OPTIMIZATION OF FLASK CULTURE MEDIUM AND CONDITIONS FOR HYALURONIC ACID PRODUCTION BY A *STREPTOCOCCUS EQUISIMILIS* MUTANT NC2168

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ABSTRACT

A mutant designated NC2168, which was selected from wild-type *Streptococcus equisimilis* CVCC55116 by ultraviolet ray combined with ^{60}Co-γ ray treatment and does not produce streptolysin, was employed to produce hyaluronic acid (HA). In order to increase the output of HA in a flask, the culture medium and conditions for NC2168 were optimized in this study. The influence of culture medium ingredients including carbon sources, nitrogen sources and metal ions on HA production was evaluated using factional factorial design. The mathematical model, which represented the effect of each medium component and their interaction on the yield of HA, was established by the quadratic rotary combination design and response surface method. The model estimated that, a maximal yield of HA could be obtained when the concentrations of yeast extract, peptone, glucose, and $MgSO_4$ were set at 3 g/100 mL, 2 g/100 mL, 0.5 g/100 mL and 0.15 g/100 mL, respectively. Compared with the values obtained by other runs in the experimental design, the optimized medium resulted in a remarkable increase in the output of HA and the maximum of the predicted HA production was 174.76 mg/L. The model developed was accurate and reliable for predicting the production of HA by NC2168.Cultivation conditions were optimized by an orthogonal experimental design and the optimal conditions were as follows: temperature 33ºC, pH 7.8, agitation speed 200 rpm, medium volume 20 mL.

Key words: *Streptococcus equisimilis* mutant; hyaluronic acid; optimization; culture medium; culture conditions

INTRODUCTION

Hyaluronic acid (HA), a linear, unbranched polysaccharide consisted of alternating N-acetyl-D-glucosamine and D-glucuronic acid, exists in many mammalian connective tissues such as joints, vitreous bodies, umbilical cords, cartilages, skins, and combs of fowls as a constituent; it plays an important role in many biological processes such as in tissue hydration, proteoglycan organisation in the extracellular matrix, and tissue repair (14). HA is a valuable biopolymer in

ophthalmics, orthopaedics, wound healing, tissue engineering, drug delivery, cosmetics, and as a vaccine aid (2, 6, 15).

In recent years, instead of extracting HA from rooster combs and umbilical cord, preparing HA through microbial fermentation is being used more, because the risk of cross-species viral infection can be avoided and the isolation process of high purity and high molecular weight HA can be simplified(5). This polymer is produced commercially by fermentation of the Lance field group C streptococci, *Streptococcus equi* subspecies *equsimilis* and *Streptococcus equi* subspecies *zooepidemicus* which can produce a capsule that is composed of hyaluronic acid polymers identical to that found in mammalian connective tissues (10). However, *Streptococcus* spp. strain can produce streptolysin which causes hemolysis. Thus, it is very important that streptolysin is absent in the hyaluronic acid obtained by cultivation of the microorganism. According to the reports (8, 10, 12), NTG treatment was an effective induced measure to obtain a mutant which does not produce streptolysin. However, in this study, a mutant designated NC2168, which was selected from wild-type *Streptococcus equisimilis* CVCC55116 by ultraviolet ray combined with $^{60}C_o$-γ ray treatment and does not produce streptolysin, was employed to produce HA.

Because no general medium works best for a new strain being tested, the optimization of the medium for the new mutant is necessary (9). Meanwhile, large numbers of experiments in stirred controlled vessels are not practical. However, the best medium chosen from shake flask data can build a foundation for the large scale stirred tank, thus it's essential to improve the HA production through the optimization of shake flask medium and cultivation conditions.

As a collection of statistical techniques for experiment designing, model developing and factors evaluating, response surface methodology (RSM) has been extensively applied in optimization of medium composition, conditions of enzymatic hydrolysis, fermentation and food manufacturing processes (3, 11, 13, 17,). In this study, to increase the yield of HA, the optimum medium composition was carried out by RSM and the

optimum cultivation conditions including temperature, rotate speed, pH, and medium volume were investigated through orthogonal experimental design.

MATERIALS AND METHODS

Microorganism and Medium

The strain used in this study was *Streptococcus equisimilis* CVCC55116, from Chinese Veterinary Culture Collection. Microorganisms were grown in a solid culture medium consisting of 1 g glucose, 16 g ox-brain extract powder, 4 g ox-heart extract powder (Brain Heart Infusion, BHI), 10 g yeast extract and 20 g agar. The blood agar (Joint Logistics Department, Beijing Military Area Command) was used for the isolation of the mutant without hemolysin. The selected mutant was cultivated in the fermentation medium consisting of 20 g glucose, 16 g ox-brain extract powder, 4 g ox-heart extract powder (BHI), 10 g peptone, 2 g $MgSO_4$ and 2 g K_2HPO_4 per liter of distilled water. The cultivation conditions were: temperature 37ºC, pH 7.4, and agitation speed 180 rpm.

Mutagenesis

Streptococcus equisimilis CVCC55116 was incubated at 37°C for 24 h on the agar plate. A single colony was inoculated in a 250 mL flask containing 50 mL of fermentation medium; it was incubated at 37°C for 12h. The grown cells were collected by centrifugation at 3,000 g for 20 min and washed with distilled water. The collected cells were resuspended in distilled water and performed ultraviolet ray mutagenesis in a dark room. After ultraviolet ray treatment, the cells were incubated at 37°C for 12 h in the fermentation medium and plated on the blood agar plate for the selection of nonhemolytic mutants. The mutant treated by ultraviolet ray was irradiated by $^{60}C_O$-γ ray with dose rate of 31.42 Gy/min. The irradiation doses of the two mutagenesis methods were based on irradiation time and corresponding the fatality rate. Through repeated screening, a high-producing hyaluronic acid mutant designated NC2168 was obtained.

Analytical Methods

CTAB Turbidimetric method was applied to determine the HA content (1). Ultraviolet and visible spectrophotometer (Rili, China) was used here.

Experiment design

Based on the single factor experiments, the carbon and nitrogen source and inorganic salt were determined, and the response surface methodology (RSM) was employed to optimize the medium for the mutant NC2168.Then, 23 runs were carried out using a factorial central composite design to study four independent variables each at five levels. The four independent variables X_1 (yeast extract), X_2 (peptone), X_3 (sucrose), and X_4 ($MgSO_4$) at five levels and the experimental design were given in Table 1. A mathematical model, describing the relationships between the process indices (the output of HA) and the medium component contents in a quadratic equation, was developed. The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination R^2. As for the optimization of the culture conditions, the experiment was arranged based on the 3 levels of orthogonal experimental design with 4 factors and 9 runs as shown in Table 3. Four independent variables representing temperature, rotate speed, initial pH and medium volume were employed respectively. In each case, the output of HA was monitored and all the experiments were carried out in triplicate for the average calculation.

Statistical analysis

Statistical evaluations were performed using F-test. A value of $p < 0.05$ was considered significant. The quadratic model was obtained by a standard multinomial regression method for four factors using the software Statistica, version 6.0. The extremum was also acquired by differential coefficient calculation and canonical analysis which was used to predict the shape of the curve generated by the model. Variance analysis on the orthogonal experiment results was performed by SAS, version8.0.

RESULTS AND DISCUSSION

Optimization of culture medium

Effect of nitrogen sources on the output of HA: The effect of organic and inorganic nitrogen sources on the yield of HA is shown in Figure 1a. It indicates that the output of HA based on inorganic source $(NH_4)_2SO_4$ was the lowest while those based on most of organic nitrogen sources were superior. The main reason might be that organic sources were more easily taken up, transported and absorbed while inorganic nitrogen could restrain the metabolism of the strain to some extent. It is easy to see that BHI was the best organic nitrogen source to cultivate the mutant NC2168 and the highest output of HA based on BHI touched 169.20 mg/L, even with the medium containing 1 g/100 mL BHI, the output was higher than that based on other nitrogen sources. However, the cost of BHI is several times higher than that of other nitrogen sources so that it is essential to find out a substitute to BHI. A composite medium containing multiple growth factors might meet the higher demand of *Streptococcus* on nutrition. As shown in Figure 1a, when the concentrations of yeast extract and peptone were 3 g/100 mL and 2 g/100 mL, the output of HA got 114.25 mg/L and 101.23 mg/L, respectively, which were the two better nitrogen sources besides BHI. The composite nitrogen source consisting of yeast extract and peptone might be a promising nitrogen source which could provide more necessary growth factors.

Based on the previous experiments, 3 g/100 mL yeast extract and 2 g/100 mL peptone were fixed respectively, and added 0.5, 1.0, 1.5, 2.0 and 2.5 g/100 mL peptone and yeast extract into the medium to constitute the composite nitrogen sources. The results in Figure 1b shows that 141.64 mg/L yield of HA was obtained using 3 g/100 mL yeast extract and 1.5 g/100 mL peptone, which was increased by 23.97% compared with 114.25 mg/L, using yeast extract only.
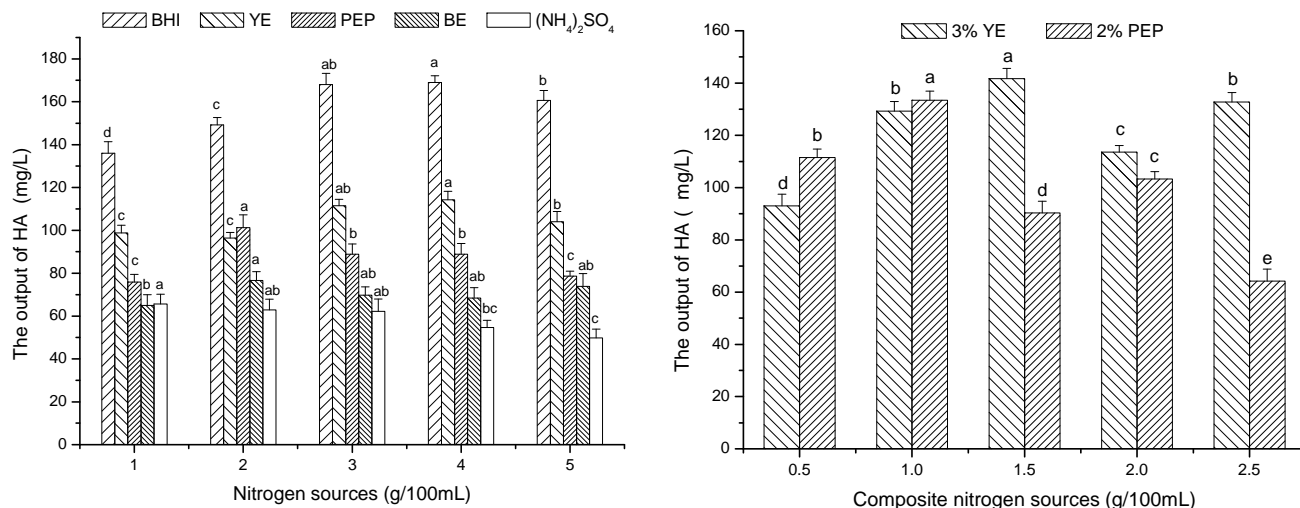
Figure 1. Effect of nitrogen sources on the output of HA "BHI" brain heart infusion, "YE" yeast extract, "PEP" peptone, "BE" beef extract. The ""a-e"" in Figure1-3 represented the significantly difference ($p < 0.05$) between the different concentration of the same medium component.

Effect of carbon sources on the output of HA: In this study, glucose, fructose, galactose, sucrose, maltose, xylose were used as the carbon sources respectively to enhance the yield of HA. Their influences at different concentrations are presented in Figure 2. It shows that the output of HA could be affected greatly by the kind and quantity of carbon sources. As shown in Figure 2, the highest output of HA was based on 0.5 g/100 mL glucose as the carbon source. Other carbon sources including maltose at 1.5 g/100 mL, fructose at 0.5 g/100 mL

and sucrose at 0.5 g/100 mL could obtain a higher output of HA. However, the galactose and xylose could not be utilized well and the corresponding output of HA was relatively low. We speculate that both UDP glucuronic acid and N-acetamide uridine diphosphoglucose are the synthesize precursors of HA and thalli. Maltose and sucrose could be easily decompounded into glucose by the thalli and fructose could be easily transformed into glucose so that they could be utilized better.
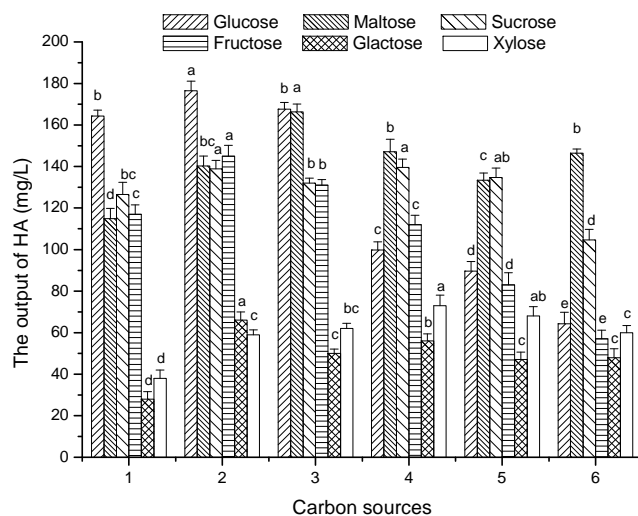

Figure 2. Effect of carbon sources on the output of HA. The numbers listed in the X axis represented 0.1, 0.5, 1.0, 2.0, 3.0, 4.0 g/100 mL carbon sources respectively.

Effect of metal ions on the output of HA: As for a cell, many metal ions are indispensable for active centers of an enzyme. During the process synthesizing HA, the HA synthetase is a key enzyme, which catalyzes the precursor of HA to synthesize HA (16). In this study, the effects of Mg^{2+} and K^{1+} on the output of HA were explored considering their possible roles in the HA synthetase. As shown in Figure 3, the highest output of HA was achieved up to 198.86 mg/L at $MgSO_4$ concentration of 0.15 g/100 mL and the difference of output based on the different concentration of $MgSO_4$ was pronounced ($p<0.05$). However, as for KH_2PO_4, the output of HA based on the different concentration was almost the same, thus the effect of KH_2PO_4 concentration was negligible in the assigned range while Mg^{2+} was selected to be performed in the following experiment.
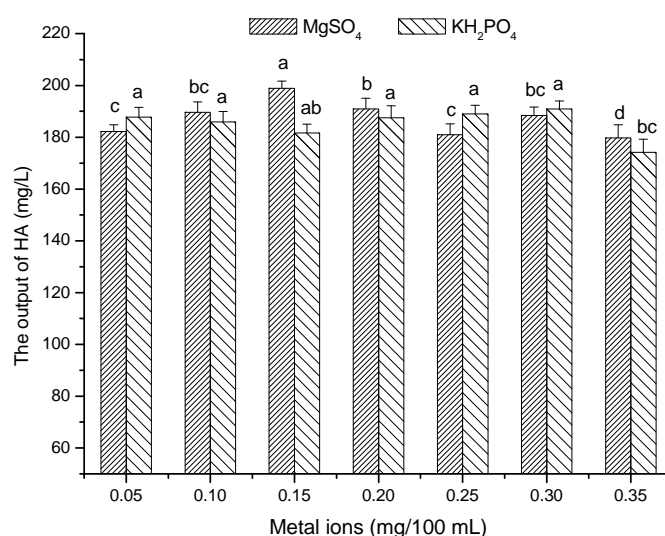

Figure 3. Effect of metal ions on the output of HA

Orthogonal rotatable central composite design

In the previous experiments, the suitability of various nitrogen sources, carbon sources, and metallic ions for the superior production of HA was evaluated. After the influence factors were determined, response surface method was used to optimize the culture medium for enhancing the output of HA through the quadratic rotary combination design (4, 7). A set of 23 experiments were required with four variables, and each variable being at five levels ($\alpha = 1.682$). The independent and dependent variables were analyzed to get regression equation, which was an empirical relationship between the HA output and the test variable in coded units. The regression equation obtained for the output of HA is as follows:

$$Y=174.76-3.39X_1+2.78X_2+6.09X_3+1.26X_4$$

$$-9.27X_1^2-12.93X_2^2-14.36X_3^2-12.21X_4^2$$
$$+1.03X_1X_2-0.32X_1X_3+0.91X_1X_4+0.91X_2X_3-0.32X_2X_4-.03X_3X_4 \quad (1)$$

Y represents the response, which is a dependent variable, the output of HA (mg/L), and X_1, X2, X_3, and X_4 represent values of the independent variables, yeast extract, peptone, glucose and $MgSO_4$, respectively.

Each of the observed value was compared with the predicted value which was calculated from the model, as depicted in Table 1. The statistical significance of the quadratic model equation was checked by analysis of variance (ANOVA), as shown in Table 2. The multiple coefficient of correlation R=0.9537 indicates a close agreement between experimental and predicted value of the HA output. The total determination coefficient, $R^2 = 0.9095$ implies that 90.95% of the variability in the response could be explained by the model.

Table 1. Experimental design matrix of RSM and the responses of the dependent variables

	Design matrix (g/100 mL)				Output of HA（mg/L）	
Run	**Yeast extract**	**Peptone**	**Glucose**	**MgSO$_4$·7H$_2$O**	**Experiment value**	**Fitted value**
1	1（3.83）	1（2.60）	1（0.74）	1（0.23）	136.11	135.97
2	1（3.83）	-1（1.41）	1（0.74）	-1（0.07）	124.39	120.13
3	-1（2.17）	1（2.60）	1（0.74）	-1（0.07）	140.83	137.39
4	-1（2.17）	-1（1.41）	1（0.74）	1（0.23）	136.6	134.83
5	1（3.83）	1（2.60）	-1（0.26）	-1（0.07）	126.67	118.91
6	1（3.83）	-1（1.41）	-1（0.26）	1（0.23）	121.46	115.39
7	-1（2.17）	1（2.60）	-1（0.26）	1（0.23）	128.13	122.81
8	-1（2.17）	-1（1.41）	-1（0.26）	-1（0.07）	131.88	122.49
9	-1.68（1.60）	0（2.00）	0（0.50）	0（0.15）	111.53	123.90
10	1.68（4.40）	0（2.00）	0（0.50）	0（0.15）	143.28	144.39
11	0（3.00）	-1.68（1.00）	0（0.50）	0（0.15）	147.02	154.24
12	0（3.00）	1.68（3.00）	0（0.50）	0（0.15）	136.6	142.84
13	0（3.00）	0（2.00）	-1.68（0.10）	0（0.15）	125.36	133.51
14	0（3.00）	0（2.00）	1.68（0.90）	0（0.15）	137.57	142.86
15	0（3.00）	0（2.00）	0（0.50）	-1.68（0.01）	127.97	138.11
16	0（3.00）	0（2.00）	0（0.50）	1.68（0.29）	139.04	142.34
17	0（3.00）	0（2.00）	0（0.50）	0（0.15）	189.00	174.76
18	0（3.00）	0（2.00）	0（0.50）	0（0.15）	183.16	174.76
19	0（3.00）	0（2.00）	0（0.50）	0（0.15）	176.16	174.76
20	0（3.00）	0（2.00）	0（0.50）	0（0.15）	172.25	174.76
21	0（3.00）	0（2.00）	0（0.50）	0（0.15）	170.30	174.76
22	0（3.00）	0（2.00）	0（0.50）	0（0.15）	170.62	174.76
23	0（3.00）	0（2.00）	0（0.50）	0（0.15）	177.62	174.76

The numbers in brackets were the actual values of the variables.

Table 2. Variance analysis for quadratic model and parameter estimates

Variables	**SS**	**DF**	**MS**	**F**	**p-value**
X_1	157.2006	1	157.2006	1.22456	0.30063
X_2	105.4269	1	105.4269	0.82125	0.39129
X_3	506.7100	1	506.7100	3.94716	0.08219
X_4	21.5302	1	21.5302	0.16772	0.69290
X_1X_1	1286.6569	1	1286.6569	10.02277	0.01328
X_2X_2	2555.3209	1	2555.3209	19.90538	0.00211
X_3X_3	3170.9927	1	3170.9927	24.70132	0.00109
X_4X_4	2271.0275	1	2271.0275	17.69079	0.00297
X_1X_2	16.9127	1	16.9127	0.13175	0.72603
X_1X_3	1.5939	1	1.5939	0.01242	0.91402
X_1X_4	13.1225	1	13.1225	0.10222	0.75737
X_2X_3	13.1225	1	13.1225	0.10222	0.75737
X_2X_4	1.5939	1	1.5939	0.01242	0.91402
X_3X_4	16.9127	1	16.9127	0.13175	0.72603
X_2X_3	13.1225	1	13.1225	0.10222	0.75737
Model	10327.2038	14	737.6574	F2=5.746	0.00229
Residual	1026.9872	8	128.3734		
Lack of fit	735.7965	2	367.8982	F1=7.581	0.01423
Pure error	291.1908	6	48.5318		

R= 0.9537; R^2= 0.9095; R^2(Adj) = 0.8191; SS, sum of squares; DF, degrees of freedom; MS, mean squares

The significance of each coefficient was checked using the variance ratio test (F-test) and *p*- value in Table 2. On the one hand, the F-test result shows that $F_{0.05}(2, 6) = 5.14 < F_1 = 7.581 < F_{0.01}(2, 6) = 10.92$, which implies that the Lack of Fit was significant wherein $\alpha = 0.05$ level but not significant wherein $\alpha = 0.01$ level. Based on the analysis of the culture medium composition, it manifests that the existence of the Lack of Fit factors were mainly due to the high order interaction. On the other hand, $F_2 = 5.746 > F_{0.01}(14, 8) = 5.56$ indicates that the regression equation was significant at $\alpha = 0.01$ level.

The *p*-value less than 0.05 indicates that the model terms were significant. The corresponding variables will be more significant if the absolute F-value becomes larger and the *p*-value becomes smaller. In this case, it can be seen that the variables with the remarkable effects on the level of HA production were the quadratic term of glucose (X_3X_3), peptone (X_2X_2), MgSO$_4$ (X_4X_4), yeast extract (X_1X_1). The *p*-value greater than 0.10 indicate that the model terms were not significant.

The maximum of the model was obtained from the equation when the concentration of yeast extract, peptone, glucose and MgSO$_4$ were at 3.00 g/100 mL, 2.00 g/100 mL, 0.5 g/100 mL and 0.15 g/100 mL, respectively. The maximum of

the predicted HA production was 174.76 mg/L. The triplicate experiments were also carried out to verify the accuracy of the result for HA production in a similar way as that of production model. Under the calculated optimal culture condition, the mean value of HA production was 178.53±3.50 mg/L, which was in agreement with the predicted value well. The average relative error between actual value and predicted value was 4.95%. Therefore, the model developed was considered to be accurate and reliable for predicting the production of HA by NC2168.

The 3-D response surface and the 2-D contour plots were the graphical representations of the regression equation (13) and showed the effect of each independent variable and also their mutual effects on the production of HA. The response surface curves and also contour plots for variations in HA production were depicted in Figure 4. The response surface representing HA yield was a function of the concentrations of the two medium components with other ingredients being at an optimal level. By using the response surface plot, the interaction between two variables and their optimum levels can be easily understood and located. The HA output was influenced by all the four independent variables. The maximum point was achieved when glucose, peptone, yeast extract and MgSO$_4$ concentrations were at their zero levels.
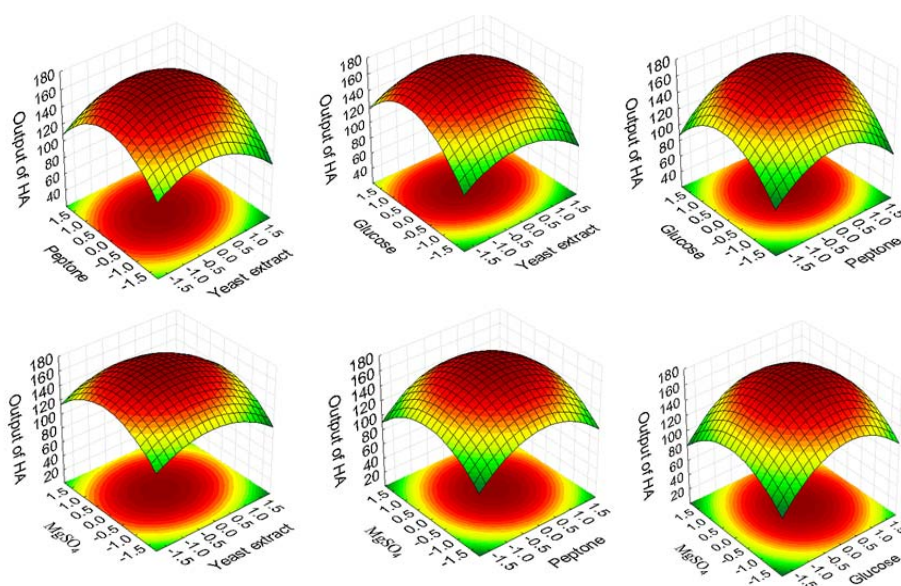

Figure 4. Response surface and contour plot of each independent variable effect and also their mutual effects on the production of HA

Optimization of culture condition

The effect of four factors: temperature, rotate speed, original pH and medium volume on the output of HA under the orthogonal experimental design are presented in Table 3. The intuitionistic analysis shows that the maximum HA production was 196.73 mg/L, which was got by the fifth run. The optimum culture condition was: initial pH=7.8; medium volume=20 mL; temperature=33ºC; rotate speed=150 rpm. The range analysis manifests that the factors order for the effect was: initial pH>

medium volume > temperature > rotate speed.

The variance analysis (Table 4) showed that the effects of pH and medium volume were extremely significant ($p<0.01$) and the effect of temperature was significant ($p<0.05$). The result was in agreement with the range analysis. The validated experiment was run at the optimum condition mentioned above. HA output touched 224.52 mg/L, which was also in agreement with the orthogonal experiment result.

Table 3. Orthogonal design and responsive value

Run	Temperature (ºC)	Rotate speed (rpm)	pH value	Medium volume (ml)	Output of HA (mg/L)
1	1(31)	1(100)	1(7.0)	1(20)	103.06
2	1(31)	2(150)	2(7.4)	2(30)	109.91
3	1(31)	3(200)	3(7.8)	3(40)	147.43
4	2(33)	1(100)	2(7.4)	3(40)	128.40
5	2(33)	2(150)	3(7.8)	1(20)	196.73
6	2(33)	3(200)	1(7.0)	2(30)	96.90
7	3(35)	1(100)	3(7.8)	2(30)	129.31
8	3(35)	2(150)	1(7.0)	3(40)	75.21
9	3(35)	3(200)	2(7.4)	1(20)	144.59
R	24.31	9.48	66.1	36.09	

Table 4. Variance analysis on the orthogonal experiment results

Source	Degr. of f	F	p-value	Significance
block	2	0.15	0.8653	
Temperature	2	5.72	0.0133	*
Rotate speed	2	3.44	0.0571	
pH	2	75.81	<.0001	**
Medium volume	2	18.68	<.0001	**

CONCLUSION

As to the new mutant *Streptococcus equisimilis* NC2168 without producing streptomycin, of which the optimum culture medium for maximum HA production was carried out with orthogonal rotatable central composite design and RSM. The model developed was considerably accurate and reliable for predicting the production of HA by NC2168 and a maximum of 174.76 mg/L HA was obtained using the optimal culture medium as follows: yeast extract=3 g/100 mL, peptone=2 g/100 mL, glucose=0.5 g/100 mL, $MgSO_4$=0.15 g/100 mL. The optimized culture condition was: temperature 32℃, rotate

speed 200 rpm, initial pH 7.8, and medium volume 20 mL. The maximum HA production was 224.52 mg/L under the optimum condition. It is evident that the output of HA was affected significantly by culture medium and conditions optimization. The NC2168 showed 149.08% increase in HA production under the optimum culture medium and conditions. This design proved to be crucial in achieving increase in the production of HA.

REFERENCES

1. Chen, Y. H.; Wang, Q. (2009). Establishment of CTAB Turbidimetric method to determine hyaluronic acid content in fermentation broth.*Carbohyd. Polym.* 78: 178–181.

2. Crescenzi, V. (1995). Microbial polysaccharides of applied interest: ongoing research activities in Europe. *Biotechnol. Prog.* 11: 251-259.

3. Cui, F. J.; Li, Y.; Xu, Z. H.; Xu, H. Y.; Sun, K.; Tao, W. Y. (2006). Optimization of the medium composition for production of mycelial biomass and exopolymer by Grifola frondosa GF9801 using response surface methodology. *Bioresour. Technol.* 97: 1209-1216.

4. Feng, Y. Y.; He, Z. M.; Ong, S. L.; Hu, J. Y.; Zhang, Z. G.; Ng, W. J. (2003). Optimization of agitation, aeration, and temperature conditions for maximum β-mannanase production. *Enzyme. and Microbial. Techonol.* 32: 282-289.

5. Fong Chong, B.; Blank, L. M.; Mclaughlin, R.; Nielsen, L. K. (2005). Microbial hyaluronic acid production. *Appl. Microbiol. Biotechnol.* 66: 341-351.

6. Fong Chong, B.; Nielsen, L. K. (2003). Amplifying the cellular reduction potential of *Streptococcus zooepidemicus*. *J. Biotechnol.* 100: 33-41.

7. Hanan, M. I.; Wan, M.; Wan, Y.; Aidil, A. H. (2005). Optimization of medium for the production of β-cyclodextrin glucanotransferase using Central Composite Design (CCD). *Process. Biochem.* 40: 753-758.

8. Hosoya, H.; Kimura, M.; Endo, H. (1994). Novel production process of hyaluronic acid and bacterium strain therefore as well as cosmetic composition containing hyaluronic acid. Eur. Pat. 0, 244, 757.

9. Kennedy, M.; Krouse, D. (1999). Strategies for improving fermentation medium performance: a review. *J. Ind. Microbiol. Biotechnol.* 23: 456-475.

10. Kim, J. H.; Yoo,S. J.; Oh, d. k.; Kweon Y. G.; Park D. W.; Lee C. H., Gil G. H. (1996). Selection of a *Streptococcus equi* mutant and optimization of culture conditions for the production of high molecular weight hyaluronic acid. *Enzyme. Microbial. Technol.* 19: 440-445.

11. Mao, X. B.; Eksriwong, T.; Chauvatcharin, S.; Zhong, J. J. (2005). Optimization of carbon source and carbon/nitrogen ratio for cordycepin production by submerged cultivation of medicinal mushroom Cordyceps militaris. *Process. Biochem.* 40: 1667-1672.

12. Nimrod, A.; Greenman, B.; Kanner, D. (1988). High molecular weight sodium hyaluronate.U.S.A. Pat. 4, 784, 990.

13. Oh, B. R.; Seo, J. W.; Choi, M. H.; Kim, C. H. (2008). Optimization of Culture Conditions for 1,3-Propanediol Production from Crude Glycerol by Klebsiella pneumoniae Using Response Surface Methodology. *Biotechnol. Bioprocess. Eng.* 13: 666-670.

14. Phrem, P. (1983). Synthesis of hyaluronate in differentiated teratocxcinoma cells: Characterization of the synthase. *Biochem. J.* 211: 181-189.

15. Radaeva, I. F.; Kostina, G. A.; Zmievskii, A.V. (1997). Hyaluronic acid: biological role, structure, synthesis, isolation, purification, and applications. *Appl. Biochem. Microbiol.*33:111-115.

16. Stoolmiller, A. C.; Dorfman, A. (1969). The Biosynthesis of hyaluronic acid by *Streptococcus*. *J. Biol. Chem.* 244(2): 236-246.

17. Wang, Y. X.; Lu, Z. X. (2005). Optimization of processing parameters for the mycelial growth and extracellular polysaccharide production by *Boletus* spp. ACCC50328. *Process. Biochem.* 40: 1043-1051.