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Study of Complex Nutrients, Temperature and Salts on Hyaluronic acid Production In *Streptococcus zooepidermicus* ATCC 43079

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ABSTRACT

The hyaluronic acidproducing *Streptococcus zooepidermicus* ATCC 43079 was used to study the hyaluronic acid production. We used the Plackett-Burman design of optimum multifactorial experiments to estimate the level effects of different factors such as peptone, meat extract, yeast extract, glucose, tween 80, dipotassium phosphate, sodium acetate, ammonium citrate, magnesium sulphate and manganese sulphate on hyaluronic acid production. As a result, meat extract (0.2%), yeast extract (0.1%) and glucose (0.4%) gave the highest production in *Streptococcus zooepidermicus* at temperature (40^{o}C). In order to confirm the estimated hyaluronic acid yield, the designated experiments were done. The maximum yield of hyaluronic acid in *Streptococcus zooepidermicus* ATCC 43079 was 42.38 mg/l while the predicted HA was 42.32 mg/l. The results also showed the Plackett - Burman can be used to predict the HA yield.The study was the first report of HA production affected by complex nutrient, temperature and salts.

INTRODUCTION

Hyaluronic acid (HA) is a commercially valuable medical biopolymer increasingly produced through microbial fermentation (Naoki *et al.,* 2008; Schmidt *et al.,* 1996). Being a major structural component of the matrix, HA has used in wound healing and analgesic effects (Allemann *et al.,* 2008; Andre, 2004) and is also being used as a vehicle for drug delivery (Beasley *et al.,* 2009). HA is produced through the extraction from rooster comb or fermentation of certain attenuated strains of group C *Streptococcus*, which synthesize HA as part of their outer capsule. In rooster comb, HA is complexed with proteoglycans, making its isolation tediously and costly. Besides, rooster comb-based HA products for human therapeutics is being met with the risk of cross-species viral and other adventitious agent infection. HA produced from attenuated pathogen *Streptococci*, on the other hand, may have the potential to be contaminated with pathogen factors. Up to now, there was only the study on HA in *Streptococcus thermophilus* that was safe (Naoki *et al.,* 2008). Although the cell-free HA biosynthesis was achieved 40 years ago, the amount of HA was still too small (Chong *et al.,* 2009). Therefore, the optimization of the hyaluronic acid production from microorganisms to improve the yield was necessary. There were many studies about the culture affecting on the molecular weight of HA (Duan *et al.,* 2008; Kim *et al.,* 1996) or the stable production of hyaluronic acid in *Streptococcus zooepidemicus*chemostats operated at high dilution rate (Blank *et al.,* 2008). Besides, the hyaluronic acid production by *Streptococcus equi* subs. *zooepidemicus* strain deficient in β-glucuronidase in laboratory conditions has been done (Krahulec *et al.,* 2006). Recently, hyaluronic acid production by *Streptococcus zooepidemicus* in marine by-products media from mussel processing waste waters and tuna peptone gave the high yield (Vazquez *et al.,* 2010). Although the optimization of medium components for high-molecular-weight hyaluronic acid production by *Streptococcus* sp. ID9102 via a statistical approach was also studied (Im *et al.,* 1996). Although there were many such studies on HA, the effects of multi-factor on the HA production did not focused. The optimization of the hyaluronic acid production based on Plackett - Burman matrix (Plackett and Burman, 1946) in hyaluronic acid - producing *Streptococcus zooepidermicus* ATCC 43079 was not focused. The optimization of the fermentation process to get the high yield and large scale are very ideal in the industry of hyaluronic acid production. Plackett - Burman matrix was used to design the medium that gave a low cost but effective method for interactions and estimation of the values in multi-factor research.

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For the above stated reasons, we optimized the factors based on Placket - Burman design and the experiments were done to determine the hyaluronic acid yield by *Streptococcus zooepidermicus* ATCC 43079.

MATERIALS AND METHODS

Microorganisms and growth cultures

Hyaluronic acid producing *Streptococcus zooepidermicus* ATCC 43079 was used to study. The MRS medium contains peptone, glucose, yeast extract, tween 80, di potassium phosphate, sodium acetate, ammonium citrate, magnesium sulphate and manganese sulphate (De Man *et al.*, 1960). Cetylpyridium chloride, carbazole, glucuronic acid were purchased from Merck.

Plackett – Burman design

To maximize the production of Hyaluronic, the MRS medium was selected as the basal medium. The Plackett-Burman strategy was implemented along with the polynomial function to analyze the relationship between each ingredient with the HA productivity.

There were 11 factors used in 15 experiments (Table 1 and Table 2).

Table. 1: The income variables and theirs levels for the Placket – Burman.

Income Variables	Symbols	Code levels		
		-1	0	+1
Peptone	A	0 g/l	10 g/l	20 g/l
Meat	B	0 g/l	10 g/l	20 g/l
Yeast	C	0 g/l	5 g/l	10 g/l
Glucose	D	0 g/l	20 g/l	40 g/l
Tween 80	E	0 g/l	1 ml/l	2 ml/l
K2HPO4	F	0 g/l	2 g/l	4 g/l
Acetate.Na.3H2O	G	0 g/l	5 g/l	10 g/l
Amonium Citrate	H	0 g/l	2 g/l	4 g/l
MgSO4	J	0 mg/l	200 mg/l	400 mg/l
Mn	K	0 mg/l	50 mg/l	100 mg/l
Temperature	L	34°C	37°C	40°C

Table. 2: The Plackett-Burman design and the experimental response values.

Experiments	A	B	C	D	E	F	G	H	J	K	L
1	-1	1	1	1	-1	-1	-1	1	-1	1	1
2	-1	-1	-1	1	-1	1	1	-1	1	1	1
3	1	1	-1	1	1	1	-1	-1	-1	1	-1
4	-1	-1	1	-1	1	1	-1	1	1	1	-1
5	0	0	0	0	0	0	0	0	0	0	0
6	1	-1	1	1	-1	1	1	1	-1	-1	-1
7	-1	1	-1	1	1	-1	1	1	1	-1	-1
8	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
9	1	-1	1	1	1	-1	-1	-1	1	-1	1
10	0	0	0	0	0	0	0	0	0	0	0
11	-1	1	1	-1	1	1	1	-1	-1	-1	1
12	1	1	-1	-1	-1	1	-1	1	1	-1	1
13	1	-1	-1	-1	1	-1	1	1	-1	1	1
14	0	0	0	0	0	0	0	0	0	0	0
15	1	1	1	-1	-1	-1	1	-1	1	1	-1

Uronic quantitative analysis

The HA amount produced in microorganisms were determined according to the glucuronic acid content by reaction with carbazole. The color changed into purple red that indicated the positive result of uronic acid (Bitter and Muir, 1962).

RESULTS AND DISCUSSION

Analysis of Plackett – Burman design in hyaluronic production

The impact level of 11 factors of the culture medium toward the amount of hyaluronic acid produced from *S. zooepidemicus* was depicted in table 3.

While factor with negative impact cause the decreasing in the amount of hyaluronic acid produced from *S. zooepidermicus* and positive impact cause the increasing in the amount of hyaluronic acid produced from *S. zooepidermicus*. Based on table 3, yeast extract and temperature had the strongest impact on the the amount of hyaluronic acid yield produced from *S. zooepidermicus*. The predicted hyaluronic acid yield (µg/ml) by *S. zooepidermicus* was shown in table 4.

Table. 3: Factors of the culture medium and their impacts.

Factor		Concentration			Impact	p-Value
		-1	0	+1		
Peptone	A	0 g/l	10 g/l	20 g/l	4.34	<0.0001
Meat	B	0 g/l	10 g/l	20 g/l	0.93	<0.0001
Yeast	C	0 g/l	5 g/l	10 g/l	0.22	<0.0001
Glucose	D	0 g/l	20 g/l	40 g/l	0.33	<0.0001
Tween 80	E	0 g/l	1 ml/l	2 ml/l	-0.48	<0.0001
K2HPO4	F	0 g/l	2 g/l	4 g/l	0.12	<0.0001
CH3COONa.3H2O	G	0 g/l	5 g/l	10 g/l	1.85	<0.0001
Amonium Citrate	H	0 g/l	2 g/l	4 g/l	5.1	<0.0001
MgSO4	J	0 mg/l	200 mg/l	400 mg/l	0.56	<0.0001
MnSO4	K	0 mg/l	50 mg/l	100 mg/l	0.93	<0.0001
Temperature	L	34°C	37°C	40°C	2.02	<0.0001

Table. 4: The actual and predicted hyaluronic acid yield (µg/ml) by *S. zooepidermicus.*

Experiments	actual value	predicted value
1	42.38	42.32
2	29.55	29.51
3	38.74	38.72
4	29,55	29.54
5	8.6	8.7
6	30.28	30.19
7	33.82	33.89
8	26.58	26.52
9	29.54	29.59
10	8.7	8.73
11	37.5	37.55
12	38.69	38.71
13	32.53	32.53
14	8.9	8.96
15	42.23	42.24

Quantitation of hyaluronic acid production

According to uronic quantitative analysis, 15 experiments showed the hyaluronic acid production. The collected hyaluronic acid was tested to quantitative. The purple color showing the uronic acid reaction with carbazol was illustrated in figure 1. All the conditions designed in the matrix of Plackett – Burman did not inhibit the HA production.

As expressed in the figure 2, the actual values (allocated at the horizontal axis) and the predictedvalue (allocated at the vertical axis) were match together, proved that the obtained actual

optimization can be firstly established by basing on Plackett-Burman design. The effect of each factor was expressed from highest to lowest order as peptone, ammonium citrate, temperature, sodium acetate, manganese sulfate, meat extract, glucose, yeast extract, di potassium hydrogen phosphate (Table 4). The highest yield of hyaluronic acid by *Streptococcus zooepidermicus* was 42.38 mg/l under meat extract (0.2%), yeast extract (0.1%) and glucose (0.4%), temperature (40^{o}C) condition for the experiment 1.

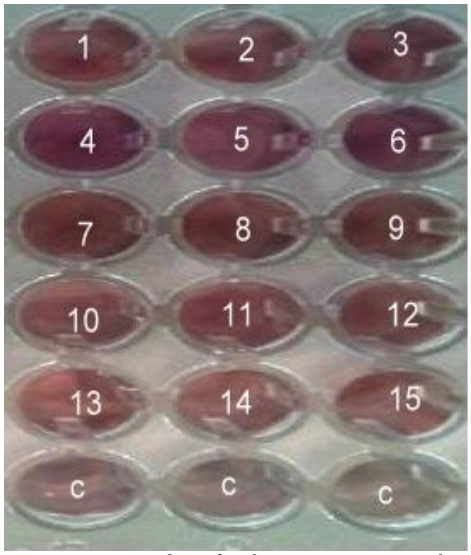

Fig. 1: Uronic quantitative analysis for the 15 experiments. The results of 15 experiments were numbered in each well. The remaining wells were the control.

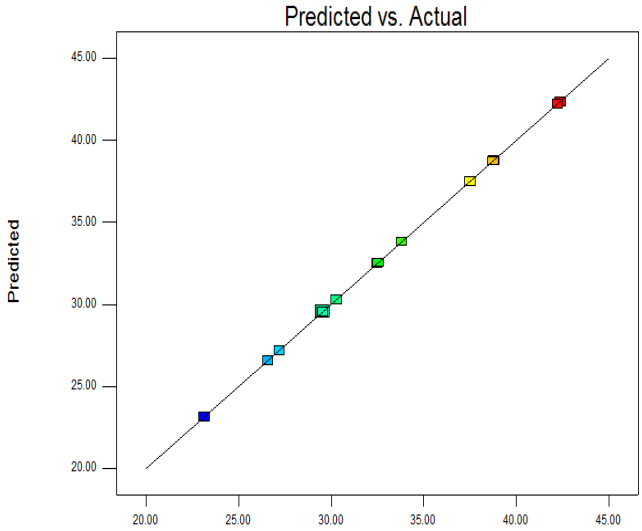

Fig. 2: Comparisons between the actual and predicted hyaluronic acid yield (µg/ml) by *S. zooepidermicus.*

The peptone, ammonium citrate, temperature, sodium acetate, manganese sulfate, di potassium hydrogen phosphate did not present the relation to the hyaluronic acid yield in the experiment 1. For the experiment 15, the actual amount was 42.24 mg/l (Table 4) due to meat extract (2%), yeast extract (1%) and peptone (2%), ammonium citrate (0.4%), temperature (34^{o}C), sodium acetate (1%), manganese sulfate (0.01%), magiesium sulfate (0.04%). Therefore, manganese sulfate, magiesium sulfate affected on the HA production, probably. Remarkably, temperature also related to the yield. In the experiment 3, peptone (2%), meat extract (2%), yeast extract (0%) gave 38.74 mg/l while *Streptococcus zooepidemicus* in marine by-products HA produced approximately 30.83 mg/l (Vazquez *et al.,* 2010). Combination of the results in experiment 1, 3, 15 yeast extract should be required in the culture. The experiment 4 without the peptone and meat extract, but yeast extract contained about 2% in the medium gave the yield of 29.55 mg/l. The temperature at 40^{o}C showed the higher the yield that was tested in the experiments 1, 2, 11, 12, 13 (Table 4). In the contrast, in the results of the experiments 5, 10 and 14, the HA production was very low at 37^{o}C while the HA was higher at 34^{o}C in the experiment 8. In the comparison with the different conditions in 15 experiments, the yeast extract and temperature had a lot of effects on HA production. Moreover, manganese sulfate and magiesium sulfate were also considered in HA production. Since 1994 and 1995, there was researches in molecular and genetics of HA biosynthesis (Crater *et al.,* 1995; O'Rehan *et al.,* 1994), but the HA amounts were still lower than this study. Even in *Bacillus*, the HA was only reported about the increased production (Chien and Lee, 2007). The lowest yield in this study obtained 8.6 mg/l while the HA yielded in *Streptococcus thermophiles* was 8 mg/l (Naoki *et al.,* 2008). The actual and predicted hyaluronic acid yield (µg/ml) by *S. zooepidermicus* was showed in table 4 and were analyzed as in figure 2. Their curves were likely overlapped (R^2= 0.99). It was meant that the Plackett - Burman design could estimate the HA production perfectly and the different effects could be studied at the same time.

CONCLUSION

Based on the Plackett-Burman design and optimium experiments, we calculated that peptone (2%), meat extract (2%), manganese sulphate (0.1%), temperature (40^{o}C), di potassium phosphate, (0.04%), yeast extract (1%), glucose (0.4%), ammonium citrate (0.4%), sodium acetate (1%) gave the high yield in *Streptococcus zooepidermicus*. The maximum yield of hyaluronic acid *S.zooepidermicus* ATCC 43079 was 42.38 mg/l. The study would study more in the mechanism of HA production in the complex-nitrogen and salts affecting on HA production, not like the previous study about the complex-nitrogen-limited growth but increase HA yield (Cooney *et al.,* 1999). By actual experiments, the obtained results were similar to the predicted yield. Therefore, the Plackett – Burman design could be applied to estimate the multi-factor effect on the production. However, the further study should be done more to investigate the effects of these factors.

REFERENCE

Allemann IB, Baumann L. Hyaluronic acid gel (Juvederm) preparations in the treatment of facial wrinkles and folds. ClinInterv Aging, 2008; 3: 629-634.

Andre P. Hyaluronic acid and its use as a rejuvenation agent in cosmetic dermatology.SeminCutan Med Surg, 2004; 23: 218-222.

Armstrong DC and Johns MR. Effect of culture conditions on molecular weight of hyaluronic acid produced by *Streptococcus zooepidemicus*.ApplEnvMicrobiol. 1997; 63: 2759-2764.

Beasley KL, Weiss MA, Weiss MD. Hyaluronic acid fillers: a comprehensive review. Facial PlastSurg, 2009; 25: 86-94.

Bitter T, Muir HM. A modifieduronicacidcarbazolereaction. Anal Biochem. 1962; 4(4): 330-334.

Blank LM, McLaughlin RL, Nielsen LK. Stable production of hyaluronic acid in *Streptococcus zooepidemicus*chemostats operated at high dilution rate. BiotechnolBioeng 2008; 20: 685-693.

Chien LJ and Lee CK. Enhanced Hyaluronic acid production in *Bacillus* subtilis by co-expressing bacterial hemoglobin. BiotechnolProg. 2007;23:1017-1022.

Chong BF, Blank LM, McLaughlin R, Nielsen L. Microbial hyaluronic acid production. ApplMicrobiolBiotechnol.2005; 66: 341-351.

Cooney MJ, Goh LT, Lee PL and Johns M R. Structured Model-Based Analysis and Control of the Hyaluronic Acid Fermentation by *Streptococcus zooepidemicus*: Physiological Implications of Glucose and Complex-Nitrogen-Limited Growth. BiotechnolProg. 1999;15: 898-910.

Crater DL and Van De Rijn I. Hyaluronic acid synthesis operon (has) expression in group *A. Streptococci*. J Biol Chem. 1995; 270: 18452-18458.

De Man JC, Rogosa M, Sharpe, and Elisabeth.A medium for the cultivation of*Lactobacilli*.Jounal of Applied Bacteriology. 1960; 23: 130-135.

Duan XJ, Yang L, Zhang X, Tan WS. Effect of oxygen and shear stress on molecular weight of hyaluronic acid.J MicrobiolBiotechnol. 2008; 18: 718-724.

Im JH, Song JM, Kang JH, Kang DJ. Optimization of medium components for high-molecular-weight hyaluronic acid production by *Streptococcus* sp. ID9102 via a statistical approach.J IndMicrobiolBiotechnol. 2009;36: 1337-1344.

Naoki I, Tomoko H, Ryoko I, Harumi M, Kazumasa K and Katsuyoshi C. *Streptococcus themophilus* produces exo-polysaccharides including hyaluronic acid. J bioscibioeng.2009; 107(2): 119-123.

O'Rehan M, Martini I, Crescenzi F, De Luca C, Lansing M. Molecular mechanisms and genetics of hyaluronic acid biosynthesis. *IntBialMacromol. 1994;* 16: 283-286.

Plackett, Burman. The Design of Optimum Multifactorial Experiments.Biometrika. 1946; 33(4): 305-325

Schmidt KH, Gunther E and Courtney HS. Expression of both M protein and hyaluronic acid capsule by group A *Streptococcal* strains results in a high virulence for chicken embryos. Med MicrobiolImmunol. 1996; 184: 169-173.

Vazquez JA, Montemayor MI, Fraguas J, Murado MA. Hyaluronicacidproductionby*Streptococcuszooepidemicus* in marine by-products media frommusselprocessingwastewaters and tuna peptoneviscera. Microb Cell Fact. 2010;9:46.

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