6). The second-order regression equation provided the levels of production of hyaluronic acid as a function initial values of pH, beef extract and yeast extract, which can be predicted by following equation:

$$Y=-7.66+4.93X_1-0.78X_1^2+0.49X_2-0.02X_2^2+123.39X_3 -2966.21X_3^2+1.13X_1X_3+0.23X_2X_3 \quad (2)$$

where, Y represents hyaluronic acid yield g/L, X_1-pH (2-4 g/l with 3 g/l as central value), X_2-beef extract (10-20 g/l with 15 g/l as central value) and X_3-yeast extract (0.020-0.040 g/100 ml with 0.030 as central value). The model efficacy was checked and it was found to be appropriate; the virtue of the fit of the model was expressed

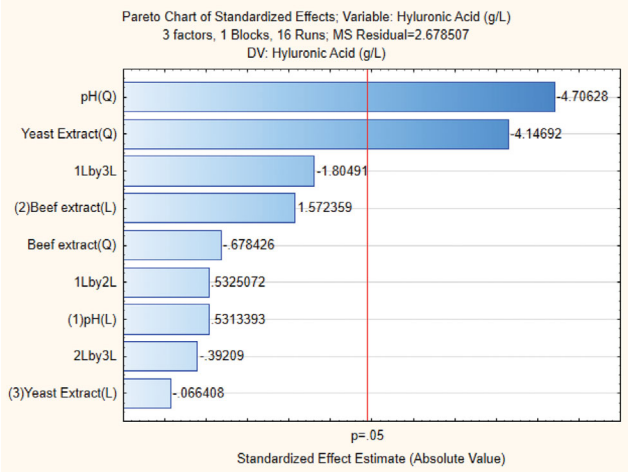

Fig. 10. Pareto chart of standardized effects of independent variables on the production of hyaluronic acid by *Streptococcus equisimilus* (MK156140). Note: Factors are significant as they cross p=0.05 line.

by the coefficient of determination R^2, which was calculated to be 0.86799, indicating that 96% of the variability in the response could be explained by the model.

The p-value, 0.003, was obtained for the significant variables (Fig. 10). This proves that the model equation as expressed in Eq. (2) provides a suitable model to describe the response of the experiment pertaining to the production of hyaluronic acid. Fig. 11(a) & (b) explains the surface response plot of the model equation, which depicts the critical values such as pH 7.38, beef extract 12.15% and yeast extract 7.64%. The model predicted maximum response for hyaluronic acid concentration of 7.12% for this combination. To confirm the predicted results of the model, experiments using the medium representing this maximum point were performed and a

Table 6. Central composite design for hyaluronic acid production (observed vs predicted value)

Run no	pH	Beef extract (g/L)	Yeast extract (g/L)	Hyluronic acid (g/L) observed	Hyluronic acid (g/L) predicted
1	4.00	4.00	4.00	0.22	0.21
2	4.00	4.00	12.00	0.165	0.18
3	4.00	12.00	4.00	0.34	0.32
4	4.00	12.00	12.00	3.4	2.85
5	10.00	4.00	4.00	0.405	1.12
6	10.00	4.00	12.00	0.195	0.11
7	10.00	12.00	4.00	5.78	5.62
8	10.00	12.00	12.00	0.64	1.12
9	1.95	8.00	8.00	0.035	0.11
10	12.05	8.00	8.00	0.225	0.21
11	7.00	1.27	8.00	6.16	6.21
12	7.00	14.73	8.00	6.36	6.73
13	7.00	8.00	1.27	0.405	0.51
14	7.00	8.00	14.73	1.56	0.54
15	7.00	8.00	8.00	7.16	6.93
16	7.00	8.00	8.00	6.36	6.45

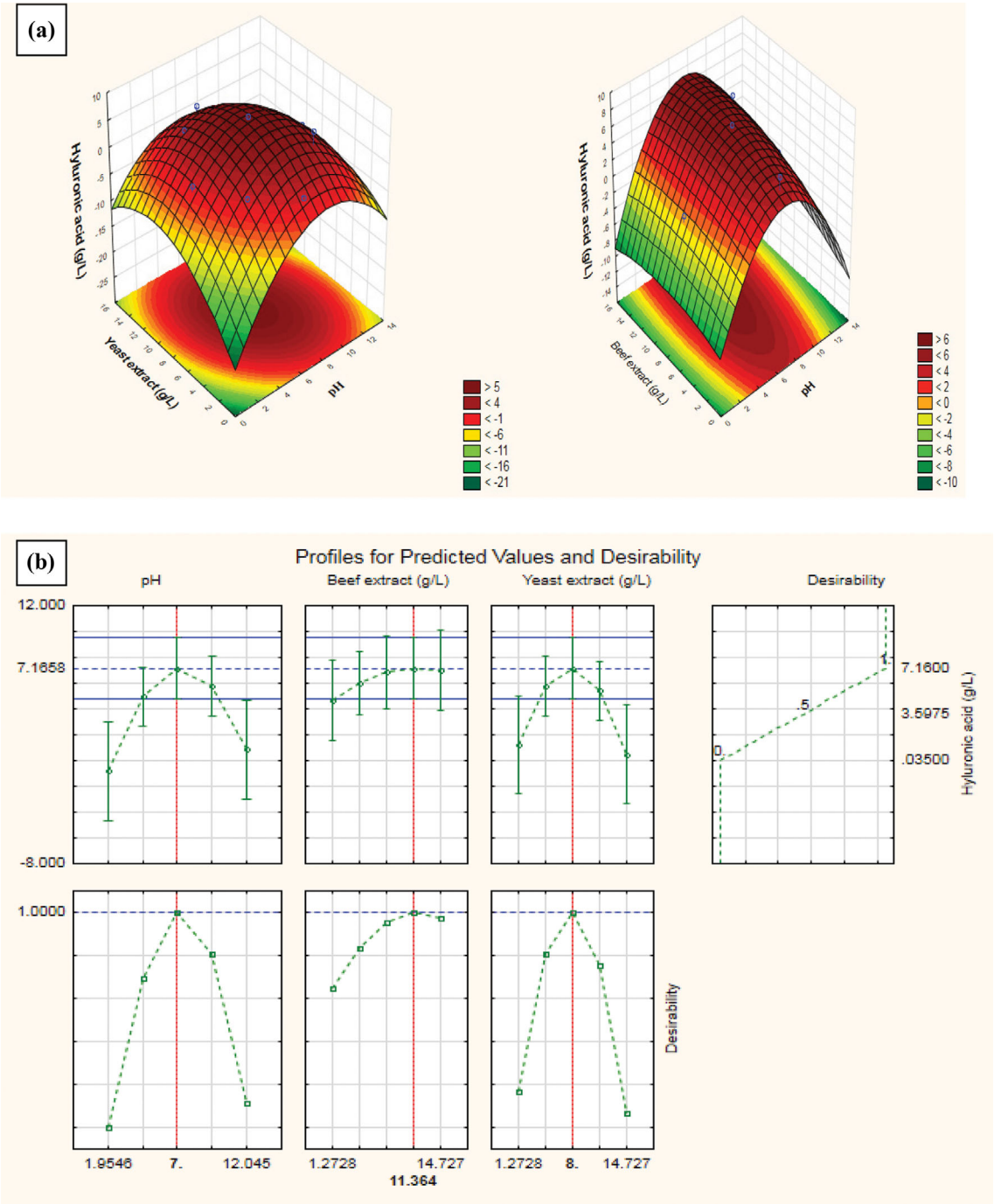

Fig. 11. (a) Contour graphs showing interaction between variables such as Beef extract, yeast extract and pH (b) desirability and predictive values profile.

value of 7.12 (Triplicate experiments were carried out and corresponding standard deviation within ±0.077) was obtained. Thus the composition of basal media was modified by incorporating the optimum components, such as beef extract and yeast extract, 12.15 and 7.12%, respectively, and the final pH of 7.12. The batch growth kinetic profile of hyaluronic acid production from *Streptococcus equisimilus* (MK156140) was compared separately in basal medium. It was observed that in the modified media the production was observed to be high.

The Plackett-Burman design is employed to screen critical independent variables which are affecting HA production. Whereas, high resolution CCD design is used to optimize the critical condition required to higher productivity of HA from selected strain(s).

Aroskar et al. [12] reported 0.70 g/L of hyaluronic acid production from *S. equi* Subsp. *zooepidemicus* ATCC 39920 by optimizing the P10 medium. Also, they stated that there are no reports cited for statistical optimization of medium for the production of HA. In contrast to the statement, our study proposes the optimum media

components along with physical parameters for the highest production of hyaluronic acid (7.16 g/L) from *Streptococcus equisimilus* MK156140.

CONCLUSION

The approach used in this study (Placket Burman design followed by factorial central composite design) allowed screening and determination of the medium composition, which significantly enhanced the production of hyaluronic acid by *Streptococcus equisimilus* (MK156140). In both the cases, suitable models were found to describe the response of the experiments pertaining to the production of hyaluronic acid, as the values obtained experimentally are in accordance with the expected values determined by the models. The models were validated by comparing the observed and predicted values in the optimum point, with a deviation of about 0.007. In the present work, the modified medium enhanced the production of hyaluronic acid by four-fold when it is compared with that of the previous studies. Our study will throw some light on the various optimizing strategies required for the large scale production of HA in industrial scale, as this compound is one of the key components of several pharmaceutically important products.

DECLARATION

Conflict of Interest with Ethical Standards

Authors declare that there is no conflict of interest. No animals or humans were used during the present study.

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AUTHOR CONTRIBUTION

Ashwini K: Planning of work, experimental design, compiling data and manuscript writing.

Savitha J: Provided value added guidance and manuscript editing.

Lokesh N: RSM Data analysis.

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