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Optimization of medium components for hyaluronic acid production by *Streptococcus zooepidemicus* MTCC 3523 using a statistical approach

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ABSTRACT

Hyaluronic acid (HA), produced from *Streptococcus* sp., has raised interest in the medical and cosmetics industries because of the various biological functions of HA. Optimization of medium components used for HA production in *Streptococcus zooepidemicus* MTCC 3523 performed by two-step optimization. A 2^4 full factorial design was employed to study the effect of crucial factors on HA production. To get optimized levels of these factors further, a central composite design was conducted. Through these two phase experiments carried out for optimization of HA production, the medium that yielded maximum average HA comprises 4.05% of glucose, 5.12% of soyapeptone, 0.075% of $MgSO_4 \cdot 7H_2O$, and 0.25% of K_2HPO_4 approximately. The maximum average productivity of HA for this composed medium was 0.798 g/L on fermentation in shake flask. The yield of HA was increased by about 65% using these statistical techniques of media optimization.

1. Introduction

Hyaluronic acid (HA) is a uniformly repetitive, linear, high-molecular-weight glycosaminoglycan composed of 2000–25,000 disaccharides of glucuronic acid and N-acetylglucosamine joined alternately by β-1-3 and β-1-4 glycosidic bonds (Chong, Blank, Mclaughlin, & Nielsen, 2005). Owing to its variety of biological functions, HA has a wide range of applications in the fields of medicine and cosmetics, including osteoarthritis treatment, ophthalmic surgery, plastic surgery, drug delivery, skin moisturizers, and wound healing (Chong et al., 2005; Goa & BenWeld, 1994; Kogan, Soltes, Stern, & Gemeiner, 2007).

Conventionally, HA was extracted from animal tissues like rooster combs and is now increasingly produced by microbial fermentation with a lower production cost. Currently, the commonly used strain for microbial HA production on an industrial scale is *Streptococcus zooepidemicus*, which synthesizes HA as the extracellular capsule (Duan, Yang, Zhang, & Tan, 2008). Much work, such as improving the fermentation process (Armstrong & Johns, 1997; Blank, McLaughlin, & Nielsen, 2005; Duan et al., 2008; Hasegawa, Nagatsuru, Shibutani, Yamamoto, & Hasebe, 1999; Huang, Chen, & Chen, 2006; Huang, Chen, & Chen, 2008; Johns, Goh, & Oeggerli, 1994; Kim, Yoo, Oh, & Kweon, 1996; Kim, Park, & Kim, 2006; Liu, Wang, Du, & Chen, 2008b), adding lysozyme (Kim et al., 1996; Ogrodowski, Hokka, & Santana, 2005), the alkaline-stress strategy (Liu, Wang, Du, & Chen, 2008a), adding hydrogen peroxide and ascorbate (Liu et al., 2009), and changing the medium composition (Rangaswamy & Jain, 2008; Zhang, Ding, Yang, & Kong, 2006), has been done to increase the production yield of hyaluronic acid in *Streptococcus* sp. Although many studies have been performed on HA production, there are very few reports on general medium optimization higher HA production and on economically efficient conditions for HA production.

In this study, we carried out optimization of medium components to produce higher amount of HA in *S. zooepidemicus* MTCC 3523. In the first step, the effects of medium component levels, such as carbon source, nitrogen source, phosphate source, and mineral sources, on HA production were investigated by full factorial design. In the second step, concentrations of selected medium components were optimized using central composite design to improve the productivity of HA.

2. Materials and methods

2.1. Microorganism and culture medium

Streptococcus equi subsp. *zooepidemicus* MTCC 3523 was obtained from the Microbial Type Culture Collection (Chandigarh, India) as a freeze-dried culture in ampoules. The stock culture preserved in 20% glycerol solution at −86 °C was cultivated on slants of streptococcus agar containing (g/L): glucose 20, pancreatic digest of casein 20, K_2HPO_4 2, $MgSO_4 \cdot 7H_2O$ 0.1, agar 15, final pH adjusted

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Table 1
Coded levels of fermentation media ingredients for FFD.

Factor	Coded levels	
	+1 (%)	−1 (%)
Glucose (X_1)	4.5	1.5
Soyapeptone (X_2)	4.5	1.5
$MgSO_4 \cdot 7H_2O$ (X_3)	0.15	0.075
K_2HPO_4 (X_4)	0.5	0.25

Table 2
Coded levels of important media ingredients for CCD.

Coded levels of X_1 and X_2	Actual levels (%)
−1.414	0.88
−1	1.50
0	3
1	4.50
1.414	5.12

6.8 at 25 °C and incubated at 37 °C for 24 h. A loopful of cells from the agar slant was transferred to 50 ml of sterilized Todd Hewitt broth medium in a 250 ml Erlenmeyer flask as seed culture and incubated at 37 °C for 12 h. This was used as the inoculum for the production of HA. The production of HA was carried out in 500 ml Erlenmeyer flasks containing 100 ml media as per experimental design (Tables 3a and 4a). The HA production medium was aseptically inoculated with 9 ml of 12-h-old seed culture. The pH of the medium was adjusted to 7.5 before sterilization. The medium was sterilized in an autoclave for 20 min at 121 °C, except glucose. Glucose solution was sterilized by autoclaving separately and mixed aseptically with other components on cooling. The inoculated flask was incubated on a rotary shaker at 37 °C and 200 rpm for 24 h.

2.2. Analytical methods

Exopolysaccharide produced by *S. zooepidemicus* MTCC 3523 was identified and confirmed as hyaluronic acid by its chemical characterization (Patil, Patil, Chaudhari, & Chincholkar, 2011). HA produced, processed by the method of Blank et al. (2005) where fermentation broth was diluted with an equal volume of 0.1% (w/v) SDS and incubated at room temperature for 10 min to free capsular EPS which was filtered through 0.45 μm membrane. Filtrate was subjected to determine mucopolysaccharides by a turbidimetric assay (Chen & Wang, 2009; Di Ferrante, 1956; Song, Im, Kang, & Kang, 2009). Briefly, 200 μl of the filtrate was mixed with 200 μl of 0.1 mol L^{-1} acetic acid (pH 6) and 400 μl of 2.5% (w/v) cetyltrimethyl-ammoniun bromide (CTAB) in 0.5 M NaOH. The mixture was incubated for 20 min at room temperature and A_{595} was recorded. A standard curve was established using a 0.18 g/L HA stock solution prepared using standard HA of microbial origin (Focuschem, China).

2.3. Experimental designs

After optimizing culture conditions using single factor at a time (Patil, Chaudhari, & Chincholkar, 2009), the concentrations of selected media ingredients on HA production by the strain *S. equi.* subsp. *zooepidemicus* MTCC 3523 were optimized using statistical techniques. In the first phase of the experimentation, effects of glucose, soyapeptone, $MgSO_4 \cdot 7H_2O$ and K_2HPO_4 on maximum HA production were investigated using a quadratic full factorial design (2^4 FFD). Factorial designs are very effective to identify the significance of factors among many other factors. While response surface methodology is a collection of mathematical and statistical techniques that are useful for the modelling and analysis of problems in which a response of interest is influenced by several variables and the objective was to optimize this response.

2.3.1. Full factorial design (FFD)

The four factors (glucose, soyapeptone, $MgSO_4 \cdot 7H_2O$ and K_2HPO_4) examined at two levels are as listed in Table 1. The choice of levels of the factors was based on information from literature and preliminary experiments. A 2^4 full factorial experiment was conducted in triplicate with *S. equi.* subsp. *zooepidemicus* MTCC 3523. A total of 16 experiments were carried out in randomized run order. The response variable selected was the maximum concentration of hyaluronic acid. Results were analyzed using analysis of variance (ANOVA), using software MINITAB.

2.3.2. Central composite design (CCD)

RSM is an empirical modelling technique devoted to the evaluation of relations existing among a group of controlled experimental factors and observed results of one or more selected criteria. Central composite experimental design (CCD) (Box & Wilson, 1951) was used to further optimize the important media ingredients like % glucose (X_1) and % soyapeptone (X_2). The preliminary investigations from FFD had indicated that glucose and soyapeptone were highly significant variables for production of HA in our study. The effect of the two variables % of glucose and % of soyapeptone on the average HA production (Y) is modelled by fitting the following second order polynomial Eq. (1) using the data obtained from CCD experiment.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (1)$$

The levels of independent variables chosen for conducting CCD experiment and the corresponding design matrix are shown in Tables 2 and 4a, respectively. A total of 13 experiments were carried out in randomized run order. The response variable selected was the maximum concentration of HA (Y). Results were analyzed using MINITAB software.

3. Results

3.1. Full factorial design

Table 3a presents the matrix of the 2^4 full factorial design with coded values for independent variables X_1, X_2, X_3, X_4 and the corresponding HA production (response Y) for all the three replicates. Statistical analysis of full factorial experiment conducted is given in Table 3b and it shows that the marginal effects of individual factors like glucose (contribution in TSS = 7.16%), soyapeptone (contribution in TSS = 49.89%) and combined effect of all the four factors (contribution in TSS = 14.33%) on HA production was significant. Some other, second and third order interaction effects were also significant. Table 3b also indicates the ranking of significant factors glucose, soyapeptone, $MgSO_4 \cdot 7H_2O$, and K_2HPO_4 as 2nd, 1st, 4th and 3rd, respectively, to maximize the HA production. Soyapeptone and glucose have shown superior effects than $MgSO_4 \cdot 7H_2O$, and K_2HPO_4 in maximizing HA production. To check relative significance in the three significant factors X_1, X_2, and X_4 we calculated the % variation attributed by each of the significant factors and interactions with respect to the total variation in the experimental data was calculated. The factor X_2 was relatively more significant than factor X_4. Hence, the two factors soyapeptone and glucose which had higher significant effect on production of HA (by *S. equi* subsp. *zooepidemicus* MTCC 3523) were taken into consideration for further study. The remaining two factors X_3 and X_4 were fixed at their low (−1) and high (+1) levels, respectively, during subsequent experiments based on the effect plots of all the four factors (Fig. 1). MINITAB creates the main effect plot by plotting the data mean for each factor level. On connecting the points of each fac-

Table 3a
Two level 4-factor full factorial design with coded values along with the observed results.

Run	Factors				HA (g/L)		
	Glucose (X_1)	Soyapeptone (X_2)	$MgSO_4{\cdot}7H_2O$ (X_3)	K_2HPO_4 (X_4)	Replicate 1	Replicate 2	Replicate 3
1	−1	−1	−1	−1	0.172	0.189	0.175
2	1	−1	−1	−1	0.242	0.253	0.248
3	−1	1	−1	−1	0.397	0.416	0.442
4	1	1	−1	−1	0.262	0.280	0.271
5	−1	−1	1	−1	0.120	0.160	0.144
6	1	−1	1	−1	0.191	0.179	0.185
7	−1	1	1	−1	0.223	0.270	0.275
8	1	1	1	−1	0.588	0.462	0.676
9	−1	−1	−1	1	0.328	0.310	0.300
10	1	−1	−1	1	0.300	0.326	0.330
11	−1	1	−1	1	0.351	0.284	0.344
12	1	1	−1	1	0.473	0.446	0.490
13	−1	−1	1	1	0.097	0.100	0.094
14	1	−1	1	1	0.248	0.257	0.245
15	−1	1	1	1	0.493	0.455	0.446
16	1	1	1	1	0.416	0.468	0.451

Table 3b
ANOVA for FFD.

Source	DF	SS	MSS	*f*-Ratio	*p*-Value	% contribution in TSS
Repl	2	0.002391	0.001196	1.12	0.340	0.28
X_1	1	0.060185	0.060185	56.35	0.000	7.16
X_2	1	0.419490	0.419490	392.77	0.000	49.89
X_3	1	0.003139	0.003139	2.94	0.097	0.37
X_4	1	0.031657	0.031657	29.64	0.000	3.76
$X_1 \times X_2$	1	0.000111	0.000111	0.10	0.749	0.01
$X_1 \times X_3$	1	0.034033	0.034033	31.87	0.000	4.05
$X_1 \times X_4$	1	0.000001	0.000001	0.00	0.973	0.00
$X_2 \times X_3$	1	0.076773	0.076773	71.88	0.000	9.13
$X_2 \times X_4$	1	0.000307	0.000307	0.29	0.596	0.04
$X_3 \times X_4$	1	0.008518	0.008518	7.98	0.008	1.01
$X_1 \times X_2 \times X_3$	1	0.006140	0.006140	5.75	0.023	0.73
$X_1 \times X_2 \times X_4$	1	0.001670	0.001670	1.56	0.221	0.20
$X_2 \times X_3 \times X_4$	1	0.004502	0.004502	4.21	0.049	0.54
$X_1 \times X_3 \times X_4$	1	0.039367	0.039367	36.86	0.000	4.68
$X_1 \times X_2 \times X_3 \times X_4$	1	0.120509	0.120509	112.83	0.000	14.33
Error	30	0.032041	0.001068			3.81
Total	47	0.840835				100.00

tor, the line is created which states the significance. There is no main effect present when the line is flat. However, when the line is not flat, then there is a main effect present. In effect plots of our study, it was observed that the higher average HA production was obtained using high level (4.5%) of glucose, high level (4.5%) of soyapeptone, low level (0.075%) of $MgSO_4{\cdot}7H_2O$ and high level (0.25%) of K_2HPO_4.

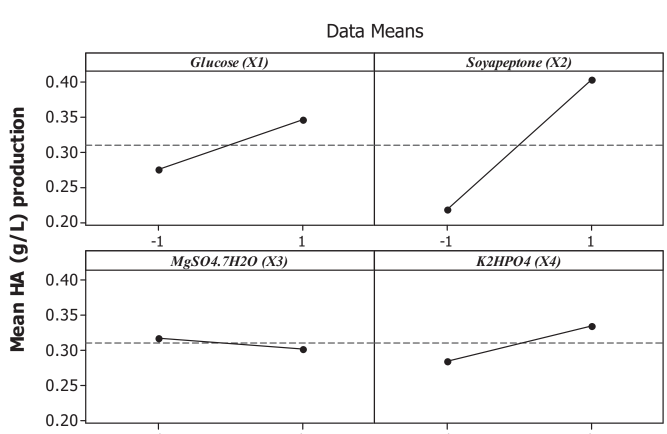

Fig. 1. Main effect plots of glucose (X_1), soyapeptone (X_2), $MgSO_4{\cdot}7H_2O$ (X_3), and K_2HPO_4 (X_4) for HA production.

3.2. Central composite design

This is a very useful tool to determine the optimal level of medium constituents and their interaction for maximizing the production of desired product/metabolite. At the first stage of the experiment, based on the FFD, two factors glucose (X_1) and soyapeptone (X_2) were selected since it significantly affected the HA production. CCD was used for further optimization. Table 2 gives the levels at which these components were supplemented to HA production. The concentration of $MgSO_4{\cdot}7H_2O$ was set at 0.075% and

Table 4a
Central composite design and experimentally observed responses.

Standard order	Glucose (X_1)	Soyapeptone (X_2)	HA produced (g/L)
1	−1	−1	0.437
2	1	−1	0.449
3	−1	1	0.505
4	1	1	0.677
5	−1.414	0	0.552
6	1.414	0	0.343
7	0	−1.414	0.364
8	0	1.414	0.863
9	0	0	0.503
10	0	0	0.484
11	0	0	0.524
12	0	0	0.470
13	0	0	0.536

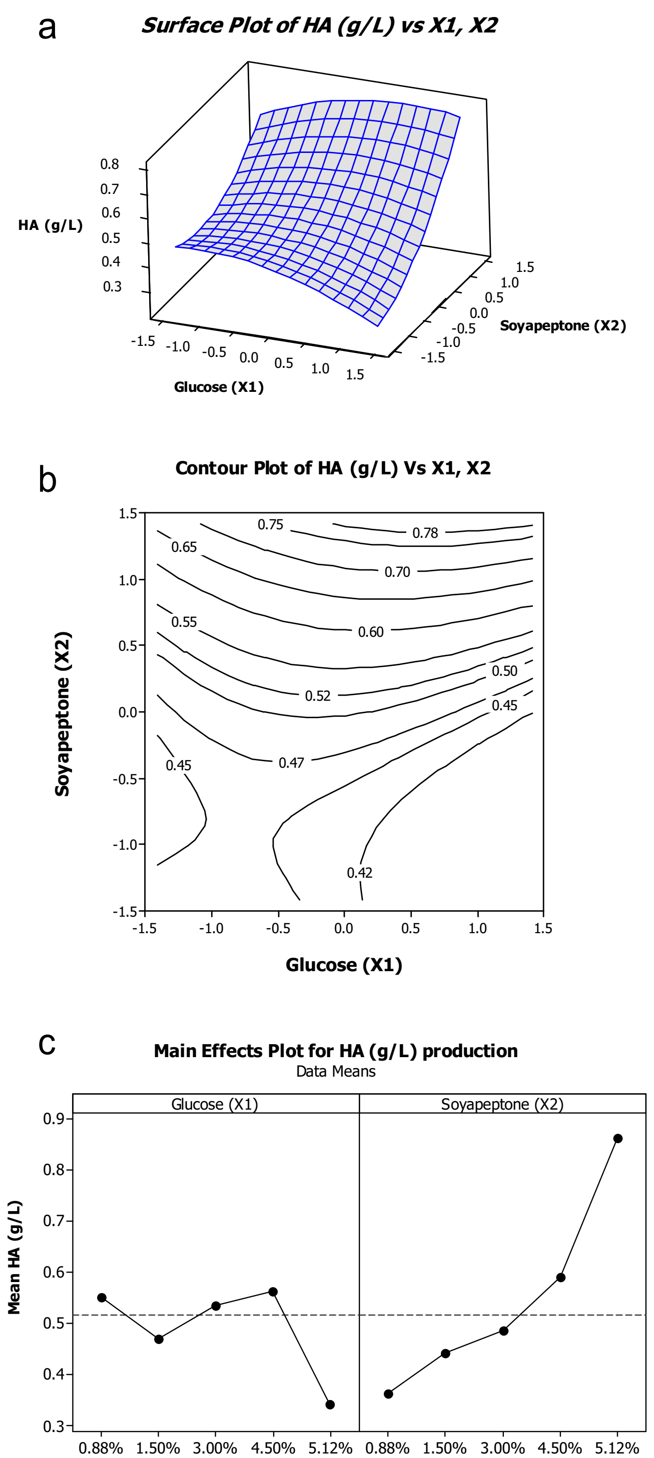

Fig. 2. (a) Response surface plot of effect of glucose and soyapeptone on HA production. (b) Contour plot of the combined effects of glucose and soyapeptone on HA production. (c) Main effect plots of glucose (X_1) and soyapeptone (X_2) for HA production.

that of K_2HPO_4 was fixed at 0.25%. Table 4a gives the design of CCD and results of experiments. The results were analyzed using analysis of variance technique (Table 4c). The regression model fitted for the data of the experiment is shown in Eq. (2).

$$Y = 0.5034 - 0.01395X_1 + 0.12521X_2 - 0.03132X_1^2 + 0.05168X_2^2 + 0.04X_1X_2 \quad (2)$$

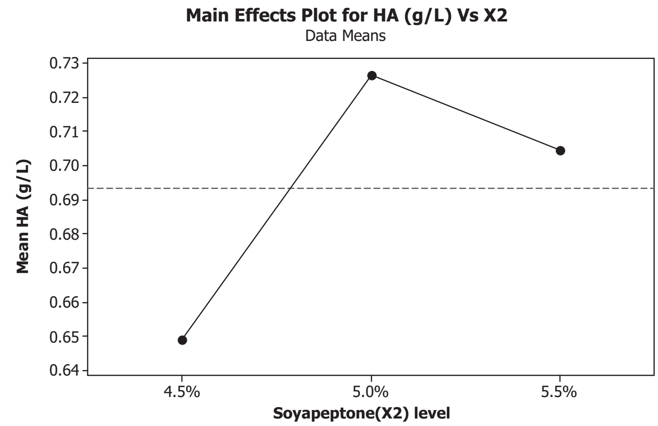

Fig. 3. Main effect plots of soyapeptone (X_2) (extended level) for HA production.

Table 4b
Estimated regression coefficients for fitted response surface model.

Term	Coefficient	Standard error of coeff.	Computed *t*-value	*p*-Value
Constant	0.50340	0.03894	12.927	0.000
X_1	−0.01395	0.03079	−0.453	0.664
X_2	0.12521	0.03079	4.067	0.005
X_1^2	−0.03132	0.03301	−0.949	0.374
X_2^2	0.05168	0.03301	1.565	0.162
X_1X_2	0.04000	0.04354	0.919	0.389

$S = 0.08707$, $R^2 = 75.3\%$, adj. $R^2 = 57.7\%$

The response surface graph corresponding to the above fitted model is shown in Fig. 2a and the contour plot of model equation is shown in Fig. 2b. The response surface graph and contour plots are graphical representations of the regression equation. It shows a visible interpretation of the interaction between two variables which states the location of optimum experimental conditions. The response surface with circular contour plot indicates the negligible interaction between the corresponding variables, whereas elliptical or saddle nature of the contour plots indicates the significant interactions between the corresponding variables. Using the response optimizer tool in MINITAB we obtained the optimal levels of glucose and soyapeptone as 4.05% and 5.12%, respectively, with the corresponding average HA production 0.798 g/L. This can also be observed from the main effect plots for the two factors glucose and soyapeptone in Fig. 2c. From the graph it seems that the average HA production may increase beyond the highest level (*i.e.* 5.12%) of soyapeptone. But by our experimental knowledge we had assurance that it may not happen. Hence to verify this we conducted another experiment in which we considered variation of % of soyapeptone in range 4.5–5.5 at an increment of 0.5 and HA production was recorded. The experiment was performed in triplicate and the corresponding results are shown in Table 5. The mean value of HA production at various % of soyapeptone is graphically shown in Fig. 3 and from this it can be seen that instead of increase in HA pro-

Table 4c
ANOVA for CCD.

Source	DF	SS	MSS	*f*-Ratio	*p*-Value
Regression	5	0.162210	0.032442	4.28	0.042
Linear	2	0.126980	0.063490	8.37	0.014
Square	2	0.028830	0.014415	1.90	0.219
Interaction	1	0.006400	0.006400	0.84	0.389
Residual error	7	0.053073	0.007582		
Lack-of-fit	3	0.050094	0.016698	22.42	0.006
Pure error	4	0.002979	0.000745		
Total	12	0.215283			

Table 5
Response of extended soyapeptone level on HA production.

Soyapeptone level (%)	HA produced (g/L)		
	Replicate 1	Replicate 2	Replicate 3
4.5	0.638	0.660	0.651
5.0	0.742	0.711	0.724
5.5	0.691	0.718	0.702

duction beyond the 5.12% level of soyapeptone it decreases. Hence we conclude that the average HA production is maximized at 4.05% of glucose and 5.12% of soyapeptone. RSM is a sequential procedure with an initial objective of leading the experimenter rapidly and efficiently to the general vicinity of the optimum. Since the location of the optimum is unknown prior to running RSM experiments, it makes sense to use a design that provides equal precision of estimation in all directions. A detailed account of the technique has been outlined by Cochran and Cox (1968). Furthermore, statistical inference techniques can be used to asses the importance of individual factors, the appropriateness of this functional form and sensitivity of the response to each factor (Mason, Gunst, & Hers, 1989). In this study, through these two phases of experiments carried out for optimization of HA production, the medium that yields maximum average HA comprises 4.05% of glucose, 5.12% of soyapeptone, 0.075% of $MgSO_4{\cdot}7H_2O$, and 0.25% of K_2HPO_4 approximately. The maximum average productivity of HA for this composed medium derived from Eq. (2) is 0.798 g/L on shake flask fermentation which is obtained using response optimizer. Among individual effect of nutrients, soyapeptone, glucose and K_2HPO_4 were found to be the most significant variables enhancing hyaluronan production (Table 3b). Glucose (X_1) and soyapeptone (X_2) were selected for further study, as carbon and nitrogen source plays vital role in fermentative production of HA. The significance of each coefficient can be seen from the *t* and *p* values listed in Table 4b. Soyapeptone (X_2) had a significant effect ($p = 0.005$) on HA yield, *Y* as it had the largest coefficient. Positive coefficient was observed for the quadratic terms of soyapeptone (X_2) and interaction term X_1X_2. However, glucose (X_1) and its quadratic term X_1^2 had a negative effect on *Y*. In conclusion, the yield of HA, which was reached to 0.485 g/L (Patil et al., 2009) by partial optimization of media using single factor at a time was enhanced to 0.798 g/L through this statistical techniques of media optimization. There was about 65% increase in the yield of HA.

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