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Evaluation studies of coconut water as replacement of serum in media: A study on BHK 21/C13 cell line

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ABSTRACT

Serum is known to be an essential evil for growth and development of any animal cells during culturing. Alternative serum replacement may be very costly, but because of very close composition of coconut water with serum, we first time have evaluated growth of BHK cell line in coconut water and serum as well. We find that coconut water can be used for growth of animal cell also but along with mixture. In coconut water, the growth rate of BHK cell line was 0.205 × 10^6 cells/ml/hrs while in 10% FBS the growth rate was 0.219 × 10^6 cells/ml/hrs. Media were optimized further for increasing overall cell growth rate. Therefore, optimized media containing coconut water using L_{18} design of Taguchi Design Of Experiment (DOE) methodology with seven media components such as FBS, Cell number, Coconut water, pH, Folic acid, rotation speed and $NaHCO_3$ and finally we obtained the optimized conditions for overall growth of cells with 28% improvement in growth of cells BHK-21/c13 cell line.

Keywords: Coconut Water, DMEM, FBS, BHK-21/C13 cell line, Cell growth rate, Cell viability, Taguchi methodology optimization.

INTRODUCTION

In animal cell culture Serum replacement is a challenge, and one of the most prominent reason its source of origin (blood) and other due to presence of undefined inhibitor which is major limitation for viability of many cells and tissue in culture [1]. Choice of coconut water is based on few reports where coconut water have been used for different purposes including cell culture, due to prevalence of various important factors (related to growth and development in the endosperm of coconut) and have been successfully used in storage and propagation of the cell line. Beside this, use of coconut water have been reported in culturing of various virus cells including vaccine formulations [2] Thomas et al, 2008 as an extender of semen for several pet animals such as dogs [3-4] and even in humans cells also [5]. The present work is first report of successful culture of BHK cell line in DMEM media containing coconut water as a replacement of FBS in DMEM media.

The media optimization is an essential part of the in cell growth and viability. Therefore many factor had been selected for media optimization. Statistical optimization using the traditional method involves the study of one variable at a time, while Taguchi design of experiments had benefit that several factor can be optimized in doing few experiments. Taguchi methodology have been used extensively in the industry for various biotechnological applications such as in the optimization of virus culture to improve rabies vaccine production (using Vero cells) in the bioreactor [6] (Rao et al, 2008) and a very good review have been published recently by [6] Rao et al, 2008 in same concern. Application of coconut water is well documented by [7] Yong, et al 2009 who identified the role of Kinetin and Kinetin riboside from coconut water. Beside this a strong role of coconut water has been reported as anti-ageing effects on human skin cells and fruitflies (*Zaprionus paravittiger*) [8] (Sharma, et al 1995) Kinetin was shown to delay the onset of several cellular and biochemical characteristics associated with cellular ageing in human skin fibroblast cultures and in the development of skin care products containing Kinetin were subsequently

developed to treat photo-damaged skin [9] since the concentration of these electrolytes in coconut water generates an osmotic pressure similar to that observed in blood [10] (Fernandes, 2000), and it also does not affect haemostasis (plasma coagulation) [11]. A very informative review is written by [7] Yong et al, 2009 on chemical composition and properties of coconut water which reported to have a variety of nutrients including vitamins, minerals (like Magnesium, Calcium And Potassium), antioxidants, amino acids, enzymes, growth factors, trace elements (Zinc, Selenium, Iodine, Sulphur, Manganese, Boron, Molybdenum) and other nutrients and it could be used as a FBS substitute as it is sterile, does not produce heat, doesn't destroy red blood cells and is really accepted by the body [2].

In the present research article, media optimization have been done for determination of effective factors and also effective concentration of coconut water and FBS either alone or in combination, in DMEM media.

MATERIALS AND METHODS

Following chemicals have been procured for cell line culture e.g. PBS, DMEM, FBS, L-Glutamine and Antimycotic antibiotic solution from the HiMedia, India, while Sodium bicarbonate, Folic acid, and Trypan blue were obtained from the Loba chemical India

CELL LINE:
BHK21-c/13 cell line (Baby hamster kidney fibroblast) was procured from the National Centre of Cell Sciences (NCCS), Pune India.

DMEM PREPARATION:
DMEM was prepared as per direction (by dissolving 4.37 gm DMEM in sterilized distilled water, 0.925 gm of Sodium bicarbonate (NaHCO3) was added externally, and the final volume was raised to 250 ml by adding distilled water). The pH was maintained at 7.2.

Media prepared from Coconut Water
Green (mature) coconut water was selected because of a high amount of sugar present as compared to other old mature coconut water. Coconut water were collected under sterile conditions inside the vertical laminar air flow in pre-sterile bottle. The sterile DMEM media and coconut water, both was further sterilized by using a disposable Millipore membrane filter having a pore size of 0.22µm. The glass wares and culture vessels were sterilized by autoclaving method.

Culturing
BHK21-c/13 cell line (initial cell no. 2.92×10^6) was inoculated into each T-flasks for monolayer culture formation using sterilized DMEM and FBS media as control. Finally 20µl of Actinomycotic antibiotic solution (contains penicillin, streptomycin and Amphotericin B antibiotics) were added in each T-flask to increase the sterility.

Culturing in coconut water
BHK-21/c13 cell line (initial cell no - 2.92×10^6) was cultured in DMEM media using green mature coconut water (2%, 4%, 6%, 8% and 10% v/v) with a fix concentration of FBS (10%) while another culture was done again different concentration of FBS were taken (control (no FBS), 2%, 4%, 6%, 8% and 10%) v/v with a fixed concentration of coconut water. Finally, a 20µl Actinomycotic antibiotic solution was added in each T-flask to avoid the growth of bacteria and fungi. Each T-flask was incubated at 5% CO_2 in CO_2 incubator at 37ºC for 24 – 60 hours maintaining humidity at 90% RH.

GROWTH STUDIES OF CELL LINE:
Growth of the BHK-21/c13 cell line was studied by counting the cells by using Haemocytometer at different time interval (24, 48 and 60 hours of incubation) Viability assay has been used by Trypan blue staining method. After the observation of the cell numbers at different time interval, the growth rate was calculated as: Growth rate Growth rate

$$r = \frac{C_i - C_f}{t_1 - t_2}$$

Where, C_i= initial cell concentration (No. of cells $\times 10^6$ per ml/hr) and C_f = final cell concentration (No. of cells $\times 10^6$ per ml/hr); t_1 = initial time (hrs); t_2 = final time (hrs)

DESIGN OF EXPERIMENTS (DOE):
The schematic representation of the Taguchi method of design of experiment (DOE) has been depicted in Fig. 1. The first step was to determine the various factors to be optimized. Factors were selected and the levels were assigned based on the relevant report of the literature. Eight essential factors (Temperature, FBS, Cell number, Coconut water, pH, Folic acid, Speed and $NaHCO_3$) were selected for L_{18} Taguchi DOE. In the present study, three levels of factor variables were considered to satisfy the L-18 OA. The total degree of freedom was equal to the number of trials minus -1, i.e. 17. Therefore, 18 experiments were performed and the results obtained were further preceded for analysis in Qualitek-4 software.

Analysis of the results:
The obtained result was processed in the Qualitek-4 software with bigger is better quality and further analysis of the results was performed according to Signal to Noise ratio (S/N) analysis. In Taguchi's method, quality is measured by the standard deviation of the characteristic from its target value, and a loss function (L(y)) is developed for the deviation as represented by L(y)) k(y - m)2, where k denotes the proportionality constant, m represents the target value and y is the experimental value obtained for each trail. In case of bigger is better quality characteristics, the loss function can be written as L(y) = k (1/y2).

Validation:
To validate the optimized conditions, cell culturing was performed again to check the cell growth rate obtained after Taguchi's application.

Microscopy:
A binocular microscope was used to attach to computer from Magnum Company India.

RESULTS AND DISCUSSION

Growth Rate in DMEM media:
Chemical compositions of coconut water [12] (Vigliar et al., 2006) have been depicted in Table 1 and Table-2.The results of growth rate in DMEM media have been depicted in the fig. 2 (a). The results obtained at different time interval (24 hrs, 48 hrs, 72 hrs) shows that the cell growth in the DMEM medium increases with the increase in FBS concentration. The significant cell number was obtained with the 10 % FBS (v/v) concentration as 17.70×10^6 cells per ml, which indicates that FBS has significant impact on the cell growth as it fulfills the requirements of cells during growth by providing them essential growth promoting components in the medium.

Growth Rate in coconut water:
The results obtained after culturing of the cell line in coconut water revealed that coconut water was able to support the growth of BHK-21/c13 cell line. After culturing of the cells in varying volume of coconut water (2%, 4%, 6%, 8% and 10% v/v), show that 6% coconut water along with 6% FBS yield 15.83×10^6 cells per ml while the growth rate was 0.206×10^6 cells per ml per hour after 72 hrs (Fig. 2 b).

Optimization of media:
Before any investigation, optimization for each factor is essential requirements for cell growth [13] (Prakasham et al., 2005). In addition the notable factors that may contribute in designing of a media are Temperature (25-37°C), FBS (Fetal Bovine Serum) (5-10 %), Cell Number (4.0 -8.0 x 10^6), Coconut water (2-6%), pH (5-7), Folic acid (4-8 mM), Speed (50-150 RPM) and Sodium bicarbonate ($NaHCO_3$) (2-6 mM) were taken into consideration (Table-3).
BHK-21/c13 (initial cell -2.8×10^6) was cultured according to the trial obtained L18 as depicted in Table 4. In addition, 20 µl of Antimycotic antibiotic solution was also added to avoid any contamination. The cultured T-flasks were incubated at 25°C inside CO_2 incubator, (5% CO_2) to check the cell growth at different time interval. The result obtained after culturing of the BHK-21/c13 cell line was entered in the (L-18 design) result column. Then after main factor analysis, Table and plot were obtained (Table 5-7 and fig. 3-6). Final analysis was done by ANOVA which give the optimum condition.

Main Effects:
The average effect of each factor, based on Signal-to-Noise ratio (S/N) is shown in Table 5. The difference between the average value of each factor at higher level and lower level (L2-L1) indicates the relative influence at individual capacities. The positive and negative values indicate the highest and lowest impact of each factor on cell growth. This gives order of the overall impact of factors, which can be confirmed by staked chart Fig. 4 pie charts depicted in figure 5.

According to the Table 5, the highest impact on cell Confluency was noticed with the Speed which scored highest positive value (2.025), while Temperature shows a maximum negative value (-1.225) preceded by pH (-0.711). However among the selected level of nutrients, Coconut water had a strong positive impact with value (1.583) whereas the impact of FBS was slightly less attain negative value (-1.461) that indicates that coconut water plays a significant role in cell growth than FBS. In case of biomass, the Cell number effect was noticed with positive value (0.844) and higher influence was achieved with level 2. The other factors like Folic acid (-0.539) and $NaHCO_3$ (-1.466) have negative values. The difference between the level 3, level 2 and level 1 of each factor indicates the relative influence of the effect. Thus larger the difference, the stronger will be the influence. Thus the order of each factor will be as speed followed by coconut water, cell number, Folic acid, pH, Temperature, FBS and $NaHCO_3$ (Table 6; fig. 4). The individual factor plot has been depicted in fig. 3.

Effect of Temperature:
Fig. 3 (a) shows the growth of the cells in culture media with different temperature levels. Two levels of temperature were selected to satisfy the L-18 condition on increasing from temperature 25°C-35°C. From the Fig. 3 (a) it can be observed that temperature range at level 1 supports the cell growth at a higher rate while within range of temperature at level 2, the result obtained indicates towards the reduction of the cell growth of BHK-21/c13 cells. It was revealed from the Fig. 3 (a) that temperature range of 25°C would supports the highest growth of the cells as comparative to 35°C.

Effect of FBS (Fetal Bovine Serum):
According to the L-18 design, the FBS concentration was selected at levels as 5%, 7% and 10%. The analysis of the FBS concentration of range within the level 1, level 2 and level 3 revealed the optimum concentration of the FBS has been depicted in the Fig. 3 (b). According to Fig. 3 (b) it was observed that the FBS range at level 1 supports the cell growth as comparative to level 2 and level 3. At level 1 cell growth was noticed as high whereas with the increase in the FBS concentration results in the gradual fall of the cell growth which would indicate the results that the level 1 concentration range of 5% supports the cell growth at maximum level.

Effect of Cell number:
Fig. 3 (c) shows the cell growth at different levels (level 1, 2, and 3) of Cell inoculum. Initial cell inoculum strongly affects the final cell confluence in vitro. Results obtained from the experiment revealed that with the increase of the initial cell inoculum, the final cell confluence also increases as shown in Fig. 3 (c). Initial cell number at level 1 supports slower growth rate while a slight increase in the initial cell number at level 2 results in the increase in the growth that further increases with the more increase in the initial cell number at level 3.

Effect of Coconut water:
The effect of each level of coconut water has been depicted in Fig. 3 (d). According to the L-18 design, levels of coconut water percentage were selected. It was observed from the figure that the cell growth increases gradually with the increase in the level of the coconut water percentage. It was clear from the figure that the coconut water percentage within the level 3 results in the highest growth rate that falls with the fall in the percentage of coconut water at level 2 and furthermore at level 1.

Effect of pH:
Fig. 3 (e) shows the growth rate at three different levels of pH range selected. It was observed that the initial cell growth rate at level 1 pH range was observed as constant whereas further in the level 2 pH range, the cell growth was reduced. A further increase in the pH range at level 3 highly increases the cell growth. Hence, it was observed from that the pH range within level 3 highly supports the cell growth as compared to level 2 and level 1.

Effect of Folic acid:
Fig. 3 (f) shows the effect of Folic acid on cell growth. Folic acid is one the important vitamin that is considered in the division of the cells at higher rates. It was observed that the concentration of the Folic acid adversely affects the growth of the cells with certain range. The graphical plot of the Folic acid revealed that the cell growth increase or decrease according to the increase or decrease the concentration of the Folic acid. At level 1 folic acid range, the cell growth was slightly slow that was further reduced to more extent at level 2. But with the slight increase in the folic acid range, gradual increase in the cell growth was observed.

Effect of Speed:
Effect of Speed on cell growth at each assigned level has been depicted in Fig. 3 (g). According to the graph plot obtained for speed, it was noticed that with the increase of the level of the speed, the effect of with each level also increases in the growth of the cells. The plot shows that at level 1 cell growth was slower that the level 2 at which

the cell growth highly increases that further continues at level 3 where the further addition in the rate of the cell growth was measured.

Effect of $NaHCO_3$:
The effect of $NaHCO_3$ on cell growth has been depicted in Fig. 3 (h). It was well studied that $NaHCO_3$ was highly interacting with the pH of the medium to adjust it at its optimum level. It was observed that level 1 would support the cell growth at higher levels as compared to level 2 and level 3 whereas level 3 was observed to support the cell growth slightly less than level 1 but supports the growth rate more than level 2. It was observed that level 2 has least effect on cell growth that was resulting in the reduction in the cells.

ANOVA analysis:
The analysis of variance (ANOVA) is used to analyses the results. It also reveals the percentage contribution of individual factor affecting cell growth. From the F-ratio at 90% confidence limit, it can be determined that all factors and interactions considered in the experimental design are statistically significant.

According to the ANOVA Table 6, the impact of each factor on cell growth. It was observed from the ANOVA Table that Coconut water has the highest percentage contribution (27.589%) followed by Cell number (23.299%), Speed (12.486%), FBS (7.389%) and pH (5.457%), the next factors showing significant percentage contribution towards the cell growth, while the Folic acid and Temperature shows 4.795% and 3.221% impact on cell growth. $NaHCO_3$ showed the least (2.351%) impact among the factors studied with the assigned variance of values (Fig. 4). Thus, the order of individual factors from highest to lowest percentage contribution can also be seen from the pie chart (Fig. 5). Thus, the overall order of all the factors according to their impact is as coconut water (27.589%) > Cell number (23.299%) > Speed (12.486%) > FBS (7.389%) > pH (5.457%) > Folic acid (4.795%) > temperature (3.221%) > $NaHCO_3$ (2.351%) has been confirmed after ANOVA.

Optimum conditions:
Optimum conditions and their performance in terms of contribution to achieving highest growth rate are shown in Table 7. The expected result at optimum condition was 20.336×10^6 cells per ml in coconut water with a total contribution from all factors being 9.977×10^6 cells per ml in coconut water with current grand average performance of 10.359×10^6 cells per ml after validation of the expected result obtained, the maximum cell growth can be achieved with temperature 25°C, FBS 5%, Cell No. 4×10^6 cells per ml, Coconut water 6%, pH 7, Folic acid 6 mM, Speed 150 RPM and $NaHCO_3$ 2% (w/v). With the consideration of these optimum culture conditions of the experiment designed, the cell confluence can be increased from 10.359 to 20.336×10^6 cells per ml as with an overall 27 % enhancement in the cell growth

OPTIMUM CONDITIONS
Expression: C.I. = sq Root {(F (1, n2)*Ve)/Ne)
Where: F (n1, n2) = 3.5
n1 = 1, n2 = 2 (Error DOF)
Ve = 1.32474 (Error Variance)
Ne = 1.13 (effective Number of Replication)
(Factor DOF's included in the estimate = 15)
Confidence level = 90
Confidence interval = +/- 2.03
Expected results at optimum = 20.336 +/- 2.03
(Low value = 18.306, high value = 22.366)
Estimate of expected results from S/N ratio
S/N = - 10 Log (MSD) = 20.336
Or MSD = 10(-(S/N)/10) = 0.009256
Where MSD = $((1/y_1)^2 + (1/y_2)^2 + \ldots.. + (1/y_n)^2)/n$
= (Avg. 1/yi)2) = $1/Y_{exp}^2$
orY_{exp} = SQR (1/MSD)
Expected performance in QC unit (or overall evaluation criteria) is:
Y_{exp} = 10.394 QC units
(Based on S/N = 20.336 at optimum)

Therefore High value of S/N means more desirable results and low value of S/N means less desirable results in all types of quality characteristics.

Improved conditions:

The expected improvement in cell growth after optimization can be viewed from fig.8 that shows the difference in the cell growth between the current and improved optimum conditions based on assumed normal performance distribution of the factors. Further validation of the proposed experimental methodology, BHK-21/c13 cell line culturing experiments was performed to observe cell growth by employing optimized conditions. The results showed an enhanced cell growth of 27 % of improvement in cell growth with the optimum culture conditions.

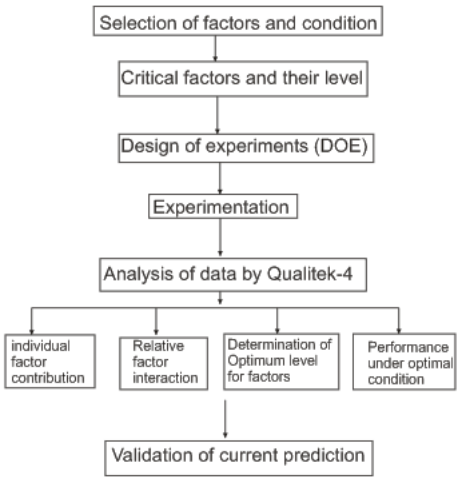


Fig 1:Experimental protocol of DOE of Qualitek-4 to design a media

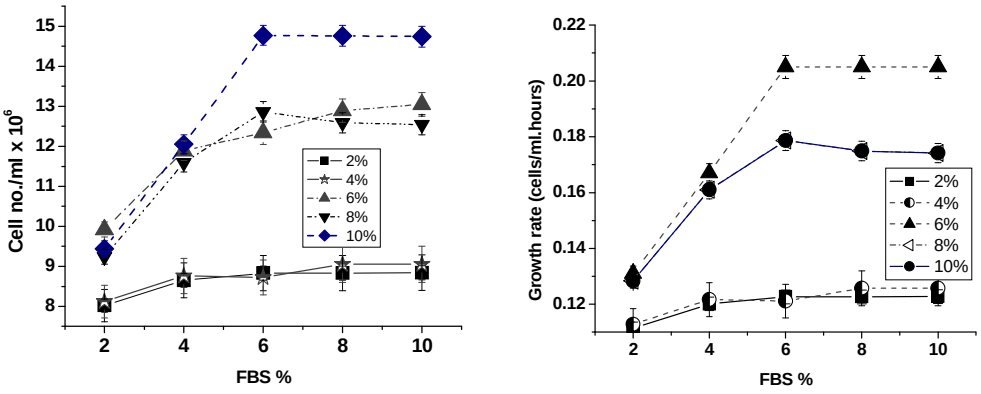
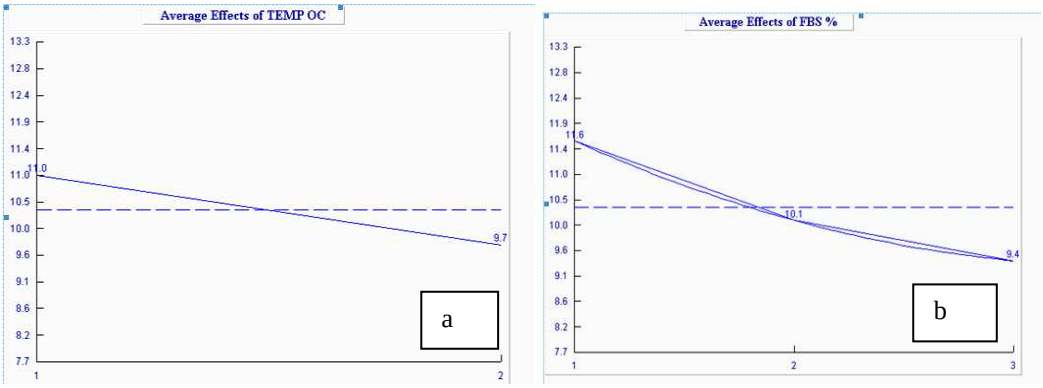


Fig. 2. Number of cells (a) at different time interval (b) at different concentration of % FBS (0,2,4,6,8,10) and coconut water 2, 4, 6, 8 % after 72 hrs



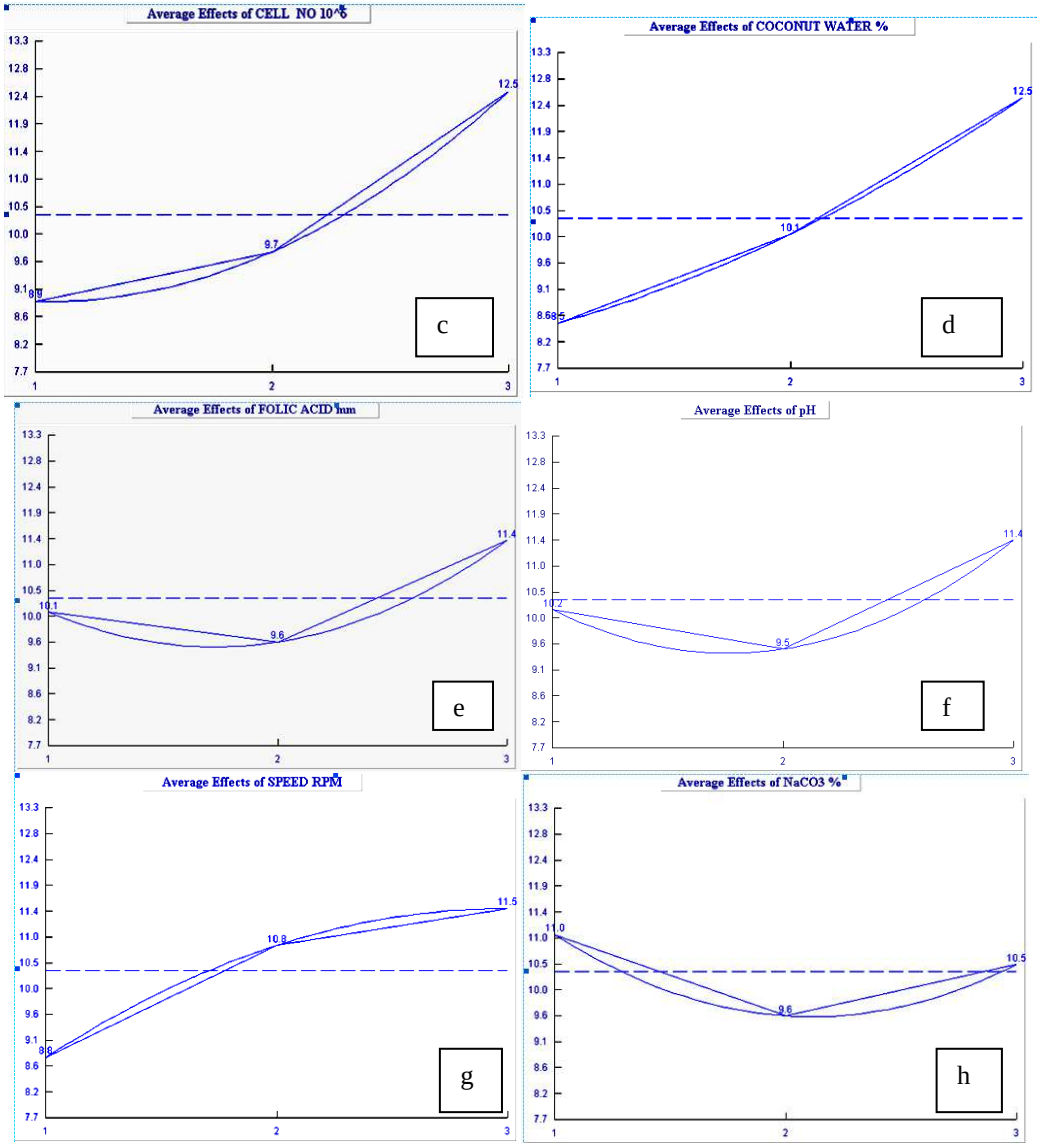


Fig 3: Impact of selected factors and their levels on cell concentration after 72 hrs

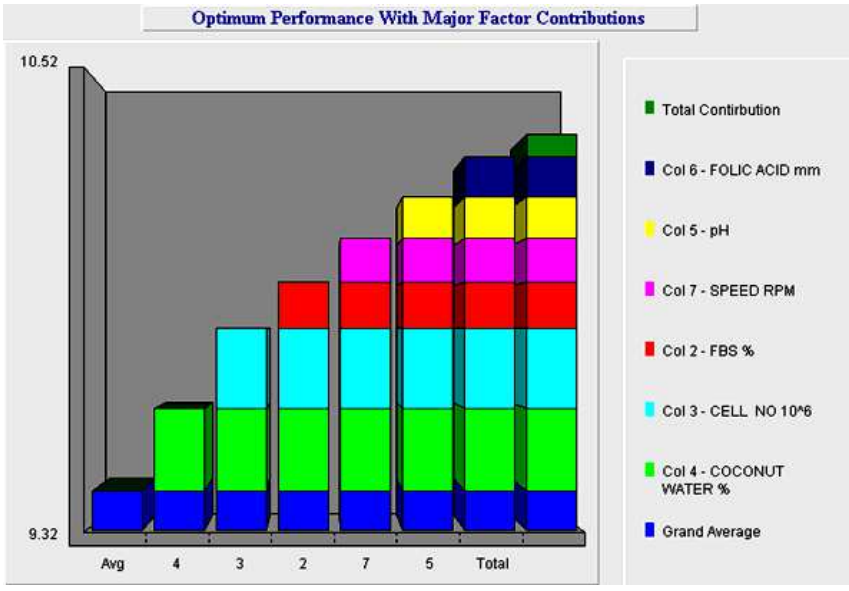


Fig 4.Optimum performance with Major factor contributions to cell growth in stacking chart

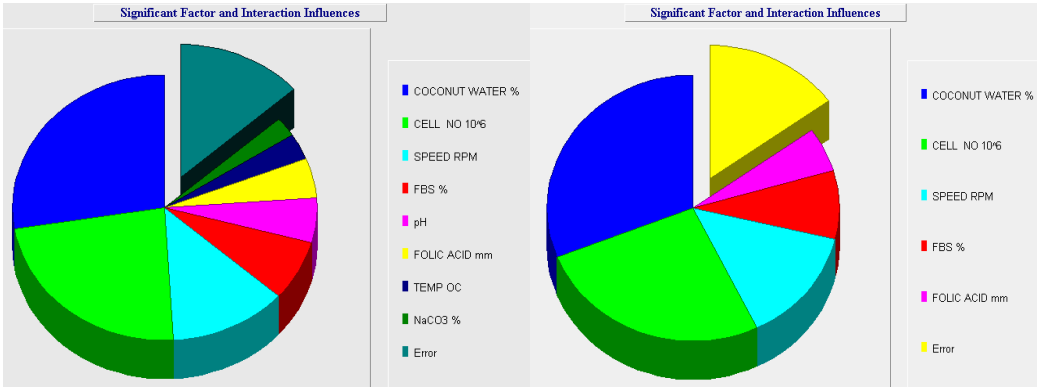


Fig 5: Significant factors and interaction influences on cell growth represented in pie chart ; after pooling representing in pie chart

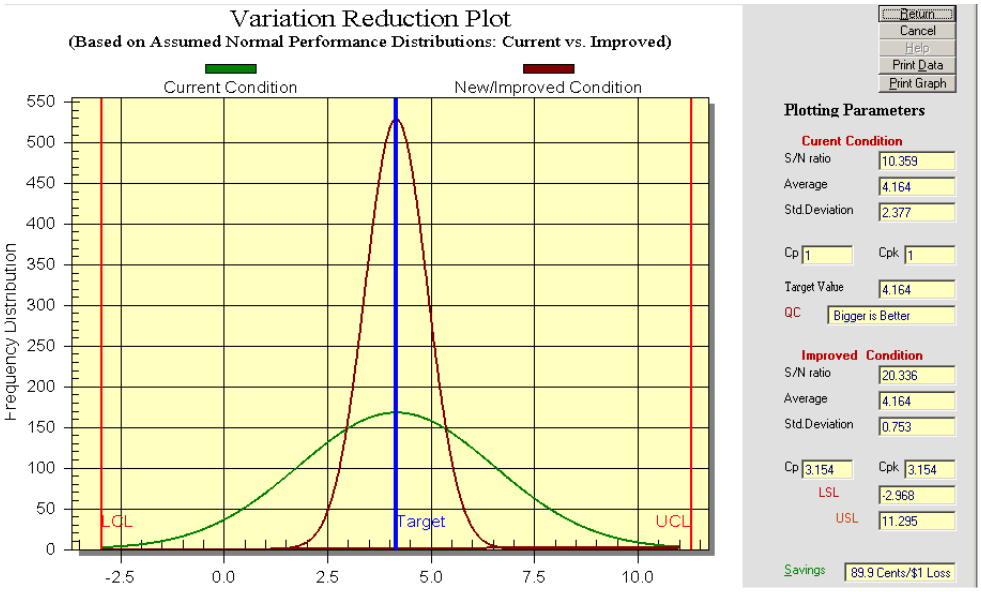
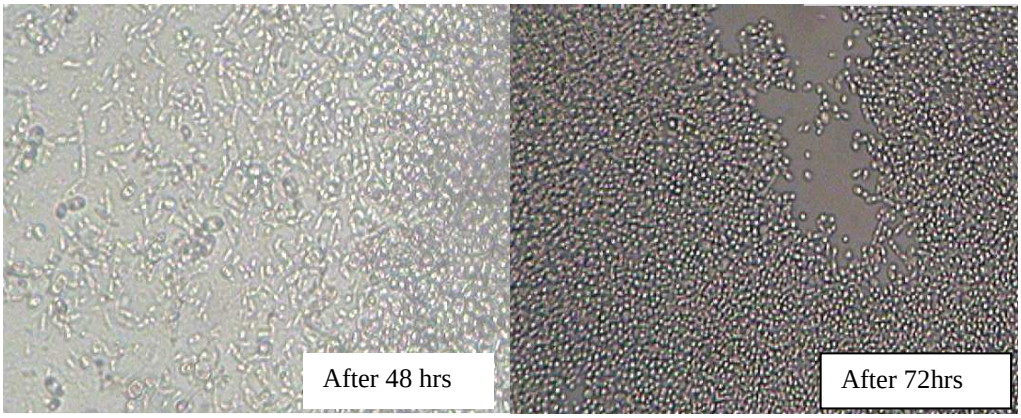


Fig 6.Variation Reduction Plot shows the performance distribution on cell growth at current and improved conditions



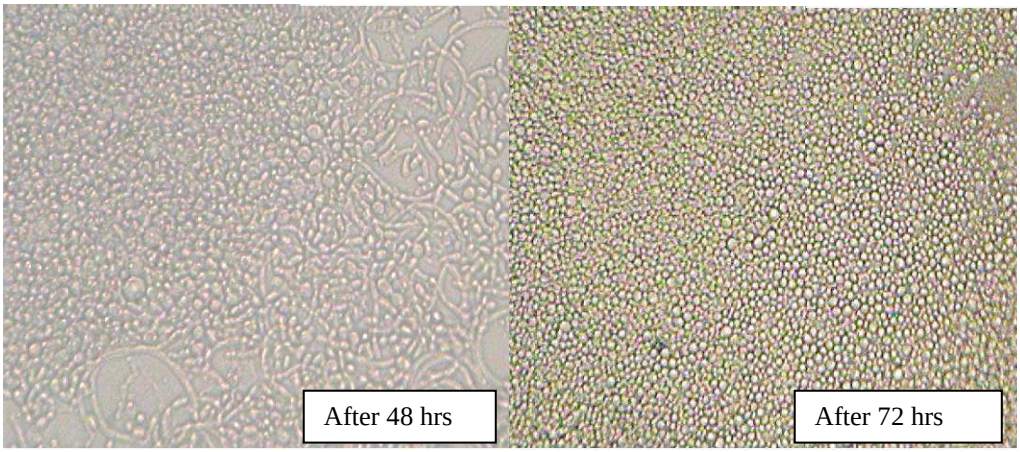


Fig 7. Initiation of the monolayer formation of BHK-21/c13 cell line after 48 hrs and 72 hrs of incubation (10X) (a) FBS added DMEM media (b) coconut water containing DMEM media after optimization.

Table 1: Chemical composition of mature and tender coconut water (Renata et al., 2006)

S. No.	Components	Mature coconut water mg (%)	Tender (green) coconut water
1.	Total solids	5.4	6.5
2.	Reducing sugars	0.2	4.4
3.	Minerals	0.5	0.6
4.	Protein	0.1	0.01
5.	Fat	0.1	0.01
6.	Acidity	60.0	120.0
7.	pH	5.2	4.5
8.	Potassium	247.0	290.0
9.	Sodium	48.0	42.0
10.	Calcium	40.0	44.0
11.	Magnesium	15.0	10.0
12.	Phosphorous	6.3	9.2
13.	Iron	79.0	106.0
14.	Copper	26.0	26.0

Table 2: Amino acid and Vitamin-B composition of coconut water (Renata et al., 2006)

S. No	Amino acid composition		Vitamin – B composition (µg/ml)	
1.	Alanine	2.41	Nicotinic acid	0.64
2.	Arginine	10.75	Pantothenic acid	0.52
3.	Aspartic acid	10.75	Biotin	0.02
4.	Cystine	0.97 - 1.17	Riboflavin	< 0.01
5.	Glutamic acid	9.76 - 14.5	Folic acid	0.003
6.	Histidine	1.95 - 2.05	Thiamine	Trace
7.	Leucine	1.95 - 4.18	Pyridoxine	Trace
8.	Lysine	1.95 - 4.57	-	-
9.	Proline	1.21 - 4.12	-	-
10.	Phenylalanine	1.23	-	-
11.	Serine	0.59 - 0.91	-	-
12.	Tyrosine	2.83 - 3.00	-	-

Table 3: Selected factors and their assigned levels selected for study (L-18 design)

S. No.	Factors	Level 1	Level 2	Level 3
1.	Temp (°C)	25	35	----------
2.	FBS (%)	5	7	10
3.	Cell No. (10^6)	2	3	4
4.	Coconut water (v/v)	2	4	6
5.	pH	5	6	7
6.	Folic acid (mM)	2	4	6
7.	Speed (RPM)	50	100	150
8.	$NaHCO_3$ (%)	2	5	10

Table 4. Experimental setup (L-18 Orthogonal Array) Columns

	1	2	3	4	5	6	7	8
Trial 1	1	1	1	1	1	1	1	1
Trial 2	1	2	2	2	2	2	2	2
Trial 3	1	3	3	3	3	3	3	3
Trial 4	1	1	1	1	2	2	3	3
Trial 5	1	2	2	2	3	3	1	1
Trial 6	1	3	3	3	1	1	2	2
Trial 7	1	1	1	1	1	3	2	3
Trial 8	1	2	2	2	2	1	3	1
Trial 9	1	3	3	3	3	2	1	2
Trial10	2	1	1	2	3	2	2	1
Trial 11	2	1	2	3	1	3	3	2
Trial 12	2	1	3	1	2	1	1	3
Trial 13	2	2	1	2	3	1	3	2
Trial 14	2	2	2	3	1	2	1	3
Trial 15	2	2	3	1	2	3	2	1
Trial 16	2	3	1	3	2	3	1	2
Trial 17	2	3	2	1	3	1	2	3
Trial 18	2	3	3	2	1	2	3	1

Table 5: Main effects of factors at levels (L2-L1) (positive and negative values shows the highest and lowest impact

S. No	Factors	Level 1	Level 2	Level 3	L2-L1
1.	Temp (°C)	10.97	9.747	0	-1.225
2.	FBS (%)	11.58	10.12	9.378	-1.461
3.	Cell No (10^6)	8.887	9.732	12.458	0.844
4.	Coconut Water (%)	8.494	10.077	12.506	1.583
5.	pH	10.179	9.469	11.429	-0.711
6.	Folic Acid (Mm)	10.106	9.567	11.404	-0.539
7.	Speed (rpm)	8.786	10.812	11.480	2.025
8.	NaHCO3 (%)	11.027	9.561	10.489	-1.466

Table 6 Analysis of Variance (ANOVA)

S. No	Factors	DOF (f)	Sums of squares (S)	Variance (V)	F- ratio (F)	Pure sum (S')	Percent (P) %
1.	Temp °C	1	6.735	6.735	5.084	5.411	3.221
2.	FBS (%)	2	15.060	7.530	5.684	12.410	7.389
3.	Cell No. (10^6)	2	41.783	20.891	15.770	39.133	23.299
4.	Coconut water	2	48.988	24.494	18.489	46.339	27.589
5.	pH	2	11.815	5.907	4.459	9.165	5.457
6.	Folic acid (mM)	2	10.704	5.352	4.040	8.055	4.795
7.	Speed (RPM)	2	23.621	11.810	8.915	20.972	12.486
8.	$NaHCO_3$ (%)	2	6.598	3.299	2.490	3.949	2.351
9.	Other /Error	2	2.469	1.324	_	_	13.413
	Total	17		167.958			100.00%

Table 7 Optimum conditions and performance in green mature coconut water for cell growth

S. No	Factors	Conc.	Level	Contribution
1.	Temp (°C)	25	1	0.611
2.	FBS (%)	6	2	1.220
3.	Cell No (10^6)	4	1	2.098
4.	Coconut water (%)	6	3	2.146
5.	pH	7	3	1.070
6.	Folic acid (mM)	6	3	1.045
7.	Speed (RPM)	150	3	1.120
8.	$NaHCO_3$ (%)	2	1	0.667

The total contribution from all factors	9.977
Current grand average of performance	10.359
Expected result at optimum condition	20.336

DISCUSSION

It is well evident from the literature that 10% FBS has the best support for animal cell growth which we also observed by 2,4,6,8,10% FBS. FBS showed some typical behavior of cell growth [14]. In 2009, Hesham et al [15]

and his co-workers reported that increasing the concentration from 5 to 20% FBS in the medium increased the total number of living cells by about 30%. Meanwhile, increasing the further FBS concentration in culture medium resulted in significant reduction in viable cell number [15]. This may be due to the presence of several undefined growth promoting components of the serum

We also observed the good effect of coconut water over BHK cell growth in the presence of FBS. Coconut water supports growth of cells but not as impressive as FBS therefore it was selected in combination owing to the presence of all the essential components present in DMEM including glucose (energy source), amino acids, vitamins (specifically Vitamin-B), minerals, sodium, potassium, Magnesium, and calcium [7]. It is also evident from our results depicted in fig.2 and fig.3. It was also observed that the 6% coconut water with 6% FBS gives the maximum cell number but in term of yield it was low therefore Further, optimization was done with DOE. We also observed that mature green coconut water gives maximum cell growth than the other types of coconut water due to the difference in the contents concentration in terms of sugars, vitamins and amino acids. Mature green coconut water contains 21.68 mg/ml of sugars (glucose (7.25 mg/ml) whereas mature coconut water contains 13.87 mg/ml [7][16]. Mature coconut water also has a higher concentration of arginine, aspartic acid, glutamic acid, Proline, Valine, tyrosine, methionine, Histidin essential for the growth of the cells but on the other hand Glutamine concentration is low in mature green coconut water [17]. so Glutamine was added to the coconut water to fulfil the demand of the cells. Coconut water has a less percentage of Folic acid. Thus the higher amount of glucose, fructose, amino acid and vitamins may provide sufficient support for growth and development of cells.

Therefore, after confirmation of the cell growth in coconut water, several physiological and nutritional factors, such as Temperature, Cell number, Coconut water, FBS (Fetal Bovine Serum), pH, Folic acid, Speed and $NaHCO_3$ were optimized by design of experiment (L-18 OA design) using Taguchi's methodology.

Analysis of the data by Qualitek-4 software depicted the results in identifying the influence of each factor on cell growth and establishing the relationship between factors and operational conditions to be selected for optimum growth of the cells. After ANOVA it has been observed that coconut water shows the highest impact (27.589%) followed by cell number (23.299%), speed (12.486%), pH (5.457%), folic acid (4.795%), temperature (3.221%), while least impact was found to be with $NaHCO_3$ (2.351%).

Coconut water supplemented with 6% FBS concentration shows the maximum cell growth but on further addition of FBS, cell growth was affected and leads to the reduction of the growth as a further addition of FBS might be show some inhibitory effect on cells due to the presence of some excessive unknown and undefined components.

According to the [18] Ozturk and Palsson, 1990, [19] Bhatt et al 2011 the final cell confluence depends upon the initial cell inoculum. Cultures with lower initial cell density resulted in lower final cell concentration. [18] Ozturk and Palsson (1990) reported that the initial cell density in the range of 10^2 to 10^5 cells per ml affects the initial cell growth as one of the factor to only 20%. In our experiment we have observed effective cell concentration as 4×10^6 and Table 6 depicts that initial cell number was the second highest impact on cell growth which indicates that the final cell confluence is highly dependent upon the initial cell inoculum.

The particular population density at which growth slows down is determined by the pH of the culture. Although pH of the culture media is one of the important parameters for the growth and survival of the cell and below the optimal pH – 7 cells show longer growth period. It was reported that pH-7 supports maximum cell growth and any variation would cause a reduction in the growth [20]. At the lower pH level population density plays an important part in limiting the rate of cell multiplication. At very high pH the restrictions on growth rate are set by depletion of nutrients in the medium. So to reduce the fluctuations in optimum pH value non-volatile buffers were used in the medium. It was observed from the present study that $NaHCO_3$ shows least impact individually (Table 6) on cell growth but shows the highest interaction influence with pH in the medium (not shown). Lactate accumulation due to glycolysis at high glucose concentration can reduce the pH throughout the culture, and that low pH can be detrimental to cell viability and growth [21]

CONCLUSION

Upon considering the optimum culture conditions of the experiments designed the cell growth could be increased from 10.359 to 20.336×10^6 per ml and hence overall 27 % enhancement in the cell growth can be achieved. It was observed that FBS cannot be completely replaced since they contain various important unknown components viz. Growth hormones, growth factors, albumin and globulins etc. which are essential for cell signaling.

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