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Effect of Various Physical Parameters and Statistical Medium Optimization on Production of Hyaluronic Acid Using *S. Equi* Subsp. *Zooepidemicus* ATCC 39920

RESEARCH ARTICLE

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Abstract

It has been shown that initial conditions for bacterial cultivation are extremely important for the successful production of hyaluronic acid (HA) by fermentation. We investigated several physical parameters that affect productivity of HA under shake flask. i.e. transfer criteria of seed, agitation and aeration of fermentation flasks. Among the various physical parameters studied, inoculum age of 8-10 h, pH 6.4, optical density (600 nm) 2.0 and 3% level inoculum transfer found to be optimum. After inoculating with *Streptococcus equi* subsp. *zooepidemicus* ATCC 39920, the temperature 37 ^{0}C and 90 rpm found optimum during growth as well as for the HA production. The fractional factorial design of six factors with two levels showed yeast extract, potassium dihydrogen phosphate and sodium bicarbonate as significant model terms. The factor potassium dihydrogen phosphate was relatively more significant than yeast extract.

Keywords: *Streptococcus zooepidemicus; Hyaluronic acid; Shake flask fermentation; Fractional factorial design; Statistical model; Physical parameters*

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INTRODUCTION

Hyaluronic acid (HA) is a high molecular weight glycosaminoglycan. Markovitz et al, (1959) reported the synthesis of HA using UDP –glucuronic acid and UDP N-acetyl glucosamine as substrates and the streptococcal -membrane-associated enzyme hyaluronate synthase as catalyst. Naturally, HA occurs in bacteria as a mucoid capsule surrounding the cell, and in soft tissues and the vitreous humor of the eye, in higher animals including human beings (O'Regan et al, 1994). It has been applied in clinical therapy and clinical diagnoses due to its high biocompability and many important physiological functions eg. arthral lubrication, diffusion & transportation of protein, maintenance of water & electrolyte and acceleration of wound healing (Swann and Kuo, 1991) .

The commercial HA can be extracted from rooster combs or produced by microbial production of group A and C *Streptococci* owing to the fact that the use of animal derived biochemicals for human therapeutics is being met with opposition because of the risk of cross species viral infection. Hence, the microbial production is gradually replacing extraction as a preferred source of HA. Currently, the commonly used strain for microbial HA production at an industrial scale is *Streptococcus zooepidemicus* which synthesizes HA as the extra cellular capsule (Duan et al, 2008). Although many studies have been performed an HA production, there are no reports on statistical optimization. Thus the present study showed the optimization of HA production at various physical parameters under shake flask conditions by *S. equi* subsp. *zooepidemicus* ATCC 39920.

MATERIALS AND METHODS

Bacterial Strain

Streptococcus equi subsp. *zooepidemicus* ATCC 39920 was obtained from American type culture collection, ATCC (USA). The strain was maintained and preserved on agar slants and in glycerol stock at 4^0 and -80^0C, respectively.

Cultivation

Seed culture development was carried out in a 250 ml Erlenmeyer flask with 50 ml of the P10 medium which contained (per liter) 10g peptone bacteriological, 5.0g yeast extract, 10.0g KH_2PO_4, 5.0g $C_2H_4NaO_2.3H_2O$, 3.0g $NaHCO_3$, and 7.5ml salt solution [The composition of the salt solution per liter: 1.0g $FeSO_4.7H_2O$, 20.0g $MgSO_4.7H_2O$, 1.0g $MnSO_4.4H_2O$, 5.0g $CaCl_2.2H_2O$, 46.0g Na_2 EDTA.$2H_2O$], pH 6.8-6.9. Total 47 ml of medium was dispensed in 250 ml conical flask and

MICROBIOLOGY

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sterilized at 121°C for 20 minutes. After sterilization, 3.0 ml of dextrose solution (50 % prepared and sterilized separately) and 2.0 ml of calcium carbonate solution (25 % prepared and separately sterilized) was added. Modification of the medium used by Vazquez et al, (2009) was used. In P10 medium concentration of potassium dihydrogen phosphate is more. In earlier reported medium of Vazquez et al, (2009) the potassium dihydrogen phosphate is 2 g/L whereas in P10 medium it is 10 g/L. Also sodium acetate, sodium bicarbonate and salt solution is additional in P10 medium which is not there in medium by Vazquez et al, (2009).The P10 medium was also used as a seed medium. Conditions for shake flask fermentation: One ml of *Streptococcus equi* subsp. *zooepidemicus* ATCC 39920 frozen glycerol stock was transferred to 50 ml vegetative medium in 250 ml Erlenmeyer flasks. The flask was incubated at 37°C and 90 rpm at 5 cm displacement for 7-12 h (Scigenics shaker). When the pH of the seed reached between 6.3-6.4 and optical density at 600 nm reached 2.0 in P10 medium, 1.5 ml of the inoculum was transferred to the 50 ml fermentation medium. The inoculated fermentation shake flasks were incubated at 37°C at 90 rpm. After 24 hours the flasks were harvested and analyzed for pH, OD and yield of HA.

Analytical Methods

Cell and optical density of the broth was measured at 600 nm with a spectrophotometer (Shimadzu, UV120-02, Japan). Total sugar was measured using Anthrone's reagent method (Trevelyan *et al.*, 1952).

HA Quantification

In the measurement of HA concentration, the fermented broth sample was first diluted with water and centrifuged at 3000 rpm for 5 minutes to remove solid mass. The resulted supernatant solution was then subjected to HA precipitation by mixing with two volumes of ethanol. The precipitate was collected by centrifugation at 3000 rpm for 20 minutes and the precipitate was redissolved in water.To this was then added disodium tetraborate solution and boiled for 10 minutes. After cooling to room temperature, carbazole solution was added and heated in water bath for 15 minutes and read at 525 nm with D-glucuronic acid as the standard (Bitter and Muir, 1962).

Effect of Various Parameters on HA production

Effect of various physical parameters on HA production like optical density (1.5-2 at 600nm), pH (5.5-6.6), temperature (30-40°C), agitation (50-150rpm) and different cultivation volume (25-100ml) were studied in P10 medium under shake flask.

Media Optimization by Two Level Factorial Fractional Designs (FFD)

Two level fractional (1/2 fraction) design was employed for P10 medium. Table 1 shows minimum and maximum concentration of P10 medium ingredient.

Table 1. Minimum and maximum concentration of media ingredients of P10 medium in fractional factorial design.

Code	Factor	Minimum (-) Concentration (g/L)	Maximum (+) Concentration (g/L)
A	Peptone	10.0	20.0
B	Yeast extract	3.0	7.0
C	KH_2PO_4	5.0	15.0
D	Sodium acetate trihydrate	3.0	7.0
E	Sodium bi carbonate	2.0	4.0
F	Dextrose	20.0	40.0

Effect of peptone, yeast extract, KH_2PO_4, sodium acetate trihydrate, sodium bicarbonate and dextrose on maximum HA production was investigated using Two level fractional (1/2 fraction) design. Factorial designs are to check the important parameters that are affecting production of HA along with interaction between the variables. The six factors (peptone, yeast extract, KH_2PO_4, sodium acetate trihydrate, sodium bicarbonate and dextrose) examined at two levels are as listed in Table 1. The choice of levels of the factors was based on information from literature and preliminary experiments. A two level fractional factorial design (1/2 frac) experiment was conducted in triplicate with *S. equi* subsp. *zooepidemicus* ATCC 39920. The total 32 experiments were carried out at randomized run order. The response variable selected was the maximum concentration of HA. Results were analyzed using analysis of variance (ANOVA) using software (Design Expert version 6.0.10, Stat-Ease, Minneapolis, MN, USA). On connecting the points for each factor, the line is created which states the significance. The parallel line to the axis represents no significant effect. However the diagonal line to the axis represents a significant effect.

RESULTS AND DISCUSSIONS

Effect of Various Physical Parameters on HA Production

The P10 medium was developed both for inoculam and shake flask fermentation. P10 medium contained required factors. Production of HA was studied using P10 medium containing glucose and peptone as carbon and nitrogen sources respectively, after 24 h, HA yield of 0.70±0.03 g/L was obtained.

When preparing inoculum the requirement is a fast growing culture where metabolic pathways are induced and then culture can immediately start multiplying in production medium. If the inoculum is suboptimal then it turns to die in the production cycle or starts to grow at the expense of producing the product thus delaying the yield time. In order to study the effect of the culture age in the seed media, experiments were conducted by transferring different age of seed (like 8 h 14 h and 20 h) to the fermentation media. The age of culture was fixed as a range from log 08 to log 14 in which the yield in fermentation media was 0.73 g/L and 0.7 g/L respectively. The maturity parameters are achieved at 08-10 h with pH 6.3-6.4 and A_{600} greater than 2.0. Huang *et al.*, (2007) proposed the same age as the log phase with greater cell number and cell mass in *Streptococcus equi* subsp. *zooepidemicus* ATCC 39920 for production of HA.

Inoculam between 2 % and 5 % has shown better yield. Higher concentrations have not proven worthwhile. Higher concentration tend to shift the fermentation to a growth cycle phenomenon and to produce other pathways, major metabolic pathway which are delicately balanced will tend to be over- ridden. If the inoculum is low then it tends to die and or take much longer time to go to log phase. The study revealed that 3% inoculum as the optimum and there was no significant difference between the 3% and 5% inoculum. When 10% inoculum was used the batch shown less yield than the batch with 3% inoculum (Table 2). Addition of high amount of inoculum such as 10% increases the sugar consumption at the initial phases and the pH drop was high in such case. Increasing the inoculum percentage allows the parameters to achieve earlier with high sugar consumption that favoring growth at the most extent. In case of HA production, the growth rate should be comparatively low than the HA production rate.

Table 2. Effect of inoculum level on HA production by *S. equi* subsp. *zooepidemicus* ATCC 39920 using P10 medium

Inoculum level (%)	HA Yield (g/L)
1	0.62 ± 0.03
2	0.65 ± 0.03
3	0.70 ± 0.04
5	0.64 ± 0.03
10	0.64 ± 0.03

± indicates standard deviation; The medium (50 ml) in 250 ml Erlenmeyer flask, after inoculating with *S. equi* subsp. *zooepidemicus* ATCC 39920 (A_{600} 2.0, pH 6.3 and 1%, 3%, 5% and 10% level) was incubated at 37°C ±1 on an orbital shaker (90 rpm) for 24 h.

Optimal oxygen must be provided such that the organism grows in a reproducible metabolic pattern, which in turn is best suited for the production of secondary desired metabolites. Higher aeration will induce undesirable pathways and metabolites that may be toxic to the organisms. Higher level of oxygen is suppressive for secondary metabolite pathways in general. The growth and production of HA was screened in the static and aerobic conditions. The aerobic conditions gave good yield of 0.70 ±0.03 g/L in the P10 medium. Under Static conditions the HA production was low reaching up to 0.50 ±0.02 g/L. The organism *Streptococcus* species require minimum critical concentration of dissolved oxygen in the medium to grow well and produce HA (Swann *et al.*, 1990). Duan *et al.* (2006) reported that under anaerobic conditions the organism do not replicate fast or produce HA in huge amounts when compared to aerated conditions.

Fermentation Period

Fermentation period will be depend on the product is being synthesized and is desirable in optimal yield. Therefore harvesting has to be product concentration related. It is not advisable to continue fermentation over long cycle unless it is a fed batch mode where periodic harvesting is used. In order to determine the fermentation period, where maximum productivity is obtained the broth was harvested at different time at regular intervals and estimated the HA yield. At log 16, the maximum productivity of HA (0.70 g/L) was observed whereas at 24 h also the HA in fermentation broth found to be 0.70 g/L. For the shake flask experiments the 24 h fully fermented broth was taken for analysis and it is kept constant for the batches to be taken in fermenter, simultaneous HA assay and viscosity

measurements were carried out to predetermine the reduction in the HA production at late hours. The organism is a fast growing organism which attains its maximum HA production at log 16 under the provided conditions (Chong *et al.*, 2005).

Determination of the Seed Maturity Parameters

Effect of Optical Density of Seed

The seed maturity parameters are optimized, especially the optical density of the seed. The seed from the lab shake flask at different optical densities (1.4, 1.6, 2.0, 2.4 and 2.8 at 600 nm) were studied by transferring them to shake flask fermentation medium. It was found that the optical density should be in the range from 1.5 to 2.2 to obtain better HA yield. Optical density greater than this limit such as 2.4 and 2.8 gave considerably less yield. The low yield of HA was obtained when optical density (A_{600}) of inoculam was 2.8 (Table 3).

Table 3. Effect of optical density of seed on HA production by *S. equi* subsp. *zooepidemicus* ATCC 39920 using P10 medium

Optical density A_{600}	HA Yield (g/L)
1.4	0.58 ± 0.03
1.6	0.66 ± 0.03
2.0	0.70 ± 0.03
2.4	0.65 ± 0.03
2.8	0.57 ± 0.02

The medium (50 ml) in 250 ml Erlenmeyer flask, after inoculating with *S. equi* subsp. *zooepidemicus* ATCC 39920 (A_{600}= 1.4, 1.6, 2.0, 2.4 and 2.8, 3% level) was incubated at 37°C ±1 on an orbital shaker (90 rpm) for 24 h.

Effect of Transfer pH of Seed

Various pH ranges 5.5, 5.8, 6.2, 6.4, 6.6 were tested for determining the transfer criteria. The fermentation media inoculated with the seed at pH 6.4 gave maximum yield of 0.70 g/L, 6.2 gave 0.68 g/L whereas the pH 5.5, 5.8 and 6.6 gave less yield of 0.55, 0.60 and 0.65 g/L respectively. The effect of lab seed pH as a transfer criterion was studied and determined that 6.2 to 6.4 as the range at which the organism reaches the active mid log phase. Hence at this stage the OD will be reaching 2.0 when measured at 600 nm. At the pH of 5.5 or 6.0 (which is highly acidic) the organism reached its late log phase and then stationary phase which are not the perfect time for the transfer to fermentation medium.

Effect of Fermentation pH

Hyaluronate synthase which catalyzed the polymerization of HA was reported to have maximum activity at pH 7.1 in cell free extracts (Stool miller and Dorfman, 1969). Akasaka *et al,* (1988) reported that pH 7.4 gave the highest viscosity compared to pH 6.0 and 7.9. The fermentation media were adjusted to different pH of 6.0, 6.5 and 7.0, in which the pH 7.0 was found as optimum for the production of HA and growth as shown in Table 4. The pH of the fermentation medium was adjusted at 7.0. The organism grows well and produces lactic acid and acetate which reduces the pH from 7.0 to 5.5 around during the harvest. Addition of calcium carbonate at a concentration of 10.0 g/L, act as a buffering agent and prevents the pH from going down towards acidic region. The neutral pH is the optimum pH for the organism to grow well and produce HA. At acidic pH the production of HA gets inhibited (Jagannath and Ramchandran, 2010).

Table 4. Effect of fermentation pH on HA production by *S. equi* subsp. *zooepidemicus* ATCC 39920 using P10 medium

Fermentation pH	HA Yield (g/L)
6.0	0.50 ± 0.02
6.5	0.65 ± 0.03
7.0	0.70 ± 0.03
7.5	0.65 ± 0.03

The medium (50 ml) with different fermentation pH in 250 ml Erlenmeyer flask, after inoculating with *S. equi* subsp. *zooepidemicus* ATCC 39920 (A_{600}= 2.0, pH 6.3, 3% level) was incubated at 37°C ±1 on an orbital shaker (90 rpm) for 24 h.

Effect of Agitation Speed

Different agitation speed of 50, 70, 90, 110 and 150 rpm was studied in a shaker with displacement 5 cm. Shaking at 70-90 rpm was found more significant for the production of HA as shown in Table 5. Increasing the turbulence more than 90 rpm increases the shear over the organism which might affect the production of HA.

Effect of Temperature

Fermentation shake flask was tested at different temperatures such as 32°C, 35°C, 37°C and 40°C among which the 37°C was found to be the optimum for the production of HA and growth of the organism as shown in Table 6. The optimum temperature for the growth and

HA production was found to be 37°C. Suboptimal temperature growth has been reported to have aided the synthesis of high molecular weight HA (Armstrong and Johns, 1997).

Table 5. Effect of agitation speed on HA production by *S. equi* subsp. *zooepidemicus* ATCC 39920 using P10 medium

Agitation speed (rpm)	HA Yield (g/L)
50	0.62 ± 0.03
70	0.69 ± 0.03
90	0.72 ± 0.03
110	0.68 ± 0.03
150	0.65 ± 0.03

The medium (50 ml) with different fermentation pH in 250 ml Erlenmeyer flask, after inoculating with *S. equi* subsp. *zooepidemicus* ATCC 39920 (A_{600}= 2.0, pH 6.3, 3% level) was incubated at 37°C ±1 on an orbital shaker (50, 70, 90, 110 and 150 rpm) for 24 h.

Table 6. Effect of temperature on HA production by *S. equi* subsp. *zooepidemicus* ATCC 39920 using P10 medium

Temperature (°C)	HA Yield (g/L)
32	0.64 ± 0.03
35	0.68 ± 0.03
37	0.70 ± 0.03
40	0.62 ± 0.03

The medium (50 ml) with different fermentation pH in 250 ml Erlenmeyer flask, after inoculating with *S. equi* subsp. *zooepidemicus* ATCC 39920 (A_{600}= 2.0, pH 6.3, 3% level) was incubated at 32, 35 37, and 40°C ±1 on an orbital shaker (90 rpm) for 24 h.

Table 7. Effect of volume of medium on HA production by *S. equi* subsp. *zooepidemicus* ATCC 39920 using P10 medium

Media volume to flask volume (ml)	Media volume to flask volume ratio	HA Yield (g/L))
25 / 250 ml	0.10	0.65 ± 0.03
50 / 250 ml	0.20	0.70 ± 0.03
75 / 250 ml	0.30	0.65 ± 0.03
100 / 250 ml	0.40	0.62 ± 0.03

Effect of Cultivation Volume

Different volume of media was used ranging from 100 ml in 250 ml, 75 ml in 250ml, 50 ml in 250 ml and finally 25ml in 100 ml. Decreasing the volume of medium in 250 ml flasks increased the productivity of HA. 50 ml/250 ml medium to volume ratio of 0.2 found to be optimum (Table 7). The addition of 75 ml or 100 ml medium to the 250 ml affected the mass transfer.

Optimization of HA production under shake flask conditions

Variations are observed while carrying out shake flask experiments. When growth and HA production started, it has been observed that pH went down which restricted growth and HA production. $CaCO_3$ was added to minimize the drop in pH to acidic side. At fermenter level we have added 3.0 N NaOH at automatic mode to maintained pH near 7.0 throughout the fermentation. It has been observed that the colony selection is an important criteria. Selection of mucoid colony is important. The culture preserved as a glycerol stock at -80°C should be used within one month. These factors are important to achieve high and reproducible yield of HA at shake flask and fermenter level.

Media optimization by two level fractional factorial designs P10 medium optimization

A two level fractional (1/2 frac) experimental design, leading to a total number of 32 experiments (Table 8) was employed for the optimization of the parameters. Table 8 also contains an experimental set-up with observed yield of HA (g/L). The coefficient of determination (R^2) was calculated as 0.9517 for HA production (Table 9), indicating that the statistical model can explain 95.87% of variability in the response. The R^2 value is always between 0 and 1. The closer the R^2 is to 1.0, the stronger the model and the better it predicts the response. This indicated a good agreement between the experimental and predicted value for HA production. The model F-value of 6.85 and values of 'prob > F' less than 0.05 indicated that the model terms are significant. For HA production B (yeast extract), C (KH_2PO_4) and E (sodium bicarbonate) along with CD (KH_2PO_4 and sodium acetate), DF (sodium acetate and dextrose), EF (sodium bicarbonate and dextrose), ABC (peptone, yeast extract and KH_2PO_4), ABE (peptone, yeast extract and dextrose), ACD (peptone, KH_2PO_4 and sodium acetate), ADE (peptone, sodium acetate and sodium bicarbonate), AEF (peptone ,sodium bicarbonate and dextrose) are significant model terms. The final response equation that represented a suitable model for HA production is given as Eq. 1. The factor KH_2PO_4 was relatively more significant than yeast extract (Figure 1). On connecting the points for each factor, the line is created which states the significance. The line is parallel to the axis then there is no significant effect. However, the line is diagonal to the axis then there is a significant effect.

Table 8. Factorial designs for components of P 10 medium in coded form and actual values with their corresponding response in terms of HA production

Run	A	B	C	D	E	F	HA Yield (g/L)± SD
1	-	+	-	+	+	+	0.426 ± 0.02
2	+	+	-	+	-	+	0.348 ± 0.015
3	+	-	-	+	+	+	0.337 ±0.015
4	-	+	+	-	+	+	0.518 ±0.02
5	+	-	+	+	-	-	0.501 ±0.02
6	-	+	+	-	-	-	0.672 ±0.03
7	-	+	+	+	-	+	0.426 ±0.02
8	+	+	-	-	+	+	0.343 ±0.015
9	+	+	+	+	+	+	0.485 ±0.02
10	+	-	+	+	+	-	0.439 ±0.02
11	-	+	-	-	-	+	0.273 ±0.01
12	+	+	+	-	+	-	0.392 ±0.02
13	-	-	+	-	+	-	0.359 ±0.015
14	+	+	+	+	-	-	0.369 ±0.015
15	-	-	+	+	-	-	0.378 ±0.015
16	-	+	+	+	+	-	0.219 ±0.01
17	+	-	-	-	-	+	0.340 ±0.015
18	-	-	-	+	-	+	0.340 ±0.015
19	-	-	-	+	+	-	0.330 ±0.015
20	-	-	+	+	+	+	0.343 ±0.015
21	-	+	-	+	-	-	0.458 ±0.02
22	+	+	-	+	+	-	0.367 ±0.01
23	+	-	+	-	-	-	0.479 ±0.01
24	-	+	-	-	+	-	0.278 ±0.015
25	+	-	-	+	-	-	0.297 ±0.01
26	+	-	+	-	+	+	0.325 ±0.015
27	+	+	+	-	-	+	0.447 ±0.02
28	+	-	-	-	+	-	0.301 ±0.015
29	+	+	-	-	-	-	0.456 ±0.02
30	-	-	-	-	-	-	0.296 ±0.015
31	-	-	+	-	-	+	0.325 ±0.015
32	-	-	-	-	+	+	0.322 ±0.01

HA= 0.38 +8.219E-003 * A +0.024 * B +0.036 * C -1.969E-003 * D -0.019 * E +2.812E- 04 * F -0.012 * A * B +4.094E-003 * A * C +5.719E-003 * A * D +3.906E-003 * A * +1.344E-003 * A * F-2.187E-004 * B * C-6.906E-003 * B * E-0.020 * C * D-0.013 * * E+8.719E-003 * D * E+0.022 * D * F+0.026 * E * F-0.018 * A * B * C+0.018 * A * B * E+0.035 * A * C * D+0.021 * A * D * E-0.028 * A * E * F

where, A-peptone, B-yeast extract, C-KH_2PO_4, D-sodium acetate trihydrate, E-sodium bicarbonate, F-dextrose

Table 9. Analysis of variance (ANOVA) for the experimental results of the Fractional factorial design for components of P 10 medium

Factors	Sum of squares	Mean square	F value	Prob > F p-value
Model	0.24	0.010	6.85	0.0042
A	2.162E-003	2.162E-003	1.41	0.2687
B	0.018	0.018	11.95	0.0086
C	0.042	0.042	27.72	0.0008
D	1.240E-004	1.240E-004	0.081	0.7831
E	0.012	0.012	7.88	0.0230
F	2.531E-006	2.531E-006	1.654E-003	0.9686
AB	4.729E-003	4.729E-003	3.09	0.1168
AC	5.363E-004	5.363E-004	0.35	0.5702
AD	1.047E-003	1.047E-003	0.68	0.4322
AE	4.883E-004	4.883E-004	0.32	0.5876
AF	5.778E-005	5.778E-005	0.038	0.8508
BC	1.531E-006	1.531E-006	1.001E-003	0.9755
BE	1.526E-003	1.526E-003	1.00	0.3471
CD	0.013	0.013	8.66	0.0186
CE	5.330E-003	5.330E-003	3.48	0.0989
DE	2.433E-003	2.433E-003	1.59	0.2429
DF	0.015	0.015	9.70	0.0144
EF	0.021	0.021	13.70	0.0060
ABC	0.010	0.010	6.71	0.0321
ABE	0.011	0.011	6.99	0.0295
ACD	0.040	0.040	26.31	0.0009
ADE	0.014	0.014	9.14	0.0165
AEF	0.026	0.026	16.80	0.0034
P<0.05				
Std. Dev.	0.039		R-Squared	0.9517
Mean	0.38		Adj R-Squared	0.8128
C.V.	10.27		Pred R-Squared	0.2271
PRESS	0.20		Adeq Precision	11.831

Figure 2 shows main interaction plots between KH_2PO_4 and sodium acetate, dextrose and sodium acetate, dextrose and $NaHCO_3$. Figure 3 is a parity plot for P10 medium by two level factorial designs. Actual value is close to the predicted value that has been given by model so the model has been fitted very well as per Table 10.

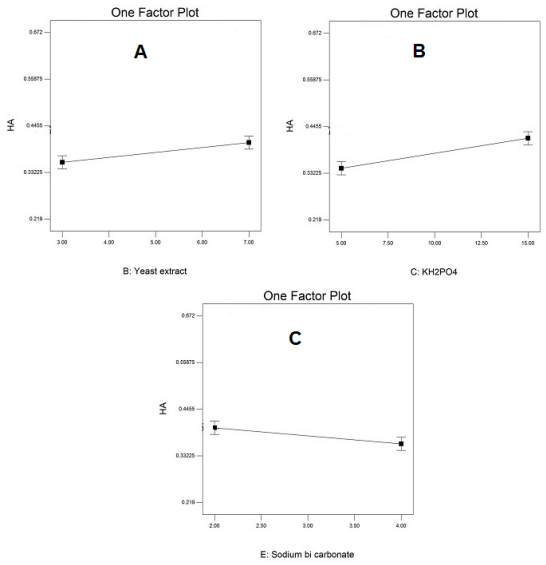

Figure 1. Main effect plot showing the effect of (A-Yeast extract,B-KH_2PO_4 , C-$NaHCO_3$)

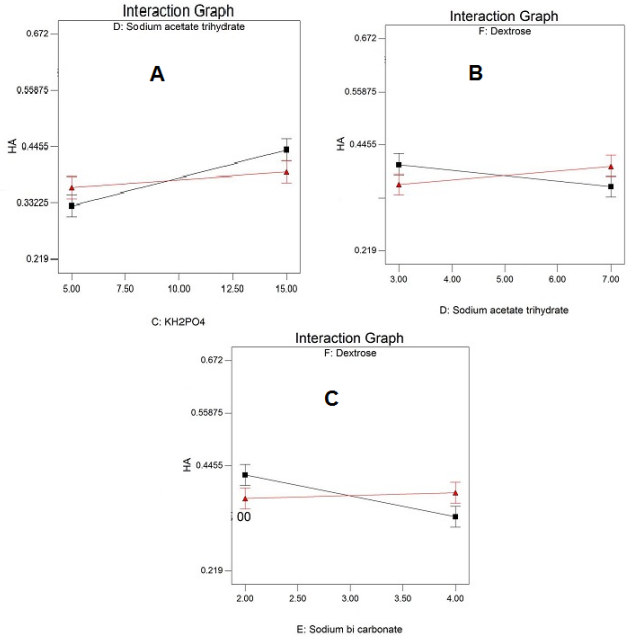

Figure 2. Main interaction plot between (A- KH_2PO_4 and sodium acetate, B-Dextrose and sodium acetate,C-Dextrose and sodium bicarbonate)

The overall production of the HA is dependent on the nitrogen sources available in the media. The carbon source value selected in the experiment was in an optimum range in which the maximum activity will be achieved (Jagannath and Ramchandran, 2010). There are very few reports on statistical optimization of the media on HA production through full factorial design, response surface methodology and other optimization designs.

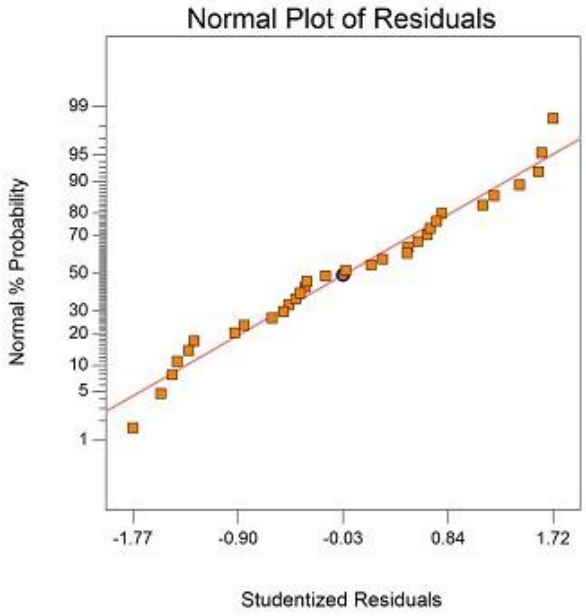

Figure 3. Normal plot Vs residulas

Radial basis function coupled with neural network quantum based particle swarm optimization (RBF-QPSO) studies on the media requirements especially over the influence of amino acids on the production of HA was carried out by Liu *et al.,* (2009). The predicted maximum HA yield was 6.92 g/L when arginine 0.062 g/L, cysteine 0.036 g/L, and lysine 0.043 g/L was added. The optimal amino acids addition allowed HA yield increased from 5.0 g/L of the control to 6.7 g/L in the validation experiments.

Table 10. Point prediction for P10 medium

Run no.	Predicted value range (g/L)	Actual value (g/L)	Mean actual value (g/L)) ± SD
1	0.18 - 0.64 (0.411)	0.356, 0.299, 0.446	0.367 ±0.070
2	0.12 - 0.58 (0.35)	0.322, 0.494, 0.419	0.447 ±0.090
3	0.14 - 0.6 (0.37)	0.549, 0.330, 0.276	0.385 ±0.140
4	0.17 - 0.63 (0.4)	0.322, 0.24, 0.334	0.299 ±0.050

The optimization of fermentation conditions was carried out by Liu *et al.,* (2009) and found that RBF-QPSO

studies gave better optimization than that of RSM. The predicted maximum HA yield by RSM and RBF-QPSO was 5.27 and 5.62 g/L, respectively, while a maximum HA yield of 5.21 and 5.58 g/L was achieved in the validation experiments under the optimal culture conditions obtained by RSM and RBF-QPSO, respectively. Production of HA from *Streptococcus zooepidemicus* via the supplement of nucleotide bases using response surface methodology (RSM) was studied by Liu *et al.,* (2009). Using a five factorial two level design among the nucleotides uracil was found as the significant variable which was further optimized using RSM.

Optimization of medium for production was using Taguchi orthogonal array design and 24 full factorial designs was reported earlier by Im et al, (2009) and Patil et al, (2011), respectively. In above studies, higher average HA production was observed using high level(4.5 %) of glucose, high level (4.5 %) of soya peptone, low level (0.075%) of $MgSO_4.7H_2O$ and high level (0.25 %)of K_2HPO_4.Soyapeptone(X_2) had a significant effect (P=0.005) on HA yield. Y as it had the largest coefficient. The maximum average productivity of HA was 0.798 g/L in shake flask.

CONCLUSION

Upon optimization of the components of P10 medium, after 24 hrs of fermentation period, HA yield of 0.70 ± 0.02 g/L was obtained. The fractional factorial design revealed that the concentration of yeast extract, $NaHCO_3$ and KH_2PO_4 were the significant factors influencing HA production. Yeast extract, potassium dihydrogen phosphate and sodium bicarbonate are three medium components that shown significant model terms. The factor potassium dihydrogen phosphate was relatively more significant than yeast extract. There are no reports cited for statistical optimization of medium for production of HA by *S. equi* subsp. *zooepidemicus* ATCC 39920.

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