Design-Expert Computational Software-Assisted Optimization adopting Plackett-Burman and Box-Benkhen Design for Production of Glycosaminoglycan

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***Abstract*— Design-Expert (DX) Software has been a leading software package in analysis and design of experiments (DOE). DX provides cutting-edge, easy-to-use tools that donot require lengthy, formal statistical training. It allows users conduct multi-variate data analysis, reducing experimental runs, thus saving time and cost of product development, while ensuring the best optimization desired production process. Glycosaminoglycan (GAG - a polysaccharide of clinical significance) is obtained from animal tissue by an expensive low-yield extraction process; however, they are highly commercially valued polysaccharides highly exploited in the biomedical market. The aim of this research was to screen and optimize production of GAG using Design-Expert computational software adopting Plackett-Burman and Box-Benkhen design. Plackett-Burman design was used to screen six media components (Sodium-acetate, cabbage peel, NH4Cl, K2HPO4, MgSO4, CaSO4 saturated solution and trace element solution). Sodium acetate (Na-acetate), cabbage peel, NH4Cl, K2HPO4, and trace element solution were assessed as critical factors and therefore subjected to response surface methodology (RSM) using Box-Benkhen design. The optimal medium composition by RSM was composed of 0.10g, 0.30g, 0.15g, 0.01g and 2.50mL for Na-acetate, Cabbage peels, NH4Cl, K2PO4 and Trace ES respectively. Maximum GAG yield was predicted to be 104.21%. Under optimized medium, GAG yield was 105%, which was similar to the predicted value of 104%. A validation experiment was conducted using some selected predicted values and results closely correspond to that of experimental values. Findings from this research revealed that GAG yield was successfully optimized using response surface methodology; hence this implies reliability, efficiency and potentiality of the optimized media for bulk synthesis of GAG for industrial application as medium for control release of drugs, cosmetics and tissue engineering.**

***Keywords*— Design Expert Software, Design of Experiment, Box-Benkhen design, Response-Surface, Plackett-Burman**

I. INTRODUCTION

Design–Expert (DX) is a Computational Software Package developed by Stat-Ease International Company. The software was specifically dedicated to performing design of experiments (DOE) for research purpose. The history of Stat-Ease commenced in the 1970s when its original principals; Pat Whitcomb, Tryg Helseth and Mark Anderson mastered design of experiments (DOE) while working for General Mills Chemical Division's Process Development group. When IBM came out with its first PC in 1981, Pat recognized an opportunity to make DOE easy for non-statisticians via a menu-driven computer program, which he named "Design-Ease" (DE). Pat then established "Stat-Ease" with the intriguing slogan "Statistics Made Easy". After several years of preparation, DE took off in 1987 when touted as "incredibly easy to use" in a widely read Journal of Quality Technology review on "Tools for Computer-Aided Experiments." In 1988, the company released its first version of Design-Expert (DX), which provided response surface methods (RSM) for process optimization. This new program complemented DE and its capability for screening main effects and characterization of interactions. DX also provided mixture designs for optimizing formulations—a big attraction for users in the process industries. In 1996 Stat-Ease incorporated all the features of Design-Ease into Design-Expert version 5 and translated it to Windows. The crucial need for a graphical user-interface led to a deluge of upgrades, the proceeds from which the three Principals reinvested into further expansion. However, in 1996 Forbes recommended Stat-Ease software for multivariable testing ("MVT") and same year www.statease.com was launched on Internet.

In the year 2000, Version 6 of DE and DX were released. In 2007, Versions 7.1 of DX and DE were also released (a major upgrade) while in 2021, Version 13 of DX was released and Stat-Ease 360 premiered [1].

DX software offers a wide range of designs, including factorials, fractional factorials and composite designs. It can handle both process variables and also mixture variables. Design Expert offers computer generated D-optimal designs for cases where standard designs are not applicable, or where pre-existing design needs to be augmented, in order to fit a more flexible model [2].

Response surface methodology (RSM) is an assortment of mathematical and statistical tools, used for designing experiments and achieving optimum conditions of independent variables/factors for desirable responses among various tools embedded within the DX computational Software [3,4]. Various approaches in response surface methodology (RSM) have been employed for screening and optimization of different media components [5]. RSM based on Box–Behnken experimental design has been widely used in various bioprocess experiments to optimize media components and process parameters [4,6].

Thiothrix nivea is a filamentous sulfur-oxidizing bacterium commonly found in activated sludge, it consists of a filament which is covered with a polysaccharide layer called a sheath. This polysaccharide layer was reported by [7] to consist of alternating units of glucose and glucosamine, forming a long chain of glycosaminoglycan (GAG). The productivity of the polysaccharide is dependent on sheath formation, thus sheath length is the same as that of a line of cells enclosed within the sheath; improvement of sheath productivity means improvement of cell growth [8]. Moreover, the glycosaminoglycan produced from T. nivea so far has not been exploited for large scale production [9]

Glycosaminoglycans (GAGs) are long-chain polysaccharides that are divided into sulphates and non-sulphates, these include chondroitin sulphate, heparan sulphate, dermatan sulphate, heparin sulphate, including non-sulphated hyaluronic acid. Some bacterial strains synthesize polysaccharides with the same backbone structure as GAGs; however, these GAG-like polysaccharides are not attached to core proteins [10]. GAGs are obtained from animal tissue by an expensive low-yield extraction process; however, they are highly commercially valued polysaccharides exploited in the biomedical market [11] utilized as: treatments in the cosmetic area, moisturizing medium, means for encapsulation and controlled release of drugs and cosmetics, in treatment of degenerative and inflammatory diseases of bone joints, in replacement of liquid synovial, in release of chemotherapeutic agents in surgical implants among others [12].

The present study is organized into five sections in the following pattern: Introduction, Methodology, Results and Discussion, Conclusion and lastly References.

II. METHODOLOGY

Screening of media components using Plackett–Burman factorial design for screening purpose, various media components were evaluated based on Plackett–Burman factorial design [13]. Each independent factor was examined in two levels: -1 for low level and +1 for high level. This refers to fraction of a two-level factorial design and allows investigation of n-1 variables in at least n experiments. Seven independent variables were screened in 12 combinations according to the design. The main effect of each variable was calculated simply as difference between the average of measurements made at high setting (+1) and the average of measurements observed at low setting (-1) of that factor. All trials were carried out in 250 ml Erlenmeyer flasks containing 50 ml of the medium. Plackett–Burman design is based on the first order model:

$$Y=\beta o+\Sigma\ \beta i\ xi \quad \text{..... (i)}$$

Where Y is the response (GAG yield), βo is the model intercept, βi is the variable estimate and xi represents the variable. The significance of variables was determined by calculating p-value through standard regression analysis.

A. Design of experiment (DOE)

For DOE selection, response surface method was chosen employing Design-Expert Software Version 11.1.0.1 (Stat-Ease Inc., USA). Box–Behnken design was chosen for optimization study, because it gave a good interaction with fiver-factors and three-level design. On the basis of factor screening studies by Plackett-Burman design, the method performance was optimized using Box–Behnken response surface design at three levels, i.e. low (− 1), middle (0) and high (+ 1). A design matrix listing 46 experimental runs was generated using Design Expert tools. Various interaction and quadratic effects were also studied.

B. Response surface methodology (Box–Behnken design)

After estimating the relative significance of independent variables in previous research, the most significant four variables were selected for further determination of their optimal level with respect to glycosaminoglycan (GAG) yield as a response. For this reason Box–Behnken design was applied for optimization studies [14]. Optimization process involves three main steps: performing statistically designed experiments, estimating coefficients of the structured mathematical model and predicting response and checking adequacy of the model. The effects of sodium acetate, cabbage peel, ammonium sulphate, potassium phosphate and trace element solution was evaluated. Levels of these factors were optimized for maximum glycosaminoglycan production (response).

Once GAG yield was measured, a second-order polynomial model was fitted to the response data obtained from the design. The polynomial equation is in the following form:

$$Y = \beta_0 + \beta_1 X_I + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_I X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_I + \beta_{22} X_2 + \beta_{33} X_3 \quad \text{(ii)}$$

where Y is predicted response, β0 is model constant; X1, X2 and X3 are independent variables; β1, β2 and β3 are linear coefficients; β12, β13 and β23 are interaction coefficients and β11, β22 and β33 are quadratic coefficients.

C. *Data Analysis*

The simultaneous effects of five most significant independent factors on each response were visualized using three-dimensional graphs generated by STATISTICA 5.0. within the Design-Expert Software. All experiments were carried out in triplicates and data represented as mean values. Various statistical parameters such as probability value (p-value), regression coefficient (R2 value), the Fisher model value (F value), and lack of fit F value were used to adapt the responses to the appropriate mathematical model developed by the design. In addition to the best fit model, quadratic polynomial response equations with key factors and interaction factors have been developed. The presented model's suitability and validity were assessed using ANOVA.

III. Results and Discussion

A. *Application of Plackett–Burman design*

Seven independent culture variables were examined for production of GAG (glycosaminoglycan). The used basal medium has the following composition (g/250mL): 0.01 g sodium acetate (Na-acetate), 0.04 g cabbage peel, 0.02 g NH4Cl, 0.01 g K2HPO4, 0.01 g MgSO4·7H2O, 2.5 mL CaSO4 saturated solution, and 0.62 mL trace element solution (EDTA -0.2g, FeSO4 x 7 H2O -0.7 g, ZnSO4 x 7 H2O -0.01g, MnSO4 x 4 H2O -0.002g, CuSO4 x 5 H2O -5µg, H3BO3 -10mg, Co(NO3) -1mg, Na2MoO4 x 2 H2O -1mg, per 1000ml of Distilled water). The uncoded design matrix along with the corresponding response (GAG yield) is shown in Table 1.

A wide variation was shown for productivity throughout different trials of the experiment. The variation ranged from 41 to 118 %. This reflects the significant effect of medium composition on GAG production. The main effect of examined variables on GAG production was calculated and illustrated graphically in (Figure 1).

TABLE 1: PLACKETT-BURMAN EXPERIMENTAL DESIGN OUTPUT FOR SCREENING MEDIA COMPONENTS FOR GAG PRODUCTION BY *Thiothrix nivea*

Std	Run	Na-acetate (A)	Cabbage peels (B)	NH_4Cl (C)	K_2HPO_4 (D)	$MgSO_4$ (E)	$CaSO_4$ Sat. solu. (F)	Trace Element solution (G)	Response GAG Yield (%)
4	1	0.02	0.04	0.05	0.02	0.01	5.00	0.62	41.81
6	2	0.02	0.08	0.05	0.01	0.02	5.00	0.62	54
8	3	0.01	0.04	0.05	0.02	0.02	2.50	1.25	82.78
7	4	0.01	0.08	0.05	0.02	0.01	5.00	1.25	94.98
10	5	0.02	0.04	0.02	0.01	0.02	5.00	1.25	52.26
11	6	0.01	0.08	0.02	0.01	0.01	5.00	1.25	68.49
5	7	0.02	0.08	0.02	0.02	0.02	2.50	1.25	85.36
2	8	0.02	0.08	0.02	0.02	0.01	2.50	0.62	90.46
3	9	0.01	0.08	0.05	0.01	0.02	2.50	0.62	87.1
12	10	0.01	0.04	0.02	0.01	0.01	2.50	0.62	29.61
9	11	0.01	0.04	0.02	0.02	0.02	5.00	0.62	47.03
1	12	0.02	0.04	0.05	0.01	0.01	2.50	1.25	71.42

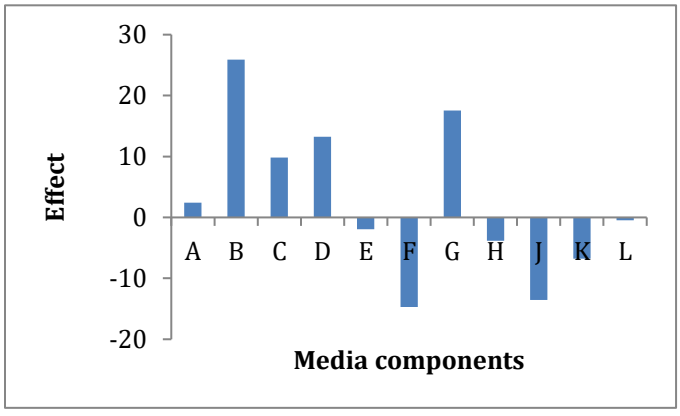

Fig. 1: Pareto chart of media component effects on GAG production from *Thiothrix nivea*

The size of a factor estimate indicates, on average, which factor setting results in higher GAG yield. A large estimate, either positive or negative close to zero means that a factor has little or no effect. On the basis of these results; it is conceivable that

GAG yield is directly related to sodium-acetate (A), cabbage peels (B), ammonium chloride (C), potassium phosphate (D) and trace element solution (G).

B. Optimization of the culture conditions by Box–Behnken experimental design

Plackett–Burman analysis is a very convenient tool at the initial phase of optimization; however, it does not reflect the interactions that may occur between the various components of the matrix, thus the need for a second multi-factorial experiment [15]. Box Behnken design (a response surface methodology) was applied to elucidate optimum level of each most significant independent variable leading to maximum production of GAG.

Each of the five factors of highest influence as elucidated through Plackett–Burman experimental design was examined at three different levels coded -1, 0, +1. The 46 different examined combinations are shown in (Table 2), where all experiments were performed in triplicates.

TABLE 2: BOX-BENKHEN EXPERIMENTAL DESIGN MATRIX FOR OPTIMIZATION OF MEDIA COMPONENTS FOR GAG PRODUCTION

STD	**RUN**	**Sodium-acetate (A)**	**Cabbage peels (B)**	**NH_4Cl (C)**	**K_2HPO_4 (D)**	**Trace Elem. Solu. (E)**	**RESPONSE (*T. nivea* % yield)**	**Predicted values**
32	1	0.08	0.30	0.20	0.01	5.00	83.00	82.24
21	2	0.08	0.20	0.10	0.01	3.75	73.00	71.24
23	3	0.08	0.20	0.20	0.01	3.75	82.00	80.60
44	4	0.08	0.30	0.15	0.01	3.75	83.79	82.25
7	5	0.08	0.30	0.10	0.02	3.75	86.00	83.51
42	6	0.08	0.30	0.15	0.01	3.75	83.41	82.25
13	7	0.05	0.30	0.10	0.01	3.75	82.00	82.29
2	8	0.10	0.20	0.15	0.01	3.75	60.77	62.71
11	9	0.08	0.20	0.15	0.01	5.00	64.00	66.47
17	10	0.08	0.30	0.15	0.01	2.50	81.00	80.46
30	11	0.08	0.30	0.20	0.01	2.50	87.00	88.12
8	12	0.08	0.30	0.20	0.02	3.75	84.00	81.92
46	13	0.08	0.30	0.15	0.01	3.75	84.98	82.25
14	14	0.10	0.30	0.10	0.01	3.75	75.00	76.34
36	15	0.10	0.30	0.15	0.01	5.00	63.00	63.00
37	16	0.08	0.20	0.15	0.01	3.75	73.00	73.08
6	17	0.08	0.30	0.20	0.01	3.75	90.40	88.43
45	18	0.08	0.30	0.15	0.01	3.75	84.45	82.25
31	19	0.08	0.30	0.10	0.01	5.00	81.00	80.88
3	20	0.05	0.40	0.15	0.01	3.75	74.97	74.31
33	21	0.05	0.30	0.15	0.01	2.50	61.00	61.66
1	22	0.05	0.20	0.15	0.01	3.75	88.00	88.00
9	23	0.08	0.20	0.15	0.01	2.50	84.00	85.36
35	24	0.05	0.30	0.15	0.01	5.00	102.0	101.78
4	25	0.10	0.40	0.15	0.01	3.75	102.6	102.83
10	26	0.08	0.40	0.15	0.01	2.50	80.00	80.51
34	27	0.10	0.30	0.15	0.01	2.50	105.0	104.21
12	28	0.08	0.40	0.15	0.01	5.00	95.00	96.63
19	29	0.08	0.30	0.15	0.01	5.00	82.00	83.09
29	30	0.08	0.30	0.10	0.01	2.50	76.00	77.75
5	31	0.08	0.30	0.10	0.01	3.75	77.50	75.12
38	32	0.08	0.40	0.15	0.01	3.75	89.88	90.47
26	33	0.10	0.30	0.15	0.01	3.75	84.00	85.03
20	34	0.08	0.30	0.15	0.02	5.00	79.00	80.03
43	35	0.08	0.30	0.15	0.01	3.75	83.99	82.25
28	36	0.10	0.30	0.15	0.02	3.75	80.00	80.52
25	37	0.05	0.30	0.15	0.01	3.75	77.10	77.10
24	38	0.08	0.40	0.20	0.01	3.75	89.00	89.75
15	39	0.05	0.30	0.20	0.01	3.75	81.00	81.15
39	40	0.08	0.20	0.15	0.02	3.75	78.00	78.76
22	41	0.08	0.40	0.10	0.01	3.75	87.00	87.39
18	42	0.08	0.30	0.15	0.02	2.50	86.00	85.40
27	43	0.05	0.30	0.15	0.02	3.75	84.00	84.92
40	44	0.08	0.40	0.15	0.02	3.75	85.40	86.67
41	45	0.08	0.30	0.15	0.01	3.75	81.00	82.25
16	46	0.10	0.30	0.20	0.01	3.75	88.00	89.20

Table 3 shows the ANOVA result output for Box-Benkhen Experimental Design. A p-value of 0.0001 implies that the model is significant. At the model level, correlation measures for estimation of regression equation are; multiple correlation coefficients R and the determination coefficient R2. The closer the value of R is to 1; the better is the correlation between the measured and the predicted values. In this experiment, the value of R was 0.9781 for GAG yield (Table 4), this result is supported by studies [5] and [16], revealing a calculated R value of 0.957 and 0.9893 respectively. A validation experiment was also conducted using the suggested media matrix on the Box-Benkhen Experimental Design Matrix (Table 5), the results clearly shows harmony between experimental outcome and the predicted values due to closeness of the percentage values obtained for GAG production.

TABLE 3: RESPONSE SURFACE QUADRATIC MODEL ANALYSIS OF VARIANCE (ANOVA)

Transform | Fit Summary | Model | ANOVA | Diagnostics | Model Graphs

ANOVA for Response Surface 2FI Model

Analysis of variance table [Partial sum of squares]

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	3781.23	15	252.08	89.29	< 0.0001	significant
A	4.40	1	4.40	1.56	0.2216	
B	639.71	1	639.71	226.59	< 0.0001	
C	137.48	1	137.48	48.69	< 0.0001	
D	3.53	1	3.53	1.25	0.2721	
E	7.56	1	7.56	2.68	0.1122	
AB	754.88	1	754.88	267.38	< 0.0001	
AC	49.00	1	49.00	17.36	0.0002	
AD	29.70	1	29.70	10.52	0.0029	
AE	1722.25	1	1722.25	610.02	< 0.0001	
BC	12.25	1	12.25	4.34	0.0459	
BD	22.47	1	22.47	7.96	0.0084	
BE	306.25	1	306.25	108.47	< 0.0001	
CD	55.50	1	55.50	19.66	0.0001	
CE	20.25	1	20.25	7.17	0.0119	
DE	16.00	1	16.00	5.67	0.0238	
Residual	84.70	30	2.82			
Lack of Fit	75.09	25	3.00	1.56	0.3287	not significant
Pure Error	9.61	5	1.92			
Cor Total	3865.93	45				

TABLE 4: PRECISION TABLE OF ANOVA ANALYSIS

Std. Dev.	1.68	**R-Squared**	0.9781
Mean	82.25	**Adj R-Squared**	0.9671
C.V.	2.05	**Pred R-Squared**	0.9489
PRESS	197.55	**Adeq Precision**	43.266

TABLE 5: VALIDATION TABLE OF EXPERIMENTAL RESULTS IN COMPARISON WITH PREDICTED VALUES

Sample	Na-acetate (g/L)	Cabbage peels (g/L)	NH_4Cl (g/L)	K_2HPO4 (g/L)	Trace ES (mL)	Experimental YIELD (%)	Predicted YIELD (%)
V1	0.01	0.28	0.19	0.02	2.52	104.1	106.64
V2	0.01	0.36	0.19	0.01	3.17	102.78	109.57
V3	0.01	0.40	0.10	0.02	3.69	94.07	97.9918
V4	0.01	0.24	0.20	0.01	2.53	104	107.39

Growth media optimization is a very complex process, which involves interactions that occurs between media components. The current study clearly shows that the type of mixture used has a significant effect on GAG production by Thiothrix nivea. The experimental findings were analyzed in form of response surface plots (Figure 2-6).

Considering the interaction between cabbage and other media components, it is evident that cabbage peel has a significant impact in GAG yield as evident in Figure 2, 4 and 5 with GAG percentage yield of 102%, 92% and 97% respectively. The plots showed areas with optimal concentrations of various media components (0.10g, 0.30g, 0.15g, 0.01g and 2.50mL of Na-acetate, Cabbage peels, NH4Cl, K2HPO4 and Trace ES respectively) support high GAG production; corresponding to the optimized media.

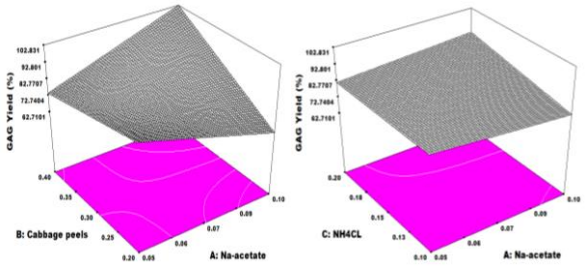

Fig 2: Three-dimensional (3D) Surface Plots of Media Components (AB and AC) Showing GAG Yield (%)

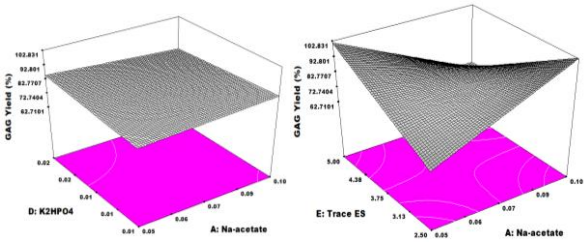

Fig. 3: Three-dimensional (3D) Surface Plots of Media Components (AD and AE) Showing GAG Yield (%)

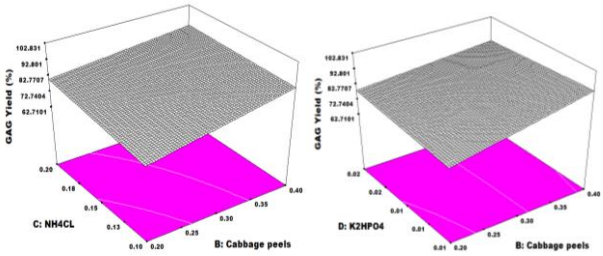

Fig. 4: Three-dimensional (3D) Surface Plots of Media Components (BC and BD) Showing GAG Yield (%)

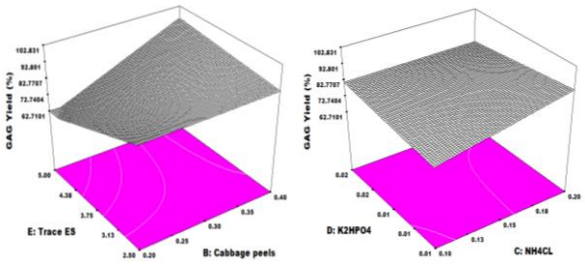

Fig. 5: Three-dimensional (3D) Surface Plots of Media Components (BE and CD) Showing GAG Yield (%)

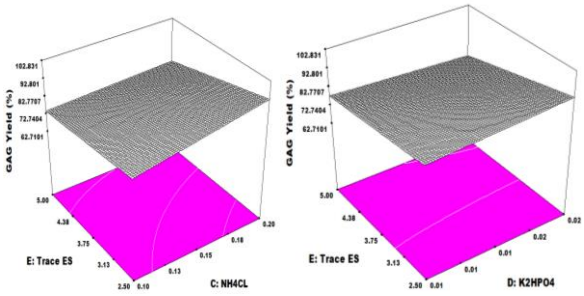

Fig. 6: Three-dimensional (3D) Surface Plots of Media Components (CE and DE) Showing GAG Yield (%)

IV. CONCLUSION

Glycosaminoglycan (GAG) yield was successfully optimized adopting Plackett-Burman and Box-Benkhen design of experiments using Design Expert software. The predicted values were in close range with the values obtained experimentally. Findings from this research revealed an attempt for bulk synthesis of GAG. Optimal combination (Na-acetate 0.10 g, Cabbage peels 0.30g, NH4Cl 0.15g, K2PO4 0.01g and Trace Element Solution 2.50 mL) of medium components was obtained with maximum GAG yield of 104.21%. ANOVA analysis in RSM showed that the developed model was remarkable and significant for optimizing media composition.These results could provide a reference to develop low-cost culture medium for bulk synthesis of GAG, which can

be utilized in functional food formulations, clinical medicine, cosmetics and biomaterial formulations. Future research should focus on optimization and characterization of biohydrogel; another polymer of clinical significance produced by Thiothrix nivea. Also, insilico studies should be carried out on GAG using various software programs, in other to ascertain and decipher its hydrodynamic nature.

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