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Cost Effective Media Optimization for the Enhanced Production of Hyaluronic Acid Using a Mutant Strain *Streptococcus zooepidemicus* 3523–7: A Statistical Approach

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Abstract

The hydrated gel hyaluronic acid (HA) comprises repeating units of glucuronic acid and N-acetylglucosamine. HA production has a lot of significance in the present day scenario owing to its clinical applications. Fermentative optimization is carried out in two steps. Statistical optimization of media components for the production of HA using Taguchi and Response Surface Methodology (RSM) was studied using a mutant strain Streptococcus zooepidemicus 3523-7. Initial optimization was carried out using L_{27} - orthogonal array. The significance of each factor with respect to HA production was identified using Taguchi design (Glucose, Yeast extract, K_2HPO_4, NaH_2PO_4, NaCl, NH_4Cl, 1M $MgSO_4$ and Trace metal mix (TMM)) and the productivity was increased 1.34 folds (738 to 988 mg/L) over native production medium. The outcome of Taguchi results indicates the glucose, yeast extract, K_2HPO_4 and NH_4Cl are most influencing factors in the production of HA. Final optimization was carried out using RSM (Central Composite Rotatable Design) and the production was significantly enhanced to 1.4 folds (988 to 1386 mg/L). For the first time statistical optimization was carried out using a mutant strain Streptococcus zooepidemicus 3523-7 and high yield (1386 mg/L) of HA was achieved.

Keywords: *Hydrated gel, Fermentative optimization, Taguchi, Response Surface Methodology*

1. Introduction

Hyaluronic acid (HA) contains repeated units of glucuronic acid and *N*-acetylglucosamine, is naturally present in human body at higher concentrations throughout connective, epithelial, and neural tissues. Approximately, 0.22% of HA is present per kg weight in different human tissues viz., umbilical cord, dermis, serum, synovial fluid and in thoracic lymph. HA is used in the prevention and treatment of symptoms related to connective tissue disorders such as fractures, hernia, glaucoma, keratoconus, detached retina, osteoarthritis, TMJ, prevention of scarring, vocal cord repair insufficiency, wrinkled skin, cartilage damage, and wound and ligament healing. Of these, hyaluronic acid is extensively used in different joint disorders, including osteoarthritis.

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Different methods for treating osteoarthritis of knee include simple analgesics, intra-articular injection of glucocorticoids, weight-relieving braces, physiotherapy and total knee arthroplasty. Among all these intra-articular injection of hyaluronic acid, is a slow-acting drug for the treatment of osteoarthritis [1]. Owing to its broad range of medical applications in biomedical and health care division hyaluronic acid attracted the attention of researchers. HA is conventionally extracted from animal tissues viz., synovial fluid, rooster combs and bovine vitreous humor [2-4]. But the production and purification of HA is economical. Hence, different types of microbes are used for the production of HA. Among all, different strains of group A and C *Streptococci* are used for the production of HA [5-9]. *S. equi* subsp. *zooepidemicus* MTCC 3523 is capable of producing exopolysaccharide, HA.

In this study, a mutant strain *Streptococcus zooepidemicus* 3523-7 is used for the production of HA using low cost medium. Based on the literature and best of our knowledge, the nutritional and environmental conditions for submerged culture of mutant *Streptococcus zooepidemicus* 3523-7 for HA production using statistical methods (Taguchi and RSM) has not been demonstrated. Initially, one-factor-at-a-time (classical method) was used to investigate the best media constituents such as carbon and nitrogen source. Later, concentration of the medium components was optimized using an orthogonal array and final optimization was carried out using RSM.

2. Materials and Methods

2.1. Media Preparation and Culture Conditions

All growth and production experiments were carried out in 500 ml conical flasks with a working volume of 100 ml. The fermentation studies were carried out in production medium containing Glucose – 50 g/L, K_2HPO_4 – 2 g/L, KH_2PO_4 - 5 g/L, NH_4Cl – 0.5 g/L, Yeast extract – 5 g/L, NaCl – 5 g/L, $MgSO_4$ – 0.5 g/L, 1M $CaCl_2$ – 0.1 mL, TMM – 1 mL ($Al_2(S0_4)_3.7H_2O$ – 10mg/L, $CuSO_4.H_2O$ – 2 mg/L, H_3BO_4 – 1 mg/L, $MnCl_3.4H_2O$ – 20 mg/L, $NiCl_2.6H_2O$ – 1 mg/L, $Na_2MoO_4.2H_2O$ – 50 mg/L, $ZnSO_4.7H_2O$ – 50 mg/L, $FeSO_4$ – 50 mg/L). The initial pH of the medium was adjusted to 7 before autoclaving at 121 ^{0}C for 15 to 20 min. The sterile medium was inoculated aseptically with 5% of overnight fresh culture.

2.2. Optimization of Production Medium Components using One-Factor-at-a-Time Classical Method

2.2.1. Effect of Carbon Source: In the production medium, glucose was substituted with seven different carbon sources viz. sucrose, fructose, xylose, maltose, lactose, starch and galactose. All carbon sources were used at 5% concentration (w/v).

2.2.2. Effect of Nitrogen Source: In the production medium, yeast extract is substituted with different organic and inorganic nitrogen sources. Organic sources such as peptone, gelatin, soya peptone, meat peptone, mycological peptone, beef extract and corn steep liquor was used. Inorganic nitrogen sources such as potassium nitrate, diammonium hydrogen orthophosphate, sodium nitrate and ammonium nitrate were used. All nitrogen sources were used at 0.5 % concentration (w/v).

2.2.3. Effect of Initial pH: The effect of initial pH of culture medium on production of HA was studied by varying the pH from pH 5–9. The pH was adjusted using 0.1 N orthophosphoric acid (OPA) or 0.1 N sodium hydroxide (NaOH). The initial pH which supports the maximum production of HA was used in further experiments.

2.2.4. Effect of Fermentation Time: Production profile of HA was calculated after the one factor- at-a-time method. Measurement of total dry mass and HA production was done after every 12 hrs up to 48 hrs.

2.3. Optimization of Concentrations of Production Medium Components using L_{27} - Orthogonal Array

The design for the L_{27}-orthogonal array was developed and analyzed using ''MINITAB 14.00'' software. Table 1 depicted the fermentation conditions and the L_{27}-orthogonal array was used in the present study. All experiments are performed in duplicates.

Table 1. L_{27} – Orthogonal Design

S. No	A	B	C	D	E	F	G	H	I	P_1 [a]	P_2 [a]
1	1 (40)	1 (0.4)	1 (4.0)	1 (0.4)	1 (4.0)	1 (1.5)	1 (0.75)	1 (1.5)	1 (75)	659	668
2	1 (40)	1 (0.4)	1 (4.0)	1 (0.4)	2 (5.0)	2 (2.0)	2 (1.00)	2 (2.0)	2 (100)	693	709
3	1 (40)	1 (0.4)	1 (4.0)	1 (0.4)	3 (6.0)	3 (2.5)	3 (1.25)	3 (2.5)	3 (125)	689	681
4	1 (40)	2 (0.5)	2 (5.0)	2 (0.5)	1 (4.0)	1 (1.5)	1 (0.75)	2 (2.0)	2 (100)	705	709
5	1 (40)	2 (0.5)	2 (5.0)	2 (0.5)	2 (5.0)	2 (2.0)	2 (1.00)	3 (2.5)	3 (125)	712	706
6	1 (40)	2 (0.5)	2 (5.0)	2 (0.5)	3 (6.0)	3 (2.5)	3 (1.25)	1 (1.5)	1 (75)	708	701
7	1 (40)	3 (0.6)	3 (6.0)	3 (0.6)	1 (4.0)	1 (1.5)	1 (0.75)	3 (2.5)	3 (125)	739	742
8	1 (40)	3 (0.6)	3 (6.0)	3 (0.6)	2 (5.0)	2 (2.0)	2 (1.00)	1 (1.5)	1 (75)	724	735
9	**1 (40)**	**3 (0.6)**	**3 (6.0)**	**3 (0.6)**	**3 (6.0)**	**3 (2.5)**	**3 (1.25)**	**2 (2.0)**	**2 (100)**	**988**	**981**
10	2 (50)	1 (0.4)	2 (5.0)	3 (0.6)	1 (4.0)	2 (2.0)	3 (1.25)	1 (1.5)	2 (100)	936	324
11	2 (50)	1 (0.4)	2 (5.0)	3 (0.6)	2 (5.0)	3 (2.5)	1 (0.75)	2 (2.0)	3 (125)	916	926
12	2 (50)	1 (0.4)	2 (5.0)	3 (0.6)	3 (6.0)	1 (1.5)	2 (1.00)	3 (2.5)	1 (75)	948	952
13	2 (50)	2 (0.5)	3 (6.0)	1 (0.4)	1 (4.0)	2 (2.0)	3 (1.25)	2 (2.0)	3 (125)	964	957
14	2 (50)	2 (0.5)	3 (6.0)	1 (0.4)	2 (5.0)	3 (2.5)	1 (0.75)	3 (2.5)	1 (75)	953	959
15	2 (50)	2 (0.5)	3 (6.0)	1 (0.4)	3 (6.0)	1 (1.5)	2 (1.00)	1 (1.5)	2 (100)	932	940
16	2 (50)	3 (0.6)	1 (4.0)	2 (0.5)	1 (4.0)	2 (2.0)	3 (1.25)	3 (2.5)	1 (75)	956	964
17	2 (50)	3 (0.6)	1 (4.0)	2 (0.5)	2 (5.0)	3 (2.5)	1 (0.75)	1 (1.5)	2 (100)	948	935
18	2 (50)	3 (0.6)	1 (4.0)	2 (0.5)	3 (6.0)	1 (1.5)	2 (1.00)	2 (2.0)	3 (125)	967	958
19	3 (60)	1 (0.4)	3 (6.0)	2 (0.5)	1 (4.0)	3 (2.5)	2 (1.00)	1 (1.5)	3 (125)	837	842
20	3 (60)	1 (0.4)	3 (6.0)	2 (0.5)	2 (5.0)	1 (1.5)	3 (1.25)	2 (2.0)	1 (75)	864	872
21	3 (60)	1 (0.4)	3 (6.0)	2 (0.5)	3 (6.0)	2 (2.0)	1 (0.75)	3 (2.5)	2 (100)	827	832
22	3 (60)	2 (0.5)	1 (4.0)	3 (0.6)	1 (4.0)	3 (2.5)	2 (1.00)	2 (2.0)	1 (75)	871	882
23	3 (60)	2 (0.5)	1 (4.0)	3 (0.6)	2 (5.0)	1 (1.5)	3 (1.25)	3 (2.5)	2 (100)	852	847
24	3 (60)	2 (0.5)	1 (4.0)	3 (0.6)	3 (6.0)	2 (2.0)	1 (0.75)	1 (1.5)	3 (125)	839	846

25	3 (60)	3 (0.6)	2 (5.0)	1 (0.4)	1 (4.0)	3 (2.5)	2 (1.00)	3 (2.5)	2 (100)	824	829
26	3 (60)	3 (0.6)	2 (5.0)	1 (0.4)	2 (5.0)	1 (1.5)	3 (1.25)	1 (1.5)	3 (125)	804	812
27	3 (60)	3 (0.6)	2 (5.0)	1 (0.4)	3 (6.0)	2 (2.0)	1 (0.75)	2 (2.0)	1 (75)	830	821

A – Glucose (g/L), B – NH_4Cl (g/L), C – Yeast extract (g/L), D – $MgSO_4$ (g/L), E – NaCl (g/L), F – KH_2PO_4 (g/L), G – TMM (mL), H – K_2HPO_4 (g/L), I – 1M $CaCl_2$ (µL).
P_1 and P_2 are production in mg/L.
Values in parentheses are uncoded values.
[a] Values are ± of two independent experiments.

2.4. Optimization of Concentrations of the Selected Medium Components by Central Composite Design

To find out the combined effect of optimum concentration of four independent variables (a): Glucose (b): Yeast extract (c): K_2HPO_4 and (d): NH_4Cl on maximum production of HA, media was optimized using Minitab. Four variables in the design were studied at five different levels, with all variables taken at a central coded value of zero. All the experiments were designed using the software, Design Expert Version 6.0.10 version (Stat Ease, Minneapolis, MN). The design expert gave (factorial portion 24 = 16 with 8 star points) 24 plus 6 centre points leading to 30 experiments. The CCRD matrix in terms of coded and actual values of independent variables is given in Table 2.

Table 2. The CCRD Matrix of Independent Variables in Coded form with their Corresponding Response from Experiments

Std Order	Run Order	Block	A (g/L)	B (g/L)	C (g/L)	D (g/L)	Experimental[a] (mg/L)	Predicted (mg/L)
1	24	Block 1	-1 (20.0)	-1 (4.00)	-1 (1.0)	-1 (1.0)	1063	1056.875
2	20	Block 1	1 (60.0)	-1 (4.00)	-1 (1.0)	-1 (1.0)	1124	1124.708333
3	12	Block 1	-1 (20.0)	1 (8.00)	-1 (1.0)	-1 (1.0)	1286	1290.375
4	23	Block 1	1 (60.0)	1 (8.00)	-1 (1.0)	-1 (1.0)	1288	1290.208333
5	8	Block 1	-1 (20.0)	-1 (4.00)	1 (3.0)	-1 (1.0)	1151	1148.708333
6	22	Block 1	1 (60.0)	-1 (4.00)	1 (3.0)	-1 (1.0)	1184	1184.041667
7	16	Block 1	-1 (20.0)	1 (8.00)	1 (3.0)	-1 (1.0)	1208	1208.208333
8	29	Block 1	1 (60.0)	1 (8.00)	1 (3.0)	-1 (1.0)	1185	1175.541667
9	17	Block 1	-1 (20.0)	-1 (4.00)	-1 (1.0)	1 (5.0)	1095	1096.708333
10	14	Block 1	1 (60.0)	-1 (4.00)	-1 (1.0)	1 (5.0)	1188	1194.041667
11	6	Block 1	-1 (20.0)	1 (8.00)	-1 (1.0)	1 (5.0)	1229	1235.208333
12	18	Block 1	1 (60.0)	1 (8.00)	-1 (1.0)	1 (5.0)	1270	1264.541667
13	28	Block 1	-1 (20.0)	-1 (4.00)	1 (3.0)	1 (5.0)	1305	1309.041667
14	**11**	**Block 1**	**1 (60.0)**	**-1 (4.00)**	**1 (3.0)**	**1 (5.0)**	**1386**	**1373.875**
15	26	Block 1	-1 (20.0)	1 (8.00)	1 (3.0)	1 (5.0)	1282	1273.541667
16	2	Block 1	1 (60.0)	1 (8.00)	1 (3.0)	1 (5.0)	1258	1270.375

17	13	Block 1	-2 (0.0)	0 (6.00)	0 (2.0)	0 (3.0)	1201	1200.416667
18	10	Block 1	2 (80.0)	0 (6.00)	0 (2.0)	0 (3.0)	1263	1265.083333
19	30	Block 1	0 (40.0)	-2 (2.00)	0 (2.0)	0 (3.0)	1090	1093.25
20	27	Block 1	0 (40.0)	2 (10.00)	0 (2.0)	0 (3.0)	1225	1223.25
21	15	Block 1	0 (40.0)	0 (6.00)	-2 (0.0)	0 (3.0)	1240	1234.416667
22	9	Block 1	0 (40.0)	0 (6.00)	2 (4.0)	0 (3.0)	1325	1332.083333
23	1	Block 1	0 (40.0)	0 (6.00)	0 (2.0)	-2 (-1.0)	1128	1132.416667
24	4	Block 1	0 (40.0)	0 (6.00)	0 (2.0)	2 (7.0)	1270	1267.083333
25	5	Block 1	0 (40.0)	0 (6.00)	0 (2.0)	0 (3.0)	1048	1050
26	7	Block 1	0 (40.0)	0 (6.00)	0 (2.0)	0 (3.0)	1052	1050
27	21	Block 1	0 (40.0)	0 (6.00)	0 (2.0)	0 (3.0)	1049	1050
28	3	Block 1	0 (40.0)	0 (6.00)	0 (2.0)	0 (3.0)	1052	1050
29	19	Block 1	0 (40.0)	0 (6.00)	0 (2.0)	0 (3.0)	1048	1050
30	25	Block 1	0 (40.0)	0 (6.00)	0 (2.0)	0 (3.0)	1051	1050

A – Glucose, B – Yeast extract, C – K_2HPO_4, D - NH_4Cl.
Values in parentheses are uncoded values.
Negative uncoded values in parentheses are set to be zero.
[a] Experiments are ± of two independent experiments.

2.5. Isolation of Hyaluronic Acid

HA produced by mutant strain of *S. zooepidemicus* 3523-7 in fermentation broth was purified as described by Bitter and Muir in 1962 [10]. Carbazole method was used for improved recovery of HA and some modifications were carried out in purification steps. The production medium containing HA was precipitated using the addition of isopropyl alcohol [11]. After precipitation, the precipitated HA was redissolved in 0.15 M sodium chloride and treated with activated charcoal (0.5 – 2%). Later stirred for 45 – 60 min and allowed for centrifugation at 10,000 rpm for 30 min at 4ºC. After centrifugation, filtration was carried out using 0.45 µ filters. The filtrate was further purified using ultrafiltration in diafiltration mode after two to three dilutions with sterile water. Finally the retentive containing HA sample was concentrated to original volume and precipitated with isopropyl alcohol and vacuum dried if necessary.

3. Results and Discussion

3.1. One Factor-at-a-time Method

The influence of different types of carbon and nitrogen sources on the production of HA by mutant *Streptococcus zooepidemicus* 3523-7 was grown in medium with continuous shaking. Individual experiments showed that glucose and yeast extract resulted the highest production of HA among different carbon and nitrogen sources tested.

3.1.1. Effect of Carbon Source: During fermentation, the carbon source act as major constituent for building the cellular material and also useful in production of HA. The

effect of different carbon sources on production of HA was shown in Figure 1. The medium was supplemented with carbon sources such as sucrose, fructose, xylose, maltose, lactose, starch and galactose. Among all glucose is found to be promising in production of HA followed by fructose (658 mg/L). The culture was able to grow in all carbon sources (sucrose, fructose, xylose, maltose, lactose, starch and galactose), but HA was production is very low in xylose (253 mg/L). In another studies, carbon sources like starch, lactose, dextrin and sucrose can be used for HA production which is similar to glucose [12, 13]. Chong *et al.*, in 2005 used maltose (20 g/L) as carbon source using *S. equi* subsp z*ooepidemicus* (ATCC 35246) by batch fermentation mode [14]. Armstrong & Johns in 1997 used glucose (60 g/L) as carbon sources using *S. equi* subsp z*ooepidemicus* (ATCC 35246) [15].

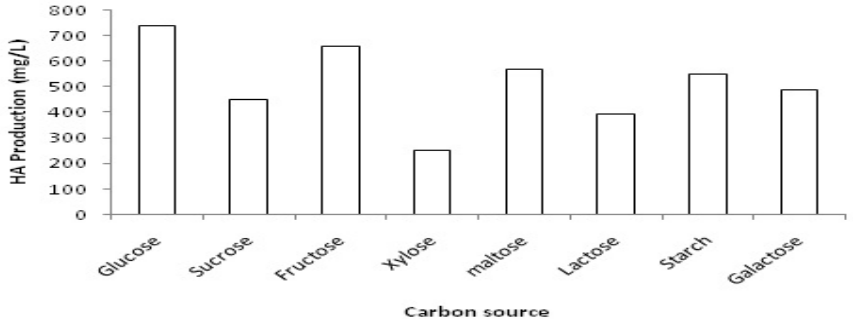

Figure 1. Effect of Different Carbon Sources on HA Production by Mutant *Streptococcus Zooepidemicus* 3523-7

3.1.2. Effect of Nitrogen Source: Different types of nitrogen sources (organic and inorganic) were evaluated for their effect on growth and production of HA was shown in Figure 2. Yeast extract was the best organic nitrogen source and sodium nitrate was found to be the best inorganic nitrogen source for the production of HA. Out of eleven nitrogen sources (peptone, gelatin, soya peptone, meat peptone, mycological peptone, beef extract, corn steep liquor, potassium nitrate, diammonium hydrogen orthophosphate, sodium nitrate and ammonium nitrate), yeast extract gave the maximum yield of 788 mg/L followed by production using beef extract (712 mg/L). With glucose as the carbon source, maximum HA production was achieved with yeast extract at concentration of 0.5%. In another study also yeast-derived nitrogen source (YE 0251) showed the highest HA production [16, 17]. Vazquez *et al.*, in 2010 used tuna peptone (8 g/L) for the production of HA using *S. equi* subsp *zooepidemicus* (ATCC 35246) by fermentation mode [18].

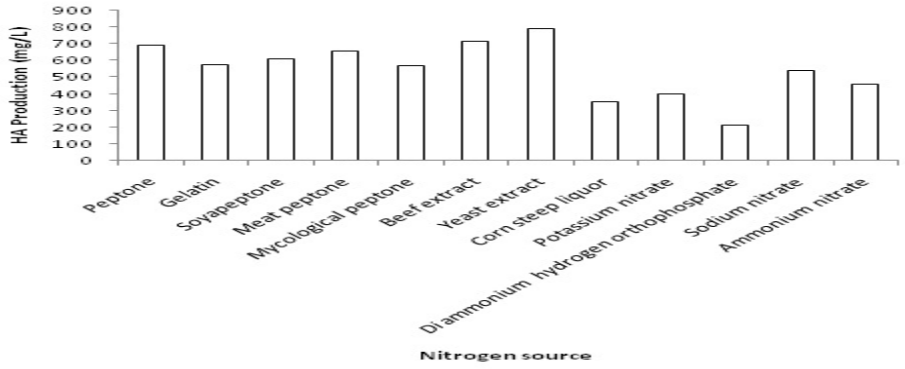

Figure 2. Effect of Different Nitrogen Sources on HA Production by Mutant *Streptococcus Zooepidemicus* 3523-7

3.1.3. Effect of Fermentation Time: The effect of cultivation time on HA production under optimum conditions were showed in Figure 3. Results indicating 24 hr is required for maximum production. After 24 hrs, total protein content and enzyme activity was decreased significantly.

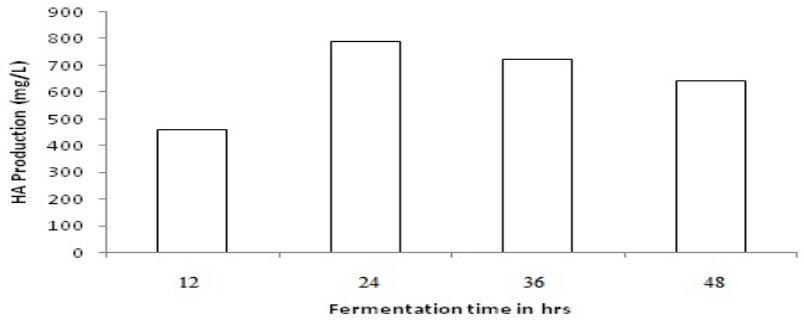

Figure 3. Effect of Cultivation Time on Production of HA by Mutant *Streptococcus Zooepidemicus* 3523-7

3.1.4. Effect of pH: Figure 4 shows the effect of pH for the production of biomass and HA at different pH and 7 is the ideal pH for the over production of biomass and enzyme production. Both in acidic and basic pH the production levels are reduced. The results in this study are similar to the results of Long *et al.*, 2011 [19].

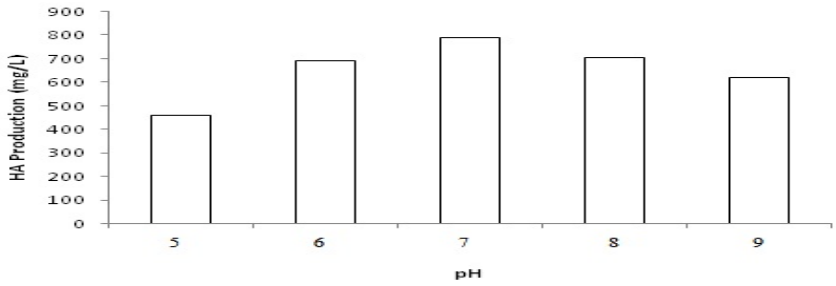

Figure 4. Effect of pH on Production of HA by Mutant *Streptococcus Zooepidemicus* 3523-7

3.2. Optimization using L_{27}–orthogonal Array

After the selection of best carbon and nitrogen sources, the medium was subjected to initial optimization using L_{27} – orthogonal array. The parameters optimized involved concentrations of Glucose, NH_4Cl, Yeast extract, $MgSO_4$, NaCl, KH_2PO_4, TMM, K_2HPO_4, 1M $CaCl_2$. Table 3 represents the response table for means (larger is better) obtained with L_{27}-orthogonal array. Table 4 represents the response table for signal to noise ratio (larger is better) obtained with L_{27}-orthogonal array. The last two rows in Tables 3 and 4 documents the delta values and ranks for the system. These delta values and ranks helps to assess which factors have the greatest effect on the response. Delta was measured by taking the difference between the highest and lowest characteristic average for a factor and higher delta value indicates greater effect of that component. On the other hand rank orders the factors from the greatest effect (based on the delta values) to the least effect on the response. The order in which the individual components selected in the present study effect the fermentation process can be ranked as glucose >yeast extract > K_2HPO_4 > NH4Cl > KH2PO4 > NaCl > MgSO4 > 1M CaCl2 > Trace metal mix (TMM) suggesting that glucose had a major effect and Trace metal mix (TMM) had least effect on HA production by mutant *Streptococcus zooepidemicus* 3523-7.

Table 3. Response Table for Means

Level	Glucose	NH_4Cl	Yeast extract	$MgSO_4$	NaCl	KH_2PO_4	TMM	K_2HPO_4	1M $CaCl_2$
1	736.1	787.5	831.3	818.0	800.4	831.7	825.2	788.3	837.1
2	913.1	837.9	786.8	835.7	831.5	798.6	836.7	867.4	822.8
3	840.6	864.3	871.6	836.0	857.8	859.4	827.8	834.0	829.8
Delta	177.0	76.8	84.7	18.0	57.3	60.8	11.5	79.1	14.2
Rank	1	4	2	7	6	5	9	3	8

Table 4. Response Table for S/N Ratio

Level	Glucose	NH_4Cl	Yeast extract	$MgSO_4$	NaCl	KH_2PO_4	TMM	K_2HPO_4	1M $CaCl_2$
1	57.28	57.47	58.31	58.17	57.61	58.33	58.27	57.49	58.38
2	58.78	58.40	57.48	58.37	58.34	57.60	58.39	58.70	57.86
3	58.49	58.68	58.76	58.00	58.60	58.62	57.90	58.36	58.32
Delta	1.50	1.21	1.27	0.37	0.99	1.02	0.49	1.21	0.52
Rank	1	4	2	9	6	5	8	3	7

Figures 5 and 6 represent the main effect plots for the system and show how each factor affects the response. MINITAB creates the main effects plot by plotting the characteristic average for each factor level and the averages are the same as those displayed in the response Table 1. When the line is horizontal (parallel to the x-axis), then there is no main effect. Each level of the factor affects the characteristic in the same way and the characteristic average is the same across all factor levels. When the line is not horizontal (parallel to the x-axis), then there is a main effect present. Different levels of the factor affect the characteristic differently. The greater the difference in the vertical position of the plotted points (the greater the deviation from the parallel x-axis), the greater is the magnitude of the main effect.

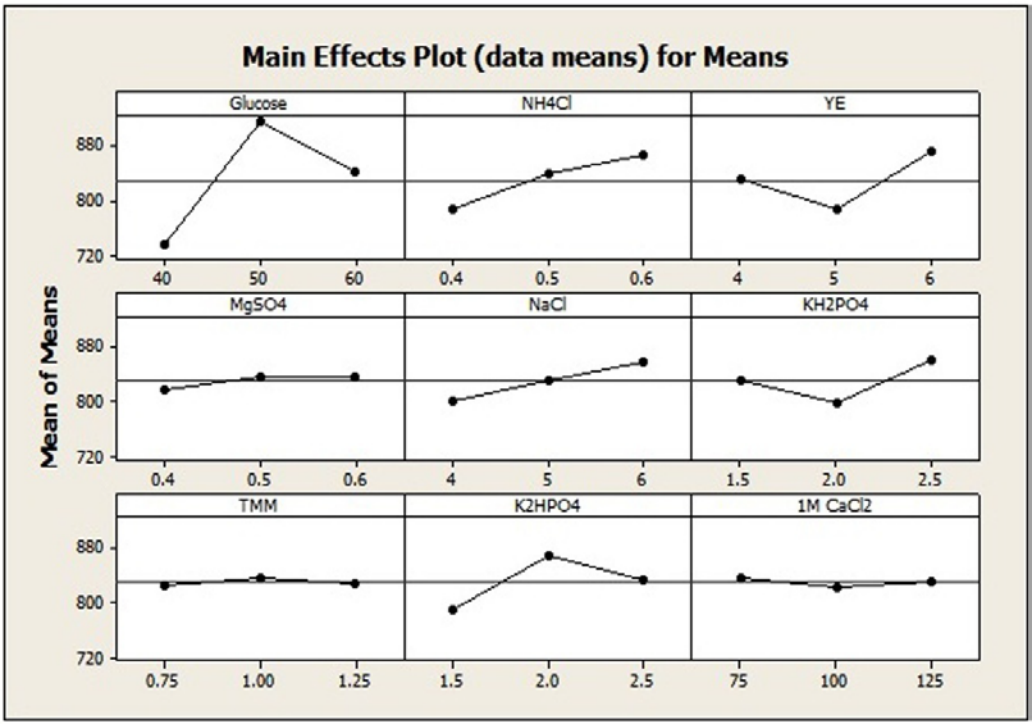

Figure 5. Main Effects Plot for Means

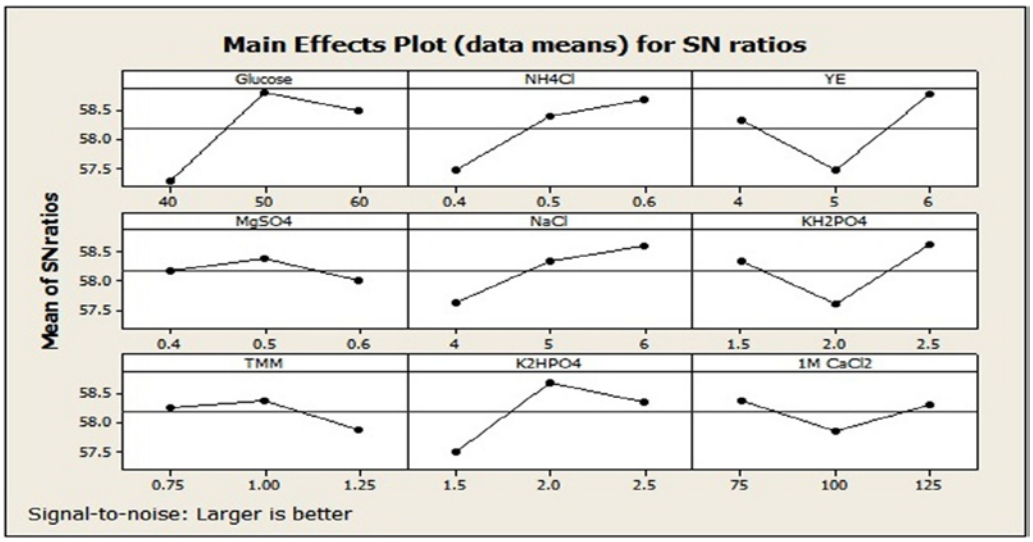

Figure 6. Main Effects Plot for S/N Ratio

3.3. Optimization of Concentrations of the Selected Medium Components by RSM

The combined effect of four independent variables A: Glucose; B: Yeast extract; C: K_2HPO_4; D: NH_4Cl; for production of HA was examined using RSM. The CCRD gave quadratic model for experimental results. The following equations represent the mathematical model relating the production of HA with independent process variables, A to D and the second order polynomial coefficient for each term of the equation determined through multiple regression analysis using the Design Expert 6.0.10.

Final equation in terms of coded factors for the production of HA (mg/L) was 1050 + 16.17 X A + 32.50 X B + 24.42 X C + 33.67 X D + 45.69 X A^2 + 27.06 X B^2 + 58.31 X C^2 + 37.44 X D^2 – 17 X A X B -8.12 X A X C + 7.38 X A X D – 43.5 X B X C – 23.75 X B X D + 30.13 X C X D.

Final equation in terms of actual factors for the production of HA (mg/L) was 1174.75521 – 5.51979 X glucose + 13.375 X yeast extract – 107.27083 X K_2HPO_4 – 41.19792 X NH_4Cl + 0.11422 X $(\text{glucose})^2$ + 6.76562 X $(\text{yeast extract})^2$ + 58.31250 X $(K_2HPO_4)^2$ + 9.35938 X $(NH_4Cl)^2$ – 0.42500 X glucose X yeast extract – 0.40625 X glucose X K_2HPO_4 + 0.18438 X glucose X NH_4Cl – 21.75 X yeast extract X K_2HPO_4 – 5. 93750 X yeast extract X NH_4Cl + 15. 0625 X K_2HPO_4 X NH_4Cl.

The experimental and predicted values of yields of HA are given in Table 2. The results were analyzed by using ANOVA, *i.e.*, analysis of variance suitable for the experimental design used. The ANOVA of the quadratic model indicated that the model is significant. The model F-value of 384.6 implies the model to be significant and is calculated as ratio of mean square regression and mean square residual. Model P-value (Prob > F) was very low (<0.0001), again signifying the model to be significant. The P values were used as a tool to check the significance of each of the coefficients, which, in turn are necessary to understand the pattern of the mutual interactions between the test variables. The smaller the magnitude of the P, the more significant is the corresponding coefficient. Values of p less than 0.05 indicate the model terms to be significant. The coefficient estimates and the corresponding P values suggests that, among the test variables used in the study, A, B, C, D, A^2, B^2, C^2, D^2, AB, AC, AD, BC, BD and CD (where A = Glucose B = Yeast extract, C = K_2HPO_4 and D = NH_4Cl) are significant model.

The fit of the model was also expressed by the coefficient of regression (R^2), which was found to be 0.9972, indicating that 99.72% of the confidence level of the model to predict the response (HA yield). The ‘‘Pred R-Squared” of 0.9843 is in reasonable

agreement with the ‘‘Adj R-Squared” of 0.9946. ‘‘Adeq Precision” measures the signal to noise ratio. A ratio greater than 4 is desirable. Here, the ratio of 62.787 indicates an adequate signal. In RSM, contour plot generation and point prediction were also studied to find optimum value of the combination of the four media constitutes for the over production of HA. The values (predicted) are verified with experimental results.

The three-dimensional graphs were generated for the pair-wise combination of the four factors, while keeping the other two at their center point levels (Figure 7-12). From the central point of the contour plot or from the bump of the 3D plot the optimal composition of medium components was identified. Figure 13 illustrated the parity plot for the distribution of predicted and experimental values for the production of HA.

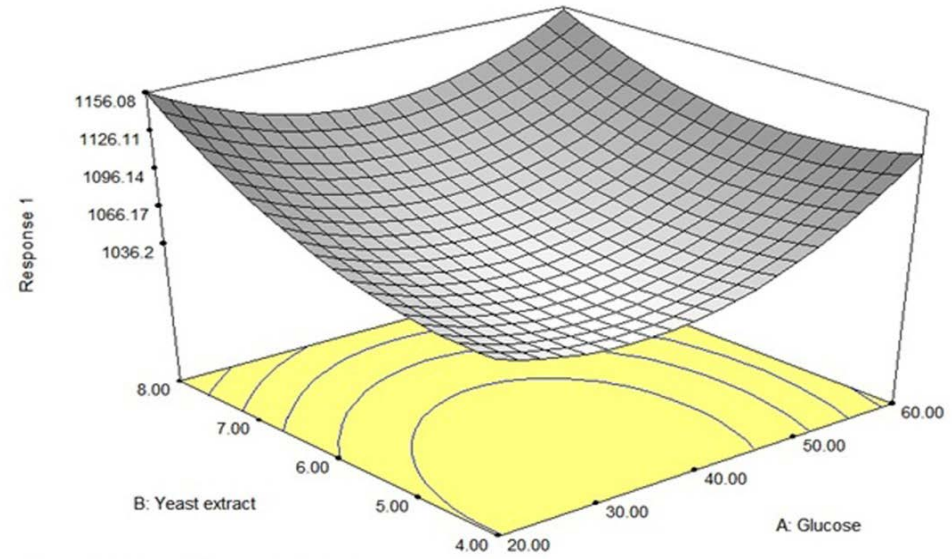

Figure 7. Illustrated the Response Surface Plot for Production of HA; Effect of Glucose and Yeast Extract

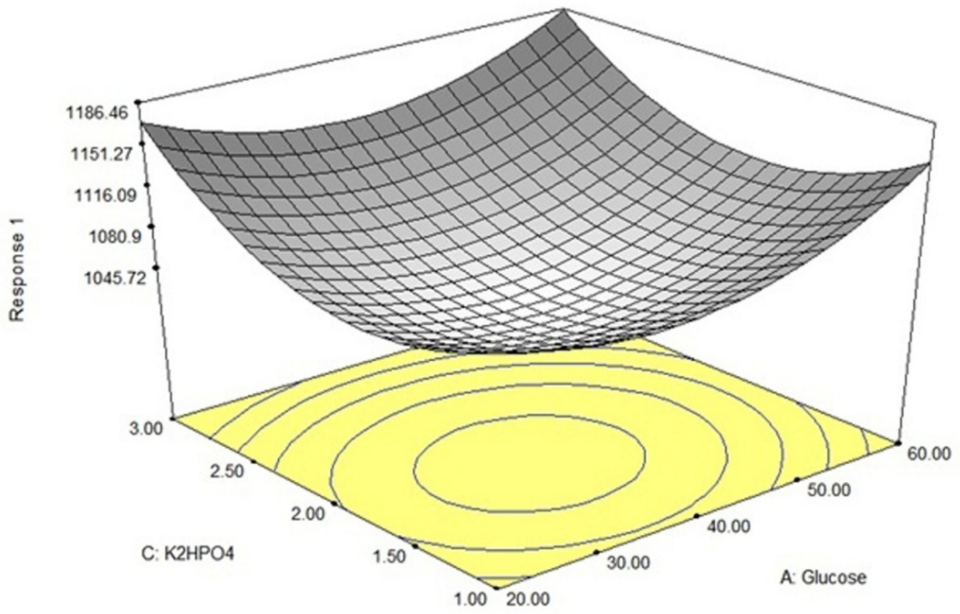

Figure 8. Illustrated the Response Surface Plot for Production of HA; Effect of Glucose and K_2HPO_4

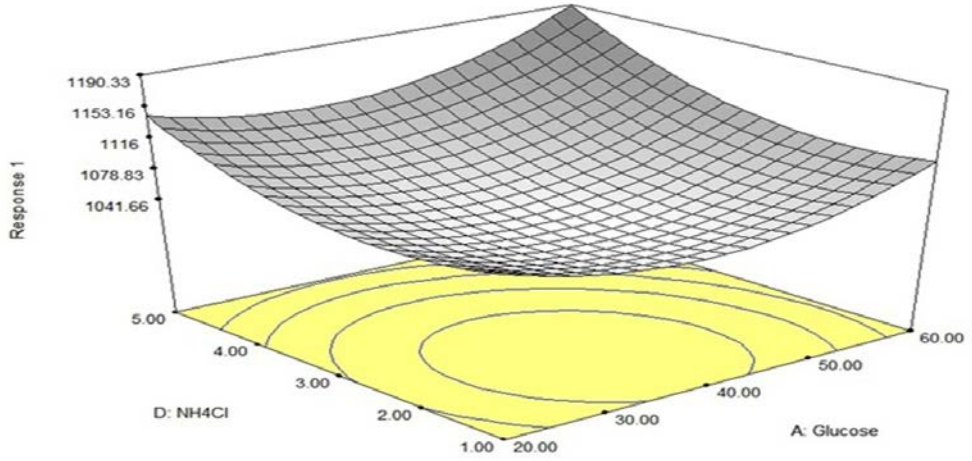

Figure 9. Illustrated the Response Surface Plot for Production of HA; Effect of Glucose and NH_4Cl

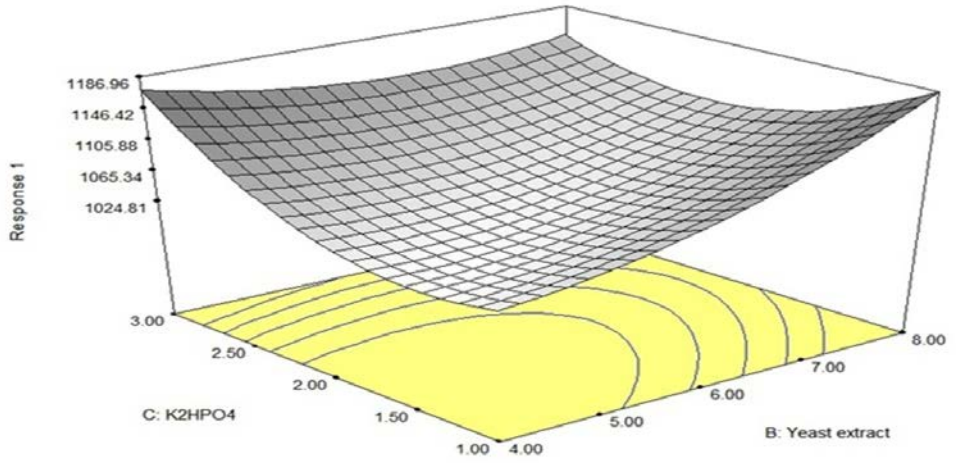

Figure 10. Illustrated the Response Surface Plot for Production of HA; Effect of Yeast Extract and K_2HPO_4

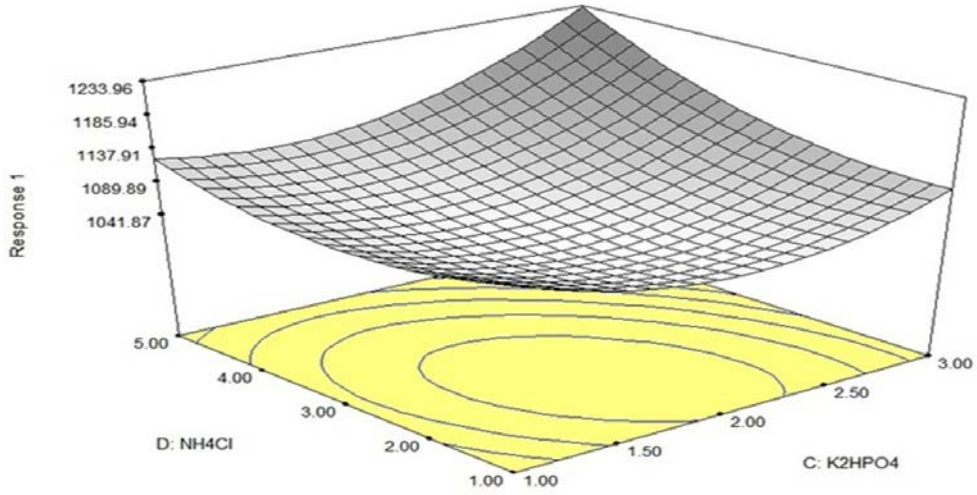

Figure 11. Illustrated the Response Surface Plot for Production of HA; Effect of NH_4Cl and K_2HPO_4

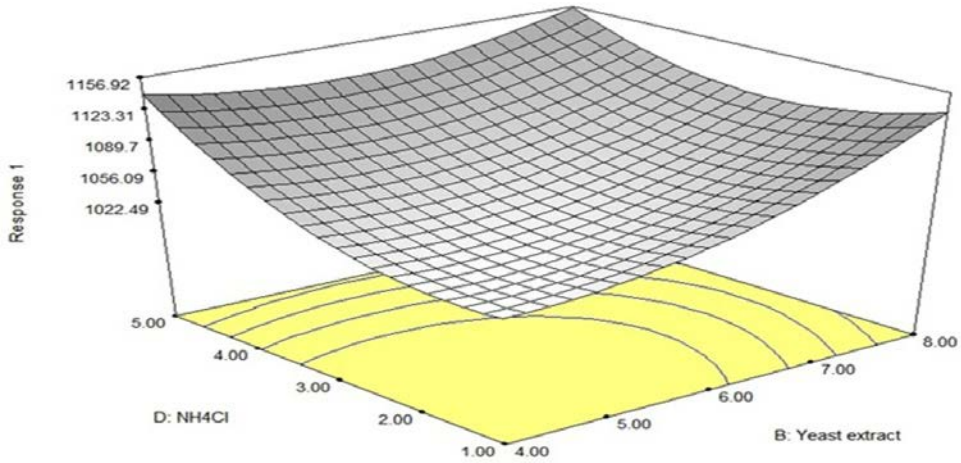

Figure 12. Illustrated the Response Surface Plot for Production of HA; Effect of Yeast Extract and NH_4Cl

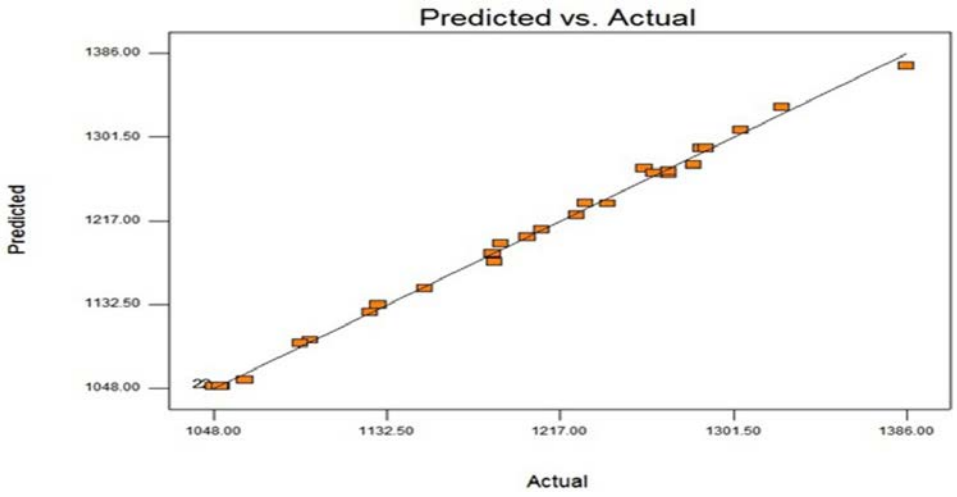

Figure 13. Illustrated the Parity Plot for the Distribution of Predicted and Experimental Values for the Production of HA

4. Conclusion

Different types of microbes are known to produce HA [20]. Now a days, fermentative production of HA using cost effective medium has gained an attractive alternative. Different types of Gram-positive and Gram-negative bacteria like *Bacillus subtilis* [21], *Lactococcus lactis* [22] and *E. coli* [23] are also known to produce HA. Among all those *Streptococcus equi* subsp. *Zooepidemicus* MTCC 3523 is one of the best producer of HA. Different methods are employed for the production of HA [24]. Liu *et al.*, [25] achieved the over production of hyaluronic acid using *Streptococcus zooepidemicus* by an intermittent alkaline-stress strategy [26]. Some studies proved the influence of hyaluronidase addition on production of hyaluronic acid by batch fermentation of *Streptococcus zooepidemicus* [25]. But production ranges are at door steps. Spontaneous mutation results, mutant strain *Streptococcus zooepidemicus* 3523-7, which produces elevated levels of HA when compared to *Streptococcus equi* subsp. *Zooepidemicus* MTCC 3523 grown in Todd Hewitt broth (THB), Brain heart infusion broth (BHI) and Veal infusion broth (VIB). In 1980, Van de Rijn and Kessler used chemically defined medium for the growth and production [11]. The medium is designed for over production of HA and components concentration was determined using taguchi method. The highest

influencing factors were run in RSM to check the influence of factors concentration on production of HA. 1.4 folds increase in production was achieved using RSM. Finally, 1.76 folds of increase were observed over native medium. The production in this study was appreciable and if run in fermentor under standard conditions this mutant strain will become ideal source for the production of HA. So in future, cost effective media composition for the over production of HA is desirable at large scale fermentation using mutant strain *Streptococcus zooepidemicus* 3523-7.

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