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RESEARCH ARTICLE

Design and Optimization of A Modified Serum-Free Medium for Chicken Myoblast Proliferation

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Abstract

The growing global population is increasing the demand for meat, while conventional livestock farming remains limited and environmentally burdensome. Cultured meat offers a more sustainable option, and serum-free media are preferred because animal sera pose cost, variability, and ethical concerns. This study aimed to develop an optimized serum-free medium for chicken myoblasts. Five serum substitutes (BSA, ST, YE, CDLC and r-EGF) were screened using a 2^{5-1} fractional factorial design (FFD), which identified BSA, ST, and YE as significant. These were optimized using a central composite design, resulting in 150 mg/L BSA, 1.0 g/L ST, 0.500 g/L YE, 0.5% (v/v) CDLC, and 0.005 mg/L r-EGF. The optimized medium supported myoblast proliferation and allowed subculture for up to 3 passages, partially supporting cell growth compared with serum-containing medium. An initial seeding density of 6×10^4 cells/cm² resulted in the highest cell proliferation, although growth remained limited due to insufficient cell attachment despite gelatin-coated culture surfaces. Overall, this study identifies key factors required for serum-free cultivation of chicken myoblasts and provides a foundation for further advancement in cultivated meat research. Further optimization will be necessary to fully enhance the performance and long-term potential of the developed medium.

Keywords: Cultured meat, Chicken myoblast cells, Design of experiments, Serum substitutes, Serum-free medium

Introduction

The world's population is expected to continue growing over the coming fifty or 60 years, reaching a peak of around 10.3 billion people in the mid-2080s, up from 8.2 billion in 2024 (United Nations Department of Economic and Social Affairs, Population Division 2024). This means that protein requirements to meet our demand should be more consistent. Meat production from livestock needs large amounts of land and water and significantly causes greenhouse gas release (≈ 14.5% of anthropogenic GHG) [1]. Although plant-based proteins are available commercially, animal meat is still a popular, high-quality protein source [2]. Cultured meat is a meat analogue and is also a potential sustainable food generated by the *in vitro* myogenesis of muscle satellite (stem) cells (MSCs). In addition, cultured meat *in vitro* has been proved to be a safe and more efficient alternative to traditional meat. The cultured meat *in vitro* has been studied for many years. With modern animal cell culture techniques and the proof of concept, the successful of culturing MSCs *in vitro* is much possible [3]. The self-renewing proliferation of satellite cells not only maintains the

stem cell population but also provides numerous myogenic cells, which proliferate, differentiate into myoblast cell, fuse, and lead to new myofiber formation and reconstitution of a functional contractile apparatus. In addition, the self-renewal and differentiation properties of MSCs are of primary interest for cultured meat production [2,4,5]. Among satellite cells to be used for cultured meat research, those derived from chickens of different ages or different species have been proved to be at the forefront of research on cultured meat [3,6,7].

Sera derived from animals can be used as the additives of cell culture media, an ill-defined attribute of such sera can lead to undesired results of cell cultures. In fact, serum serves as a source of amino acids, proteins, vitamins, carbohydrates, lipids, hormones, growth factors, inorganic salts, trace elements, and other unidentified compounds. Nevertheless, a number of disadvantages, such as batch-to-batch variation and contamination with adventitious agents (mycoplasma, bacteria, fungi, viruses, and prions), make serum not suitable for use in animal cell cultures [8]. Jiang *et al.* [9] compiled studies using plant and animal protein hydrolysates (including zein, potato, kikuyu grass, chickpea, blue whiting, black soldier fly, and sprat) in serum-free media, these protein hydrolysates can significantly increase myoblast cell proliferation and reduce serum dependence under low or no serum conditions. However, studies applying protein hydrolysates or peptones derived from plants (Bacto™ Soytone) and yeast cells (Bacto™ Yeast Extract) for the development of serum-free media for chicken myoblast cells remain limited. In this study, a modified serum-free medium for chicken myoblast cells was developed. To do this, a basal medium (DMEM-high glucose) was further modified with supplements previously described in literature. A 2^{5-1} fractional factorial design was adopted to use in screening prominent serum substitutes, and then a central composite design was applied to optimize such prominent ones according to the software Design Expert V13.

Materials and methods

Chemicals and culture media

High glucose, Dulbecco's Modified Eagle Medium (DMEM-HG), Fetal Bovine Serum (FBS), Horse Serum (HS), Chicken Serum (CS), AIM-V® medium, GlutaMAX™, Penicillin-Streptomycin (10,000 U/mL), Phosphate Buffered Saline (PBS, pH 7.4), Trypsin (1:250), Collagenase Type I, Chemically Defined Lipid Concentrate (CDLC), Recombinant Human Epidermal Growth Factor (r-EGF), TrypLE™ Select, and Soybean Trypsin Inhibitor (STI) were obtained from Life Technologies, NY, USA. Bacto™ Soytone and Bacto™ Yeast Extract were obtained from Becton, Dickinson and Company, MD, USA. Bovine Serum Albumin (BSA), Ethylenediaminetetraacetic acid (EDTA), and Dimethyl Sulfoxide (DMSO) were obtained from Sigma Aldrich, St. Louis, MO, USA. All chemicals and culture media used in this study were suitable for animal cell culture.

Isolation of chicken muscle satellite cells

Fresh pectoralis major of broiler chickens was purchased from a local farm abattoir. It was then cut into large pieces and poured into a 250 mL laboratory bottle containing 70% ethanol for cleaning. Afterwards, the bottle was immediately shaken vigorously for 5 min. Cleaning was repeated 3 times by replacing 70% ethanol. The final wash was replaced with sterile PBS, and the tissues were then transferred into a biosafety cabinet (BSC). One large piece of pectoralis major was taken into a 100 mm sterile Petri dish. The outside of the tissue previously exposed to 70% ethanol was carefully removed using sterile forceps and scissors. The inside of the tissue was cut into small pieces using another set of sterile forceps and scissors. Small pieces of tissue were transferred into a 50 mL sterile centrifuge tube of known weight using sterile forceps. Afterwards, the centrifuge tube containing the tissue was weighed, and the tissue weight was adjusted to 4 g after subtracting the known weight of the centrifuge tube. Next, small pieces of tissue were placed on a new sterile Petri dish and then added 10 mL a collection medium (CM) containing DMEM-HG, 1% (v/v) P/S, and 1% (v/v) GlutaMAX™. A piece of tissue was then cut again using forceps and scissors until they became small pieces. It was then transferred into a 50 mL new sterile centrifuge tube using a sterile spatula. Afterwards, the centrifuge tube was added with 20 mL of a dissociation medium no. 1 (DMEM-HG containing 0.1% (w/v) collagenase type I, and 1% (v/v) P/S), then incubated at 37 °C for 1 h in a water bath. During the incubation time period, the centrifuge tube was gently shook every 15 min. After

that, the sample was pipetted up and down (trituration method) thoroughly using a 10 mL serological pipette for 15 min. The tissue slurry was centrifuged at 800×g for 5 min at 4 °C to remove large non-digested tissue fragments from the cell suspension containing satellite cells and other residual cells, such as red blood cells. The supernatant was removed carefully and then added 20 mL of a dissociation medium no. 2 (DMEM-HG containing 0.1% (w/v) porcine trypsin, 0.02% (w/v) EDTA, and 1% (v/v) P/S), incubated again at 37 °C for 20 min. Afterwards, the samples were triturated and centrifuged again at 200×g for 5 min at 4 °C to further separate small remaining tissue fragments. Under these conditions, most satellite cells remained in the supernatant, thereby reducing contamination by residual tissue debris. The supernatant was filtered through a 70 μm cell strainer placed on a 50 mL new sterile centrifuge tube. The flow through was collected following centrifugation at 800×g at 4 °C for 5 min to obtain a pellet enriched in satellite cells prior to culture initiation. The cell pellet was resuspended in 5 mL of a serum containing medium (SCM) (DMEM-HG containing 5% (v/v) FBS, 10% (v/v) HS, 10% (v/v) CS, 1% (v/v) P/S, and 1% (v/v) GlutaMAX™) and then aspirated into T25 flask (T1 flask). The flask was incubated at 37 ° C, 5% CO_2 (Thermo Electron Corporation, USA) overnight. After 24 h-incubation, the culture medium in T1 flask was transferred into a new T25 flask (T2 flask) coated with 2% (w/v) gelatin solution. The SCM was replaced in T2 flask every 2 days.

Cryopreservation of chicken myoblast cells

Chicken myoblast cells in T2 flask were dissociated with 1.0 mL TrypLE™ Select per flask, flasks were then washed once with 5 mL PBS. The flasks were incubated at 37 °C, 5% CO_2 in a humidified incubator for 5 min. The dissociated cells of T2 flask were resuspended in 10 mL SCM. The suspension cells were aspirated into a 50 mL sterile centrifuge tube and then the cells were collected by centrifugation at 800×g at 4 °C for 10 min. Afterwards, the cells were resuspended in 2 mL SCM and a cell sample was taken and counted using Countess II FL automated cell counter (Thermo Fisher Scientific, MA, USA). Cryopreservative medium used in this study included 50% (v/v) FBS, 10% (v/v) DMSO and 40% (v/v) SCM. The number of total cells per one cryotube was 1×10^6 cells and it was established as passage no. 0 or P0. The cell stock was kept at –80 °C for short-term use during the study.

Culture of chicken myoblast cells in serum-containing medium (SCM)

The starter cells were obtained from those before the cell cryopreservation was carried out as described above. The cells were seeded at 1×10^4 cells/cm^2 in a 6-well plate and then 3 mL of SCM was added to each well. The experiment was carried out in triplicate. The plate was incubated in a humidified CO_2 incubator at 37 °C, 5% CO_2 for 4 days. The SCM in wells was replaced every 2 days. To subculture the cells, spent medium was removed and washed once with 3 mL PBS. The monolayer cells were dissociated with 0.5 mL TrypLE™ Select per well and incubated at 37 °C for 5 min. The dislodged cells in each well were well resuspended with 0.5 mL of 0.50 mg/mL soybean trypsin inhibitor (STI). Sample of suspension cells in each well (10 - 20 μL) was taken for cell counting. The cells were subcultured continuously until no growth was observed. Doubling time (D) and the number of generations of cell growth (x) [10] were calculated as the equations described below during each subculture.

Doubling time (D) and the number of generations of cell growth (x)

During the cell growth phase, an exponential increase in cell number can be represented in Eq. (1).

$$N = N_0\times2^x \quad (1)$$

where N = final cell concentration (cells/mL), N_0 = initial cell concentration (cells/mL), and x = number of generations of cell growth.

Doubling time (D) can be calculated from in Eq. (2).

$$D = T/x \quad (2)$$

where D = population doubling time (h), T = total elapsed time (h), and x = number of generations of cell growth.

Development of a modified serum-free medium for chicken myoblast cells

Screening experiment

A 2^{5-1} fractional factorial design (FFD) was used to screen 5 serum substitutes, namely (1) bovine serum albumin (BSA) (2) Bacto™ soytone (ST) (3) Bacto™ yeast extract (YE) (4) chemically defined lipid concentrate (CDLC) and (5) recombinant human epidermal growth factor (r-EGF). High glucose, Dulbecco's Modified Eagle Medium (DMEM-HG; Gibo, Cat. No. 11965167) was used as a basal medium. Before adding the 5 serum substitutes, DMEM-HG was first supplemented with the following components at the indicated concentrations. The functions and roles of each supplement in cell culture have been previously described in the literature; (1) 30 mg/L recombinant human insulin (SAFC, Cat. No. 91077C) [11]. (2) 10 µg/L sodium selenite (Sigma Aldrich, Cat. No. 214485) [12], (3) 550 µg/L iron (II) sulfate heptahydrate (Sigma Aldrich, Cat. No. F8633) [13], (4) 10 mg/L L-ascorbic acid 2 – phosphate sesquimagnesium salt hydrate (Sigma Aldrich, Cat. No. A8960) [14], (5) 2 mg/L ethanolamine (Sigma Aldrich, Cat. No. E0135) [15], (6) 1 mL/L trace element A (1,000×, Corning, USA) [16], (7) 1 mL/L trace element B (1000x, Corning, USA) [16], (8) 1 mL/L trace element C (1,000×, Corning, USA) [16], (9) 30 mg/L L-glutathione reduced (Sigma, USA, Cat. No. G6013) [17], (10) 0.55 mg/L thymidine (Sigma Aldrich, CAS No. 50-89-5, USA) [18], (11) 14 mg/L hypoxanthine (Sigma Aldrich, CAS No. 68-94-0, USA) [18], (12) 9 mg/L adenosine (Sigma Aldrich, CAS No. 58-61-7, USA) [18], (13) 9 mg/L cytidine (Sigma Aldrich, CAS No. 65-46-3, USA) [18], (14) 9 mg/L guanosine (Sigma Aldrich, CAS No. 118-00-3, USA) [18], and (15) 9 mg/L uridine (Sigma Aldrich, CAS No. 58-96-8, USA) [18]. For the experiment, the starter chicken myoblast cells were obtained from P0 as described above. The cell density of 1×10^4 cells/cm^2 was seeded into 12-well plates (Nunclon™, Cat. No. 150628, Denmark) containing 2 mL SCM per well. Each run was performed in triplicate. Subsequently, the plates were placed in a humidified CO_2 incubator at 37 °C and 5% CO_2 for 24 h. Afterwards, SCM was replaced with tested media (Run 1 - Run 16) except a positive control, while a negative control was replaced with serum-free DMEM-HG containing 1% (v/v) GlutaMAX™. Then, the plates were further incubated for 4 days with replacing corresponding media every 2 days. The cells were dissociated using TrypLE™ Select at 400 µL/well and resuspended in soybean trypsin inhibitor (STI) at 600 µL/well before counting the cells using the same method as described above.

Optimization experiment

To further optimize selected serum substitutes, a central composite design (CCD) was adopted. Three selected serum substitutes were BSA, ST, and YE. For the experiment, the starter chicken myoblast cells were obtained from P0 of cell stock. The cells were grown and expanded in SCM in a new T25 flask at 37 °C and 5% CO_2 for 4 days. SCM was replaced every 2 days. After the cells were grown to 80% - 90% confluence, spent medium was removed and washed once with 5 mL of PBS. Afterwards, 1.0 mL TrypLE™ Select was added into the flask to dissociate the monolayer cells. The flask was incubated at 37 °C for 5 min. Dislodged cells were resuspended with 1.0 mL of SCM before taking a cell sample for counting. The cell density of 1×10^4 cells/cm^2 was seeded into 12-well plates containing 2 mL SCM per well. Each run was performed in triplicate. Then, the plates were placed in a humidified CO_2 incubator. Subsequently, SCM was replaced with tested media (Run 1 - Run 20) except a positive control, while a negative control was replaced with serum-free DMEM-HG containing 1% (v/v) GlutaMAX™. The plates were then further incubated for 4 days with replacing corresponding media every 2 days. The cells were dissociated using TrypLE™ Select at 400 µL/well and resuspended in soybean trypsin inhibitor (STI) at 600 µL/well before counting the cells using the same method as described above.

Verification study

From the screening and optimization experiments, the optimum concentrations of serum substitutes were as follows: BSA (150 mg/L), ST (1.0 g/L), YE (0.532 g/L), CDLC (0.5% v/v), and r-EGF (0.005 mg/L). For the experiment, a direct cell adaptation was adopted. The starter cells were obtained as those used in the optimization experiment. The cells were seeded at 1×10^4 cells/cm^2 in 6-well plates and then SCM was added to 3 mL per well. The experiment was carried out in

triplicate. The plates were incubated at 37 °C, 5% CO_2 in a humidified CO_2 incubator for 24 h to allow the cells to attach and spread. Subsequently, SCM in wells was removed, except for a positive control, and replaced with an optimized medium, AIM-V® medium, and serum-free DMEM-HG containing 1% (v/v) GlutaMAX™ for a negative control. The cells were grown for 4 days with replacing corresponding media every 2 days. For cell subculture, spent media were removed and washed once with 3 mL PBS. The monolayer cells were dissociated with 0.5 mL TrypLE™ Select per well at 37 °C for 5 min. The dislodged cells in each well were resuspended with 0.5 mL of 0.50 mg/mL soybean trypsin inhibitor (STI). Sample of suspension cells in each well (10 - 20 μL) was taken for cell counting. The total cells obtained from the first culture were established as P0. The cells were subcultured using the same method used for P0 for at least 3 passages (P1 - P3).

Cell seeding density of chicken myoblast cells

The starter cells were obtained from P0 of the cell stock in which the cells were recovered and expanded in a T25 flask containing 5 mL of SCM. The cells were incubated at 37 °C, 5% CO_2 for 9 days, with SCM replaced every 2 days. Monolayer cells were dissociated with 1.0 mL TrypLE™ Select at 37 °C, and then washed once in 10 mL of an optimized medium at 200× g for 5 min. For a long-term culture of the cells, the seeding density of the cells was varied at 1×10^4, 2×10^4, 3×10^4, 4×10^4, 5×10^4 and 6×10^4 cells/cm^2. The cells were inoculated in 6-well plates containing 3 mL of the optimized medium. Cell culture was carried out in triplicate. The plates were then incubated at 37 °C, 5% CO_2 for 9 days, with the optimized medium replaced every 2 days.

Statistical analysis

In this study, the Design Expert V13 Statistical Software was adopted for design of experiments and the statistical analysis of data. Two-tailed Student's t-test was used to compare the averages, and a p-value < 0.05 was considered statistically significant.

Results and discussion

Isolation of chicken muscle satellite cells

Isolation of chicken muscle satellite cells from the pectoralis major using collagenase type I (0.1% (w/v)) in association with trypsin enzyme (0.1% (w/v) was carried out. Trituration and centrifugation were crucial steps to achieve the isolation of chicken muscle satellite cells from the pectoralis major. However, a large number of red blood cells could not be separated by such steps, and they were also observed after 24 h of incubation of the isolated chicken muscle satellite cells in a T25 flask (T1). The supernatant collected (medium + digested tissue + suspension cells) from T1, after 24 h of incubation, to a new T25 flask (T2) coated with 2% (w/v) gelatin solution, it was found that chicken muscle satellite cells could differentiate to chicken myoblast cells, a spindle-shaped cell. after 9 days of culture with replacing SCM every 2 days as shown in **Figure 1**. Such isolation and purification of chicken muscle satellite cells were similar to those previously described by Kim *et al*. [6]; Jung *et al*. [7]. However, we noticed that both trituration method and centrifugation at a given condition had benefits on the isolation of such satellite cells. Trituration method using a 10 mL serological pipette for 15 min in combination with the centrifugation allows chicken satellite cells to be isolated from most extracellular substances, such as the extracellular matrix, fat, bones, and cartilage, contained in tissue as described by Hong and Do [5]; Matsuyoshi *et al.* [19].

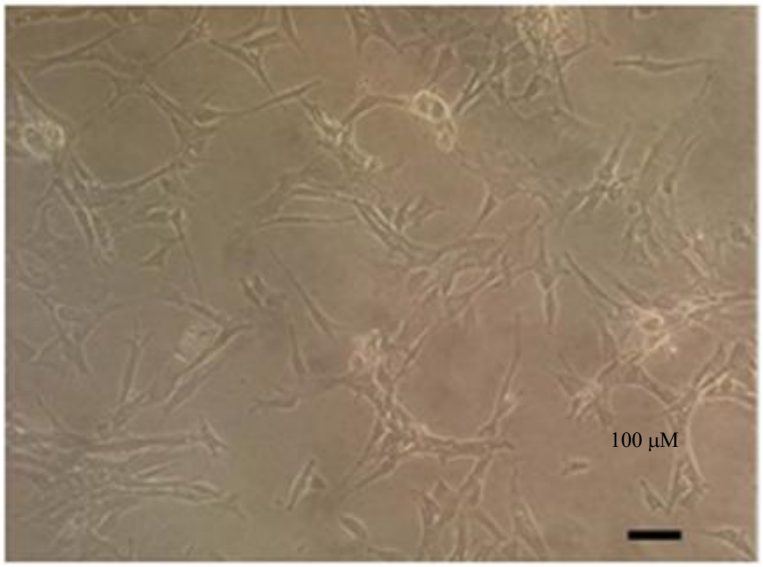


Figure 1 Chicken myoblast cells isolated from the pectoralis major in T2.

Culture of chicken myoblast cells in serum-containing medium (SCM)

Chicken myoblast cells obtained from the pectoralis major in T2 were continuously subcultured in SCM. The first dissociation of chicken myoblast cells with TrypLE™ Select was established as P0. As shown in **Figure 2**, the chicken myoblast cells could be subcultured successively for 5 passages (P5) with a difference in the number of cell generations as shown in **Table 1**. At passage 6 (P6), no growth and proliferation of chicken myoblast cells was observed, with the number of cell generations being 0. The total number of cell generations (x) of 12.37 was observed with a gradual increase of the doubling time (D) from 28.27 to 129.47 h. The growth and proliferation of chicken myoblast cells closely resemble normal primary cells isolated from tissue of interest. Although there has been the attempt to generate immortalized chicken myoblast cells by transfecting the exogenous chicken telomerase reverse transcriptase (chTERT) gene by Guo *et al*. [20], only at least 20 generations of population doublings could be maintained.

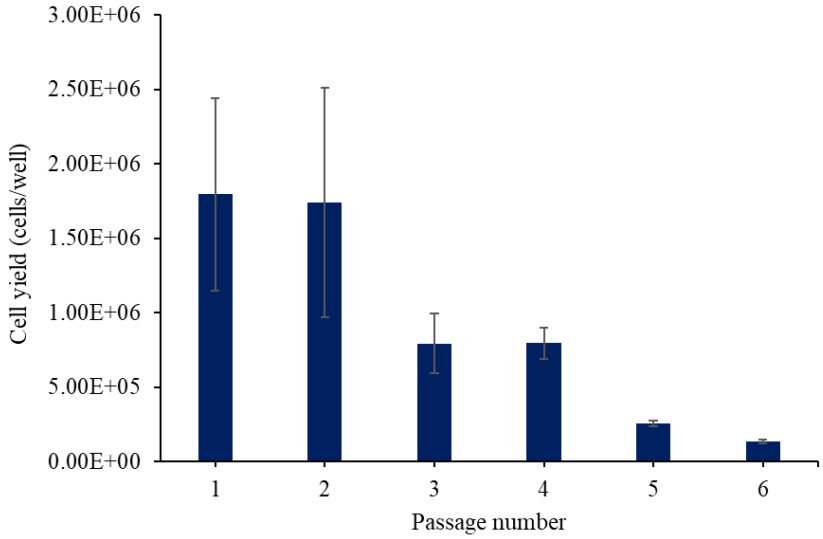


Figure 2 The cell yields obtained from a continuous subculture of chicken myoblast cells in 6-well plates containing 3 mL of SCM at 37 °C, 5% CO_2 for 4 days, with SCM replaced every 2 days. Cell yield was expressed as mean ± SD (n = 3).

Table 1 The number of cell generations (x) and the doubling time (D) of chicken myoblast cells grown in serum-containing medium (SCM).

Passage number	x	D(h)	x (on average)	D (h) (on average)
1	2.94 - 4.03	23.85 - 32.69	3.48	28.27
2	2.70 - 4.07	23.61 - 35.62	3.38	29.62
3	1.98 - 2.72	35.24 - 48.43	2.35	41.83
4	2.20 - 2.59	37.11 - 43.65	2.39	40.38
5	0.64 - 0.88	108.99 - 149.95	0.76	129.47
6	no growth	no growth	no growth	no growth

Development of a modified serum-free medium for chicken myoblast cells

Screening experiment

To develop a modified serum-free medium for chicken myoblast cell culture, 2 protein hydrolysates, namely Bacto™ soytone (ST) [21] and Bacto™ yeast extract (YE) [21], and 3 other supplements, namely bovine serum albumin (BSA) [22], chemically defined lipid concentrate (CDLC) [8] and recombinant human epidermal growth factor (r-EGF) [23] were used as serum substitutes. To screen these serum substitutes, a 2^{5-1} fractional factorial design (FFD) was adopted. Cell yields obtained from the screening experiment were shown in **Table 2**, with the range of $0.35 \pm 0.00 \times 10^5$ - $2.86 \pm 0.07 \times 10^5$ cells/well. The cell yield of a positive control was $5.04 \pm 0.40 \times 10^5$ cells/well, while that of a negative control was obviously lower at $0.17 \pm 0.02 \times 10^5$ cells/well. The maximum cell yield of $2.86 \pm 0.07 \times 10^5$ cells/well was obtained from standard run no. 14, where the concentrations of serum substitutes were BSA (150 mg/L), ST (1 g/L), YE (5 g/L), CDLC (0.5% (v/v)), and r-EGF (0.005 mg/L).

Table 2 Layout of the screening experiments using a 2^{5-1} fractional factorial design (FFD).

Experiment no	A BSA (mg/L)	B ST (g/L)	C YE (g/L)	D CDLC (% v/v)	E r-EGF (mg/L)	pH (pH value)	Osmolality (mOsm/Kg)	Cell yields ($\times 10^5$ cells/well)
1	50	1	1	0.1	0.030	7.829	339	1.61 ± 0.50
2	150	1	1	0.1	0.005	7.893	343	2.51 ± 0.03
3	50	5	1	0.1	0.005	7.829	344	1.52 ± 0.00
4	150	5	1	0.1	0.030	7.808	340	1.52 ± 0.00
5	50	1	5	0.1	0.005	7.819	344	0.35 ± 0.08
6	150	1	5	0.1	0.030	7.814	368	1.91 ± 0.50
7	50	5	5	0.1	0.030	7.749	351	1.05 ± 0.80
8	150	5	5	0.1	0.005	8.002	348	1.26 ± 0.40
9	50	1	1	0.5	0.005	7.916	354	0.62 ± 0.10
10	150	1	1	0.5	0.030	7.895	353	2.20 ± 1.00
11	50	5	1	0.5	0.030	8.060	356	1.57 ± 0.60
12	150	5	1	0.5	0.005	7.837	353	0.76 ± 0.20
13	50	1	5	0.5	0.030	8.027	359	0.56 ± 0.40
14	150	1	5	0.5	0.005	7.876	360	2.86 ± 0.07
15	50	5	5	0.5	0.005	7.739	363	0.42 ± 0.03
16	150	5	5	0.5	0.030	7.715	355	0.35 ± 0.00
Positive control						8.629	338	5.04 ± 0.40
Negative control						7.658	339	0.17 ± 0.02

Main effects plots were demonstrated in **Figure 3**. It was found that BSA had effect positively, while ST, YE, and CDLC had effect negatively. For the effect of r-EGF, there was little positive effect on the cell yield between a low level and a high level. Analysis of variance (ANOVA) for selected factorial model was shown in **Table 3**. It was found that model terms, namely A, B, C, D, AB, AC, AE, BD, and CE, were statistically significant (p-value < 0.05). Based on the effect hierarchical principle, both main effects and lower-order effects are more important than the higher-order effects, and those effects of the same order are equally important. Therefore, 3 main effects, namely A (BSA), B (Bacto™ soytone), and C (Bacto™ yeast extract) were selected to further optimize using a central composite design. Bovine Serum Albumin (BSA) is one of the most widely used protein supplements in mammalian cell culture, particularly in serum-free and chemically defined media. BSA functions as a carrier, stabilizer, detoxifying agent, and protective macromolecule that supports cell growth and viability [24]. BSA does not act as a specific signaling molecule, but it mimics several protective and transport functions of serum, helping chicken myoblast cells survive and grow better in serum-reduced or serum-free conditions. Bacto™ soytone and Bacto™ yeast extract are commonly used as complex nutrient supplements in microbial and mammalian cell culture media. They provide amino acids, peptides, vitamins, trace elements, and growth-promoting factors that support cell proliferation, metabolism, and protein production [24, 25]. Even though yeast extract is often used as a low-cost supplement, its undefined composition and biological variability can interfere with controlled proliferation of chicken myoblast cells, sometimes resulting in reduced yield compared with more defined supplements. Chemically defined lipid concentrate (CDLC) is commonly supplemented in serum-free culture media to provide essential fatty acids and cholesterol required for membrane synthesis, cellular metabolism, proliferation, and maintenance of cell viability under chemically defined culture conditions [24]. Chemically defined lipid concentrates improve chicken myoblast growth by supplying essential membrane components, supporting energy metabolism, and replacing serum-derived lipids in a controlled and reproducible way. Recombinant human epidermal growth factor (r-EGF) is a recombinant growth factor widely used in mammalian cell culture to stimulate cell proliferation, survival, migration, and differentiation, particularly in serum-free and chemically defined media. It is commonly supplemented at concentrations ranging from 5 - 20 ng/mL to support the proliferation and viability of epithelial and adherent cells [24]. In chicken myoblast serum-free culture, r-EGF is typically used as part of a growth factor cocktail rather than as a single driver, and its optimal concentration must be empirically optimized in combination with other supplements such as insulin, transferrin, and fibroblast growth factors. In this experiment, different concentrations of r-EGF did not show significant differences in chicken cell proliferation, possibly due to the medium composition where r-EGF may not have played a major role in promoting cell growth. Although the optimized medium is serum-free, it is not completely animal-free because it still contains bovine serum albumin (BSA), an animal-derived protein. Therefore, the present formulation should be regarded as a serum-free medium rather than a fully animal-free medium. While the inclusion of BSA contributed to improved cell growth and viability in the present study, it remains a limitation for applications requiring completely animal-free culture systems. Future studies should therefore focus on replacing BSA with suitable non-animal-derived alternatives while maintaining comparable cell growth and proliferation.

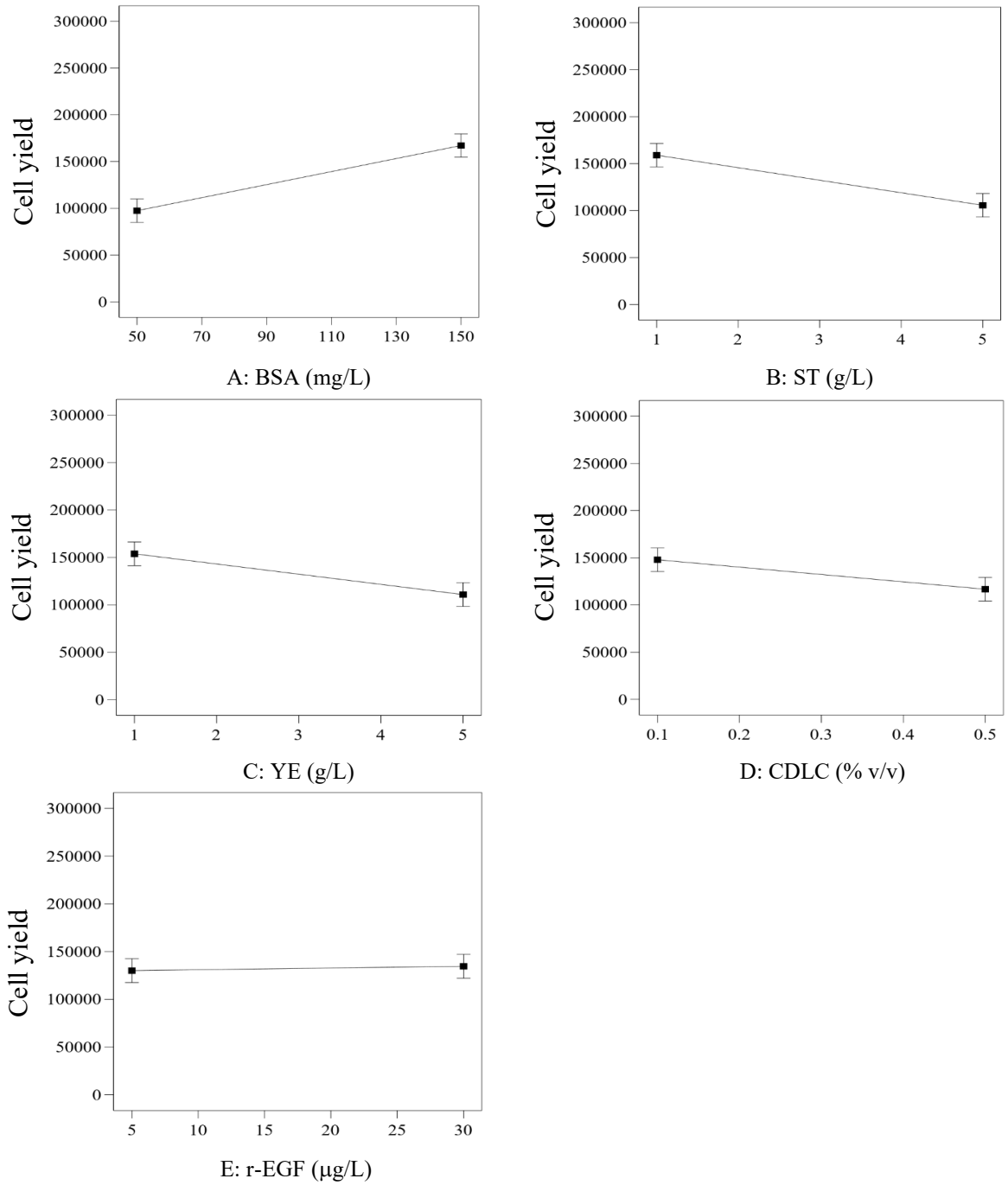


Figure 3 Main effects plots of each serum substitute obtained from a 2^{5-1} fractional factorial design (FFD). The concentration level of each serum substitute was shown in actual value.

Table 3 ANOVA for selected factorial model.

Source	Sum of squares	df	Mean Square	F-value	*p*-value	
Model	8.978E+10	11	8.162E + 09	25.11	0.0035	Significant
A-BSA	1.946E+10	1	1.946E + 10	59.86	0.0015	
B-ST	1.138E+10	1	1.138E + 10	35.00	0.0041	
C-YE	7.387E+09	1	7.387E + 09	22.72	0.0089	
D-CDLC	3.900E+09	1	3.900E + 09	12.00	0.0257	

Source	Sum of squares	df	Mean Square	F-value	*p*-value
E-r-EGF	8.327E + 07	1	8.327E + 07	0.2561	0.6394
AB	2.987E + 10	1	2.987E + 10	91.87	0.0007
AC	3.069E + 09	1	3.069E + 09	9.44	0.0372
AE	6.380E + 09	1	6.380E + 09	19.62	0.0114
BD	2.528E + 09	1	2.528E + 09	7.77	0.0494
CD	1.478E + 09	1	1.478E + 09	4.55	0.0999
CE	4.248E + 09	1	4.248E + 09	13.07	0.0225
Residual	1.300E + 09	4	3.251E + 08		
Cor Total	9.108E + 10	15			

Std. Dev. = 18030.64, R^2 = 0.9857, R^2 (Adjusted) = 0.9465.

Comparison of the *p*-values in **Table 3**, showed that only 3 factors (BSA, ST and YE) were statistically significant, with p-values below 0.05. Although CDLC also showed a *p*-value below 0.05, the interaction term between CDLC and YE exhibited a higher *p*-value (0.0999), indicating a non-significant interaction effect. In contrast, the significant factors (BSA, ST and YE) demonstrated statistical significance not only individually but also through their interaction terms (AB and AC), which had *p*-values well below 0.05. This differed from the interaction terms involving CDLC (BD and CD), which showed higher *p*-values and lacked statistical significance. Normality test, Residual analysis, Model validation, Lack-of-fit test, and Confidence intervals for 2^{5-1} fractional factorial design (FFD) were in **Supplementary data S1 (Figure S1.1-S1.2, Table S1.1)**.

The selected factors were designated as A (BSA), B (ST), and C (YE). Each factor was divided into 5 levels: −1.682, −1, 0, +1, and +1.682, which are coded units. These 5 concentration levels were then converted into uncoded units or actual values, as shown in **Table 4**, used in the preparation of the factors for the CCD experiment.

Table 4 Actual factor levels corresponding to coded factor levels.

Variables	Symbols (unit)	Code factor levels				
		−1.682	**−1**	**0**	**+1**	**+1.682**
BSA	A-BSA (mg/L)	31.00	150.00	325.00	500.00	619.00
ST	B-ST (g/L)	0.33	0.50	0.75	1.00	1.20
YE	C-YE (g/L)	0.33	0.50	0.75	1.00	1.20

Optimization experiment

Each concentration of factor A (BSA), B (ST), and C (YE) were designed by CCD for 20 runs as shown in **Table 5**, while the CDLC and r-EGF were fixed at 0.5% (v/v) and 0.005 mg/L, respectively. Cell yields were in the range of 0.84 ± 0.06×10^5 - 4.18 ± 0.30×10^5 cells/well. The cell yield of a positive control was 5.67 ± 0.10×10^5 cells/well, while that of a negative control was obviously lower at 1.70 ± 0.00 x 10^5 cells/well. The maximum cell yield of 4.18 ± 0.30×10^5 cells/well was obtained from standard run no. 3, where the concentrations of serum substitutes were BSA (150 mg/L), ST (1.0 g/L), YE (0.5 g/L). Analysis of variance (ANOVA) for Reduced Cubic model was shown in **Table 6**. However, to analyze the data (cell yields), it was necessary to transform the data using the power mode of configure analysis with a lambda value (λ) of 1 and a constant value (k) of 1. According to the result of analysis of variance (ANOVA), as shown in **Table 6**, main effects (A-BSA, B-ST, and C-YE), 2-factor interactions (AB), quadratic terms (B^2, C^2) were statistically significant (*p*-values < 0.05). The coefficient of determination (R^2 = 0.9221) suggested that 92.21% of the data (cell yield) could be explained

by the empirical mathematical model. The empirical mathematical model for estimating the cell yields corresponding to the optimum concentration (coded values) of serum substitutes was shown in Eq. (3).

$$(\text{Cell yield } +1.00)^{1} = 2.663E + 05 - 59959.17A + 27809.00B - 29381.84C - 41800.00AB + 7006.25AC - 27906.25BC + 12059.39A^2 - 49909.68B^2 - 40085.32C^2 \quad \text{Eq. (3)}$$

The model explains most of the variability in the response ($R^2 > 0.9$), making it useful for prediction or process understanding. However, the significant lack-of-fit test indicates that the model is not a perfect representation of the underlying relationship and some systematic error remains. Normality test, Residual analysis, Model validation, Lack-of-fit test, and Confidence intervals for central composite design (CCD) were in **Supplementary data S2 (Figure S2.1-S2.2, Table S2.1)**.

Table 5 Layout of the optimization experiments using a central composite design (CCD).

Experiment no	A-BSA (mg/L)	B-ST (g/L)	C-YE (g/L)	pH (pH value)	Osmolality (mOsm/Kg)	Cell yields (×10^5 cells/well)
1	150	0.5	0.5	8.055	349	1.67 ± 0.10
2	500	0.5	0.5	7.951	340	1.12 ± 0.08
3	150	1.0	0.5	7.972	352	4.18 ± 0.30
4	500	1.0	0.5	7.972	357	1.39 ± 0.10
5	150	0.5	1.0	7.960	352	1.95 ± 0.10
6	500	0.5	1.0	8.002	337	1.12 ± 0.08
7	150	1.0	1.0	7.936	361	2.78 ± 0.20
8	500	1.0	1.0	7.910	339	0.84 ± 0.06
9	31	0.75	0.75	8.005	383	3.62 ± 0.30
10	619	0.75	0.75	8.058	354	2.39 ± 0.20
11	325	0.33	0.75	8.035	347	1.12 ± 0.08
12	325	1.20	0.75	7.910	346	1.39 ± 0.10
13	325	0.75	0.33	8.001	343	2.23 ± 0.20
14	325	0.75	1.20	7.916	340	0.84 ± 0.20
15	325	0.75	0.75	8.020	343	2.78 ± 0.20
16	325	0.75	0.75	7.997	348	2.82 ± 0.20
17	325	0.75	0.75	7.980	342	2.51 ± 0.20
18	325	0.75	0.75	7.957	344	2.54 ± 0.20
19	325	0.75	0.75	8.057	354	2.70 ± 0.20
20	325	0.75	0.75	8.174	337	2.62 ± 0.20
Positive control				8.629	338	5.67 ± 0.10
Negative control				7.658	339	1.70 ± 0.00

Table 6 ANOVA for Reduced Cubic model

Source	Sum of squares	df	Mean Square	F-value	*p*-value
Model	1.519E + 11	9	1.688E + 10	13.16	0.0002
A-BSA	4.910E + 9	1	4.910E + 10	38.28	0.0001
B-ST	1.056E + 8	1	1.056E + 10	8.24	0.0167
C-YE	1.179E + 9	1	1.179E + 10	9.19	0.0126
AB	1.398E + 10	1	1.398E + 10	10.90	0.0080
AC	3.927E + 8	1	3.927E + 8	0.3062	0.5922

Source	Sum of squares	df	Mean Square	F-value	*p*-value
BC	6.230E + 9	1	6.230E + 9	4.86	0.0521
A^2	2.096E + 9	1	2.096E + 9	1.63	0.2300
B^2	3.590E + 10	1	3.590E + 10	27.99	0.0004
C^2	2.316E + 10	1	2.316E + 10	18.06	0.0017
Residual	1.282E + 10	10	1.339E + 8		
Lack of Fit	1.202E + 5	5	9.668E + 5	14.98	0.0050
Pure Error	8.027E + 8	5	1.605E + 8		
Total	1.647E + 11	19			

Std. Dev. = 35811.85, R^2 = 0.9221, R^2 (Adjusted) = 0.8521.

The optimum concentrations of serum substitutes corresponding to the empirical mathematical model were estimated by using Optimization function of the software. It was found that the optimum concentrations of A-BSA, B-ST, and C-YE were 150 mg/L, 1.0 g/L, and 0.500 g/L, respectively. The effects of serum substitutes on the cell yields in form of 3D response surfaces and contour plots were shown in **Figure 4**. The cell yield that was estimated by the empirical mathematical model was 3.82×10^5 cells/well. However, the cell yields in the range of 3.25×10^5 - 4.39×10^5 cells/well cells/well was acceptable. Concerning pH values and Osmolality values in **Tables 2** and **5**, chicken myoblast cells can survive temporary alkaline conditions (pH > 8.0) due to buffering systems, intracellular pH regulation, and cellular stress-response mechanisms. However, survival under these conditions does not indicate optimal growth, as high pH generally impairs proliferation, metabolism, protein synthesis, and differentiation efficiency. Similarly, the optimal osmolality for chicken myoblast culture is approximately 280 - 330 mOsm/kg, which supports normal morphology and metabolic activity. When osmolality exceeds 370 mOsm/kg, hyperosmotic stress can cause cell shrinkage, disrupted ion balance, impaired nutrient transport, activation of stress pathways, and reduced proliferation. Therefore, although chicken myoblast cells may tolerate moderate pH and osmotic stress for short periods, such conditions negatively affect healthy growth and expansion efficiency [24,26,27].

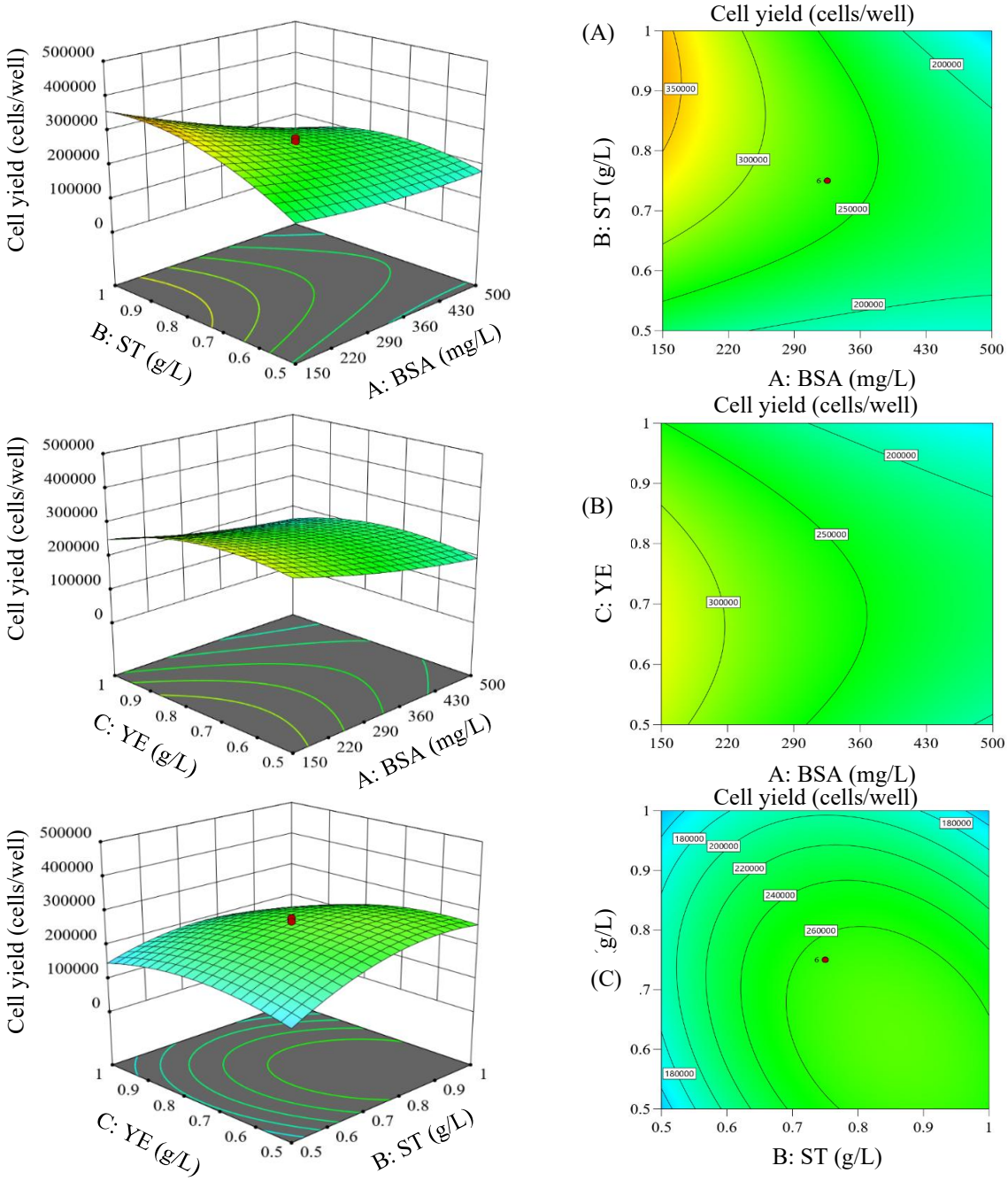


Figure 4 Viable cell yields in 3D response surfaces (left) and contour plots (right): (A) effects of A-BSA and B-ST concentration, (B) effects of A-BSA and C-YE concentration, (C) effects of B-ST and C-YE concentration. All 3D response surfaces and contour plots were created using Design-Expert® v13 software (Stat Ease, Inc. MN, USA).

Verification study

To verify the optimized serum-free medium, a direct cell adaptation was adopted. As shown **Figure 5**, the yield of chicken myoblast cells in SCM (positive control) at P0 was 4.01 ± 0.32×10^5 cells/well, with the number of cell generations (x) ~ 2, and the doubling time (D) ~ 48 h. At P1, the maximum cell yield was obtained at 5.28 ± 0.62×10^5 cells/well, with the number of cell generations (x) ~ 2.40, and the doubling time (D) ~ 38 h. After that, the capability of growth decreased dramatically at P2, with the cell yield of 2.29 ± 0.63×10^5 cells/well, and the number of cell generations (x) and the doubling time (D) were abruptly decreased at ~1.20 and ~ 80 h, respectively. At P3, little cell growth in SCM was observed with the cell yield of 1.23 ± 0.25×10^5 cells/well, and the number of cell generations (x) and the

doubling time (D) were negligible. For the optimized medium, the cell yield at P0 was 2.03 ± 0.13×10^5 cells/well, and the number of cell generations (x) and the doubling time (D) were ~ 1.02, and ~ 94 h, respectively. For AIM-V® medium, the cell yield at P0 was 2.51 ± 0.061×10^5 cells/well, and the number of cell generations (x) and the doubling time (D) were ~ 1.32, and ~ 73 h, respectively. At P2, the mean of cell yields between the optimized medium and AIM-V® medium was not statistically significant (*p*-value > 0.05). At P3, little cell growth was observed for the optimized medium (1.28 ± 0.19×10^5 cells/well), and the number of cell generations (x) and the doubling time (D) were negligible. To elucidate the condition of adaptation using a cell adaptation method, the tendency of cell growth and proliferation between a positive control and serum-free media groups (the optimized medium and AIM-V® medium) was relatively similar due to the fact that chicken myoblast cells were inherently constrained; that was a limited number of cell generations. It was noticed that the results of serum-free medium development for chicken myoblast cells derived from other groups were relatively similar to those done by our experiments [23,28].

The limited proliferation of isolated chicken myoblasts in serum-free medium may be attributed to the finite proliferative capacity of primary cells and the absence of sufficient mitogenic and attachment factors normally supplied by serum. Under serum-free or serum-reduced conditions, chicken myoblasts tend to withdraw from the cell cycle and undergo differentiation into myotubes, thereby reducing the population of proliferative cells [29]. Primary chicken satellite cells generally exhibit the highest proliferative and myogenic potential at early passages (P0 - P1), while prolonged passaging is associated with reduced stemness, differentiation capacity, and eventual cellular senescence, particularly under serum-free conditions [26,27]. However, cell viability during the subcultivations of 3 media were higher than 80% as shown in **Figure 6**.

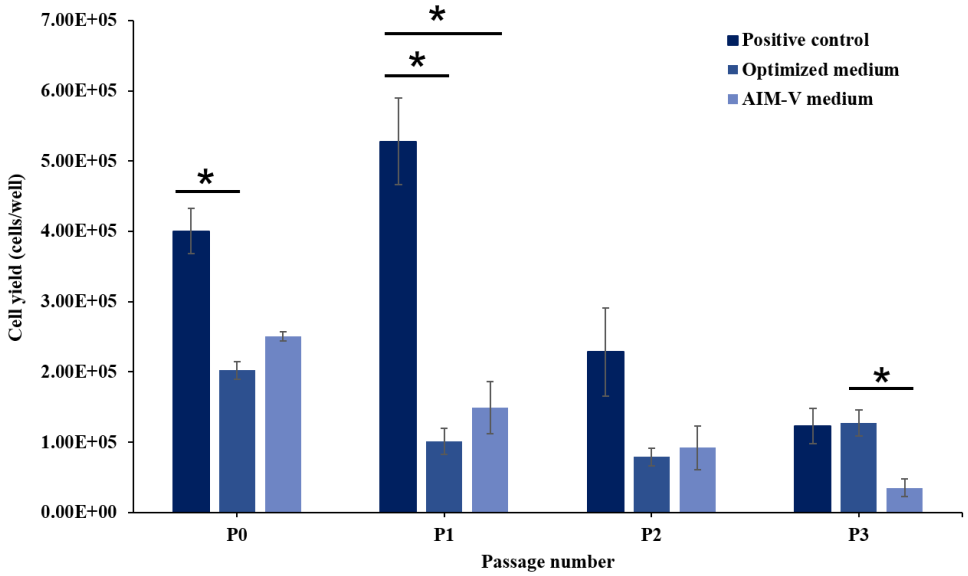


Figure 5 A direct adaptation of chicken myoblast cells in an optimized medium. Chicken myoblast cells were cultured in 6-well plates containing 3 mL of each medium at 37 °C, 5% CO_2 for 4 days, with each medium replaced every 2 days. Cell yield was expressed as mean ± SD (n = 3). **p*-value < 0.05.

Based on cost estimation, the developed medium is approximately USD 165 - 195 per L, which is considerably less expensive than commercially available serum-free media. Nevertheless, further optimization is required to improve its ability to support cell growth, ensure that all components are available in cell culture-grade quality, and evaluate its economic feasibility for large-scale manufacturing to further

reduce production costs. In addition, identifying alternative sources of medium components, particularly plant-derived amino acids and peptides, may provide a promising strategy for further lowering the overall cost of the medium.

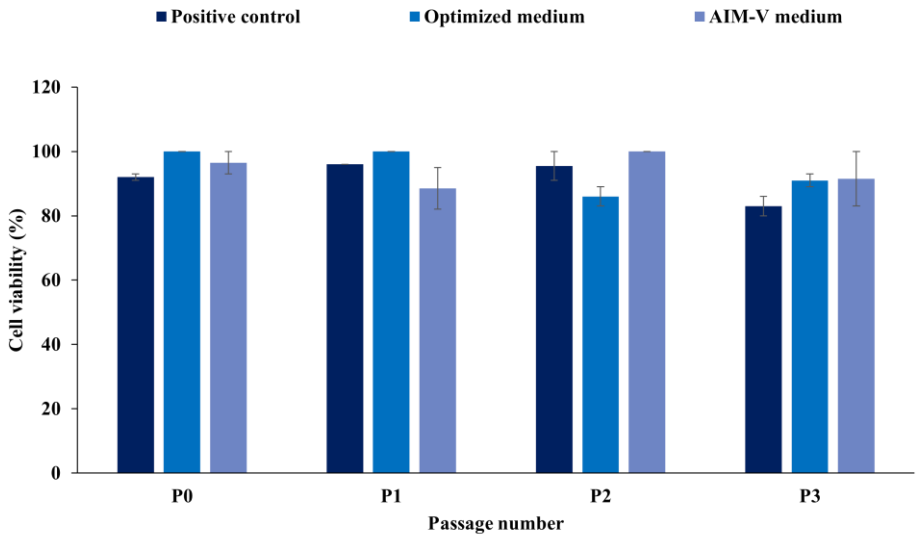


Figure 6 Viability of chicken myoblast cells were cultured in 6-well plates containing 3 mL of each medium at 37 °C, 5% CO_2 for 4 days, with each medium replaced every 2 days. Cell yield was expressed as mean ± SD (n = 3).

Cell seeding density of chicken myoblast cells

Since the goal is to produce meat for human consumption, it is essential to examine the influence of the initial cell seeding density on cell proliferation. In this study, a culture of the cells in which the seeding density of the cells was varied at 1×10^4 - 6×10^4 cells/cm^2. The final yield of chicken myoblast cells was shown on **Figure 7**. The fold increase in cell number was calculated by dividing the initial cell count by the number of cells increased. It was found that an initial cell seeding density of 6×10^4 cells/cm^2 resulted in a 1.29-fold increase. The morphology of chicken myoblast cells cultured in optimized medium (**Figure 8**) was quite different from culture in SCM (**Figure 1**), cells were round-shaped, while cells cultured in serum were elongated. Moreover, after 9 days of culture with medium replaced every 2 days, it was found that suspended growing cells were observed in all experiments and were removed out of wells of the 6-well plates every 2 days, suggesting attachment factors (e.g., fibronectin, laminin, and vitronectin) were required for supporting cell attachment [30]. Therefore, proliferation of chicken myoblast cells in cultures was limited even when the 6-well plates used were coated with gelatin solution (2% (w/v)). For this reason, the mechanism of action of gelatin might be quite different from fibronectin, laminin, or vitronectin through different integrins, as described by Mogha *et al*. [31]. However, we observed that coated wells of the 6-well plates with such gelatin solution could bring about small flocs in the culture medium when incubating at 37 °C for a long-term culture. This might be the drawback of using gelatin solution to prepare culture surface area to culture chicken myoblast cells in this study because it might be dissolved in a solution form at 37 °C and in the absence of serum.

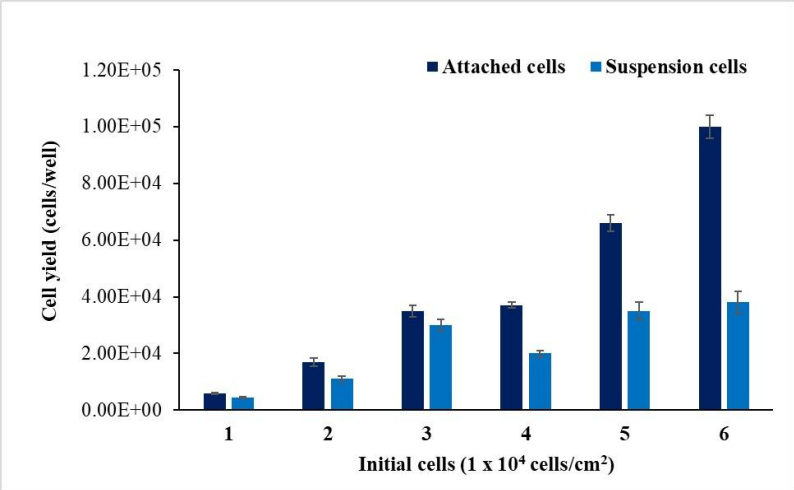


Figure 7 Cell yields obtained from culture of chicken myoblast cells in the 6-well plates with different initial cell densities harvested on day 9 of culture. Cell yield was expressed as mean ± SD (n = 3).

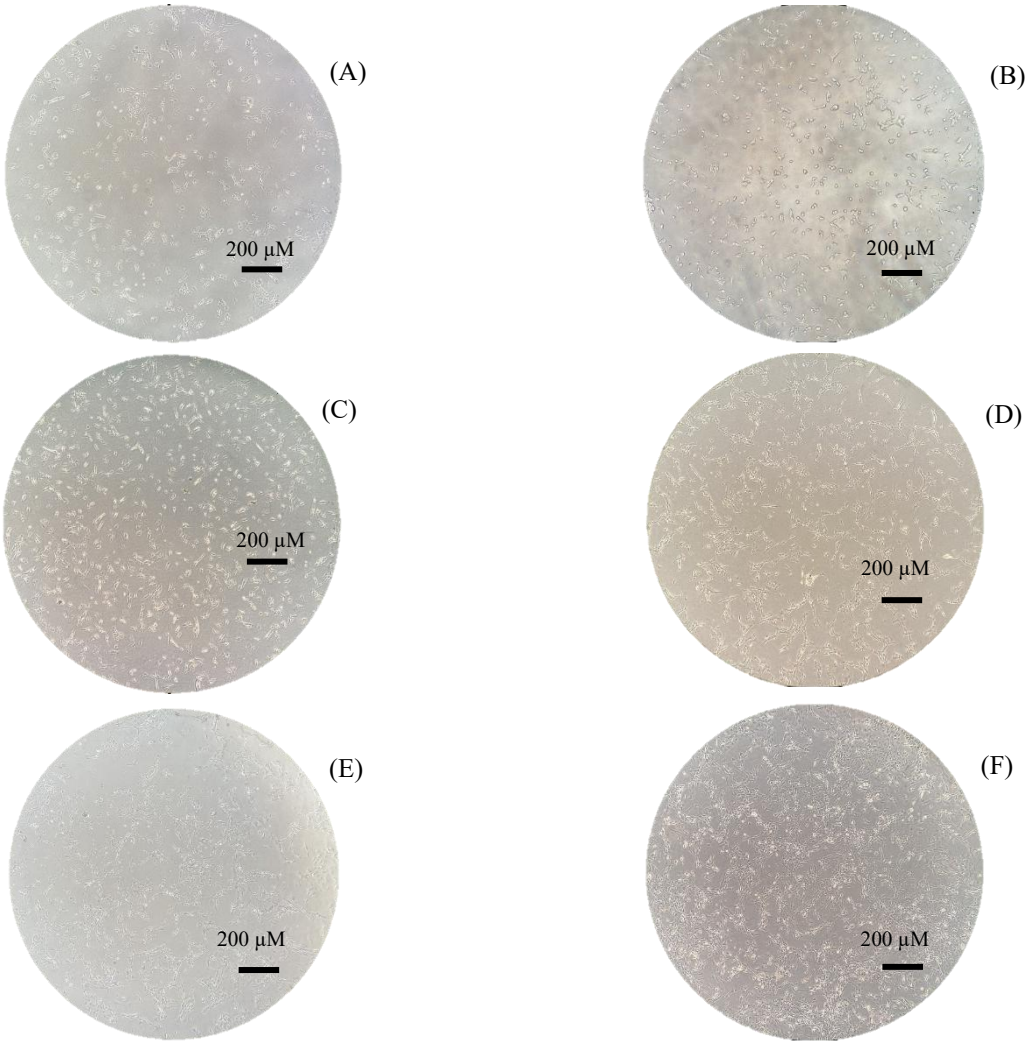


Figure 8 Chicken myoblast cells cultured on the 6-well plates coated with gelation solution at 2% (w/v) for 9 days with the optimized medium replaced every 2 days. The initial cells were as follows: (A) 1×10^4, (B) 2×10^4, (C) 3×10^4, (D) 4×10^4, (E) 5×10^4, and (F) 6×10^4 cells/cm^2. The magnification of chicken myoblast cells on day 9 of culture was at 10×.

Conclusions

This study developed a prototype serum-free medium for chicken myoblast cells using a systematic design-of-experiments approach for factor screening and optimization. The optimized formulation, containing bovine serum albumin, Bacto™ soytone, Bacto™ yeast extract, chemically defined lipid concentrate, and recombinant epidermal growth factor, supported cell expansion for up to 3 passages, although proliferation remained lower than in serum-containing medium. Therefore, the current formulation should be considered a partially optimized serum-free medium rather than a complete substitute for conventional serum-containing culture conditions. Nevertheless, these findings provide a useful foundation for further optimization of long-term proliferation and maintenance of myogenic potential. Future studies should investigate additional growth and attachment factors, including recombinant human fibroblast growth factor 2 (rhFGF-2), basic FGF (bFGF), fibronectin, laminin, and vitronectin, to further improve culture performance.

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Declaration of Generative AI in Scientific Writing

The authors used ChatGPT-4.0 for English language editing and QuillBot for plagiarism checking.

CRediT Author Statement

Seangduen Wattiwong: Methodology, Writing - original draft. **Manoch Posung**: Conceptualization, Methodology, Data curation, Writing - review & editing. **Atsadawut Areesirisuk**: Conceptualization, Methodology, Formal analysis, Writing - review & editing. **Suthida Tuntigumthon**: Resources, Methodology, Writing - review & editing. **Panarat Thongpoem**: Resources, Writing – review & editing. **Jantima Teeka**: Conceptualization, Supervision, Funding acquisition, Project administration, Writing - review & editing.

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Supplementary Materials

Supplementary data S1

To describe or explanation these questions, we use cell yields obtained from both a 2^{5-1} fractional factorial design and a central composite design in **Tables 2** and **5**. We used values reported to three decimal places, such as 1.61, for the analysis.

Normality test: For 2^{5-1} fractional factorial design

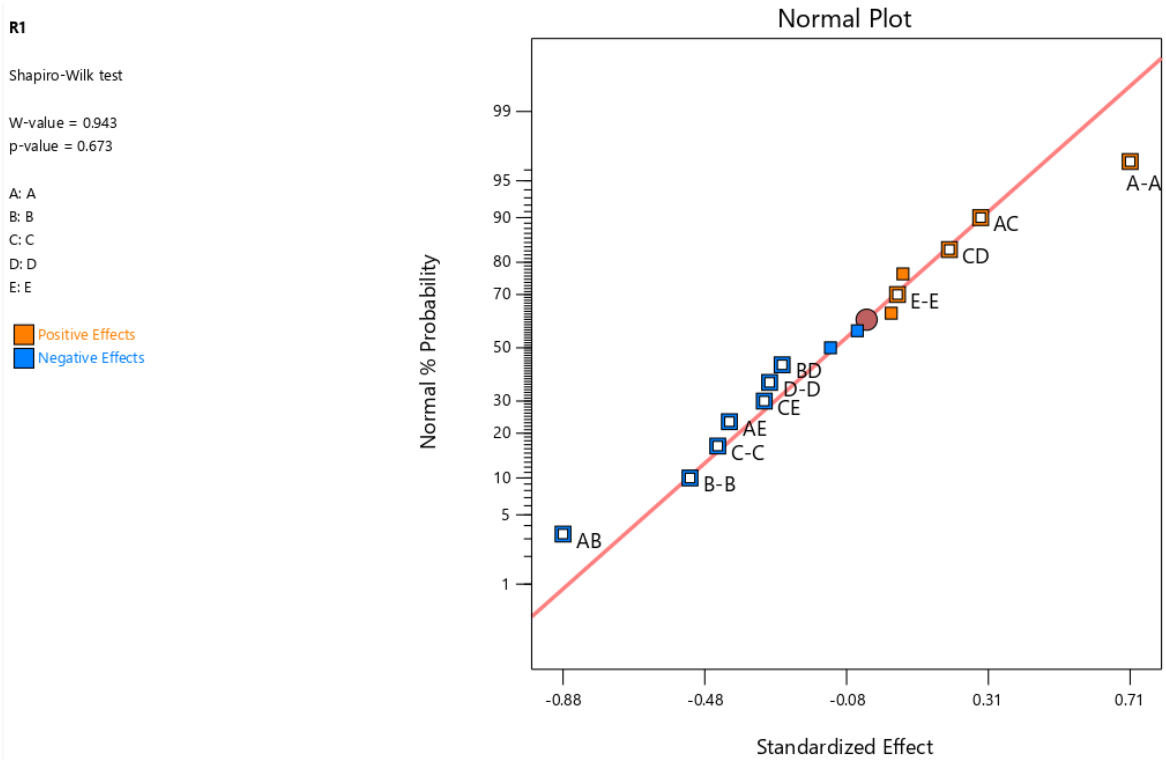


Figure S1.1 Normality test for 2^{5-1} fractional factorial design

The normal plot of standardized effects is used to identify which main factors and interactions have the greatest influence on the response variable in the experimental design. In this plot, effects that lie close to the red reference line are generally considered small or statistically insignificant, whereas effects that deviate farther from the line, particularly at the extreme left or right ends, are more likely to be significant. The Shapiro–Wilk test results (W = 0.943, p = 0.673) indicate that the effects follow an approximately normal distribution, supporting the suitability of this plot for interpretation. Based on the labeled points, factor A–A appears to be the strongest positive effect, indicating that increasing this factor substantially increases the response. Other positive effects such as AC, CD, and E–E may also contribute to increasing the response, although to a lesser extent. On the negative side, AB shows the largest negative effect, suggesting that this interaction decreases the response most strongly, while B–B, C–C, AE, CE, D–D, and BD exhibit smaller negative influences. Overall, the results suggest that only a few factors or interactions have meaningful effects on the response, while the remaining terms are relatively minor.

Residual analysis: For 2^{5-1} fractional factorial design

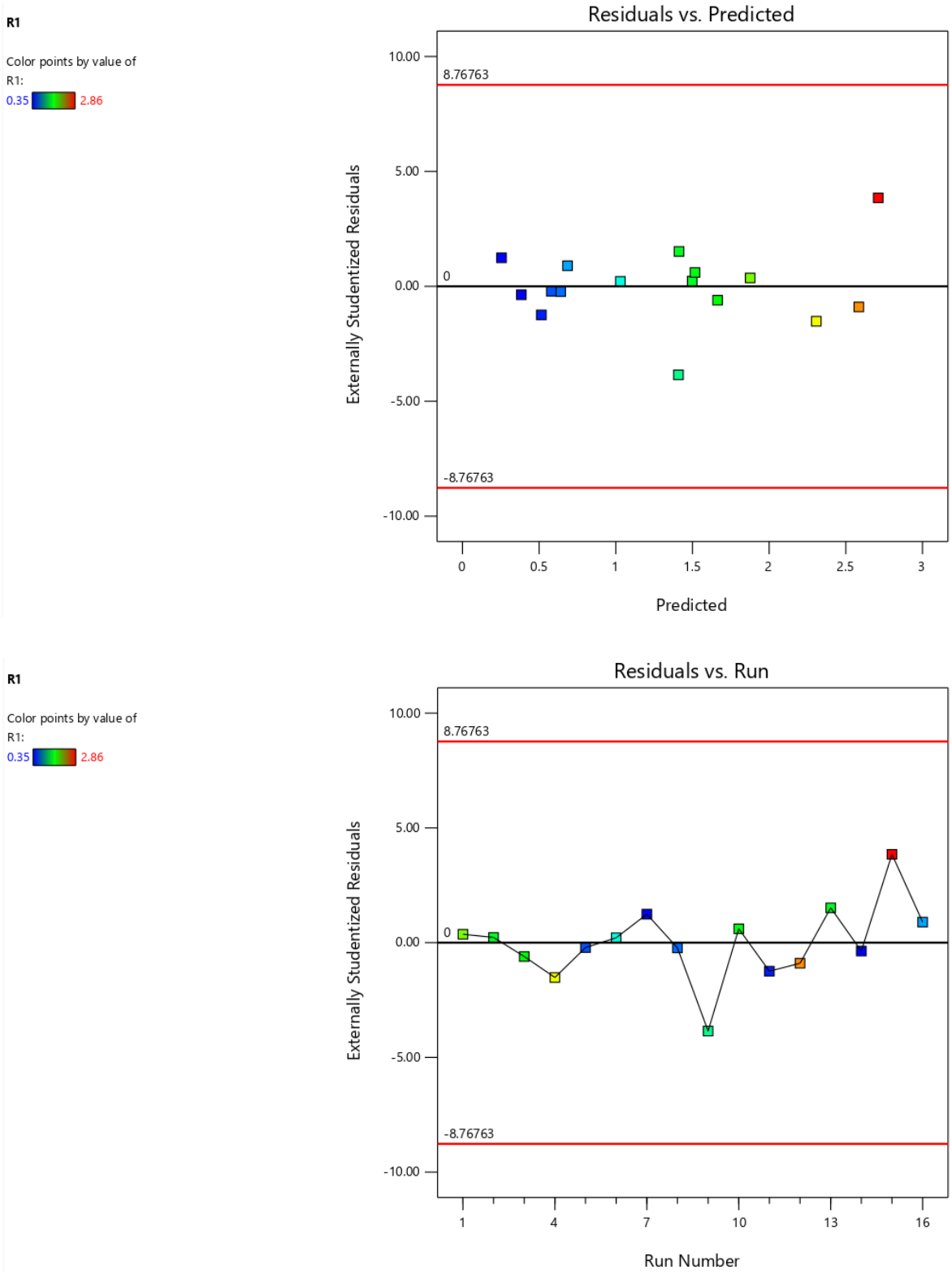


Figure S1.2 Residual analysis for 2^{5-1} fractional factorial design

All observations remain within the red control limits (± 8.76763), indicating that no severe outliers or highly influential points are present.

Model validation: As described in **Table 3**, a significant model *p*-value ($p < 0.05$) indicates that the model explains a meaningful portion of the variation in the response. Therefore, in Table3., a p-value of Model is 0.0035.

Lack-of-fit test: For the 2^{5-1} fractional factorial design, a formal lack-of-fit test was not available because the design lacked sufficient pure-error degrees of freedom. Therefore, model adequacy was assessed using ANOVA significance, residual diagnostics, and confirmation experiments.

Confidence intervals: As described in table below

Table S1.1 Coefficients in Terms of Coded Factors for 2^{5-1} fractional factorial design

Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	1.32	1	0.0408	1.20	1.43	
A-A	0.3544	1	0.0408	0.2412	0.4676	1.0000
B-B	-0.2606	1	0.0408	-0.3738	-0.1474	1.0000
C-C	-0.2219	1	0.0408	-0.3351	-0.1087	1.0000
D-D	-0.1494	1	0.0408	-0.2626	-0.0362	1.0000
E-E	0.0294	1	0.0408	-0.0838	0.1426	1.0000
AB	-0.4381	1	0.0408	-0.5513	-0.3249	1.0000
AC	0.1456	1	0.0408	0.0324	0.2588	1.0000
AE	-0.2056	1	0.0408	-0.3188	-0.0924	1.0000
BD	-0.1319	1	0.0408	-0.2451	-0.0187	1.0000
CD	0.1019	1	0.0408	-0.0113	0.2151	1.0000
CE	-0.1569	1	0.0408	-0.2701	-0.0437	1.0000

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multi-colinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

Supplementary data S2

Normality test: For central composite design

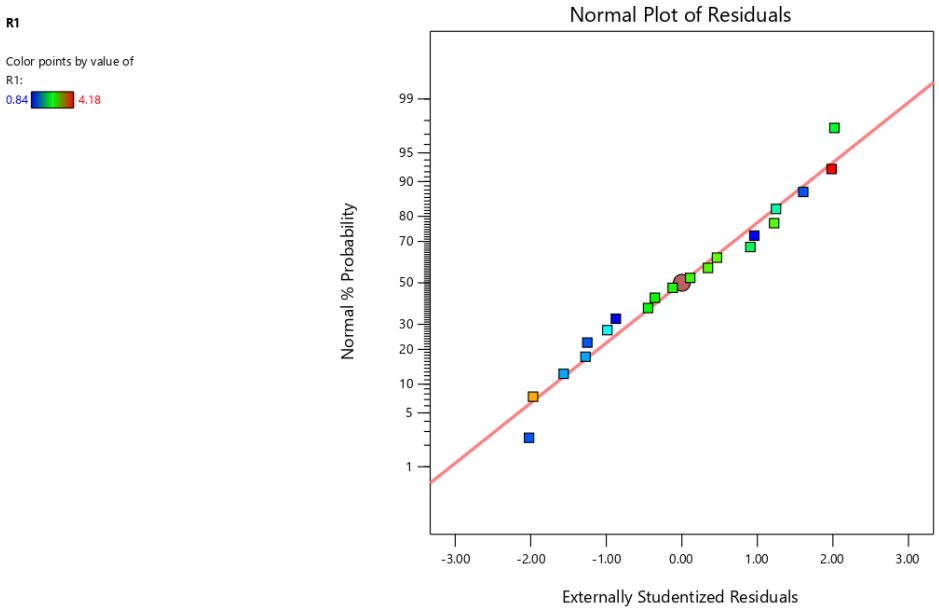


Figure S2.1 Normality test for central composite design

As shown in the figure above, normality was evaluated using the Normal Plot of Residuals in Design-Expert. The points distributed close to the straight reference line indicate that the residuals were approximately normally distributed.

Residual analysis: All observations remain within the red control limits (±4.14579), indicating that no severe outliers or highly influential points are present.

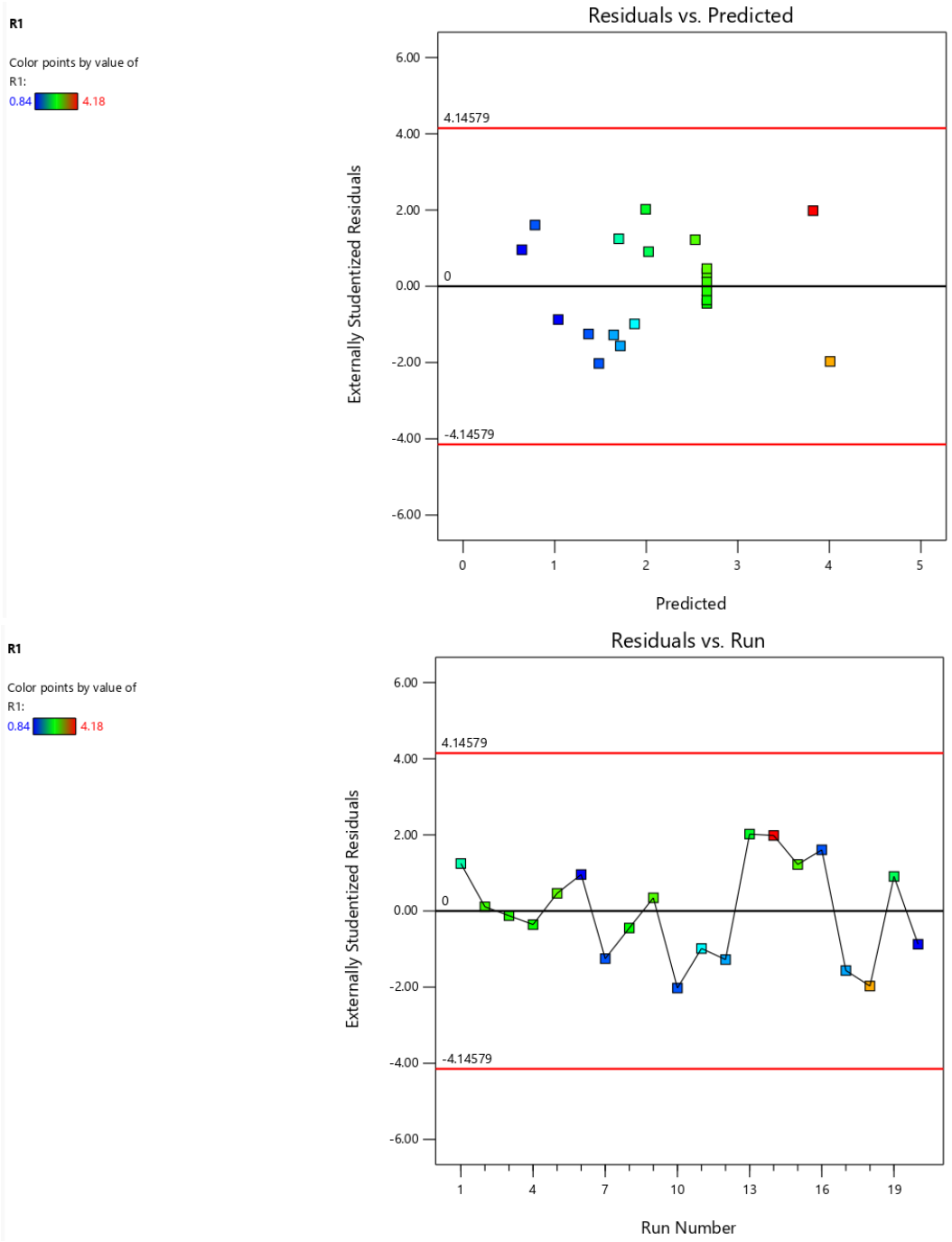


Figure S2.2 Residual analysis for central composite design

Model validation: As described in **Table 6**, the p-value of the model is less than 0.05 (< 0.0001), indicating that the model explains a meaningful portion of the variation in the response.

Lack-of-fit test: As described in **Table 6**, The p-value of lack-of-fit was greater than 0.05 (0.9412), indicating that the lack-of-fit was not significant and that the model adequately fitted the experimental data.

Confidence intervals: As described in table below

Table S2.1 Coefficients in Terms of Coded Factors for central composite design

Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	2.66	1	0.1458	2.34	2.99	
A-A	-0.5989	1	0.0967	-0.8144	-0.3834	1.0000
B-B	0.2771	1	0.0967	0.0616	0.4926	1.0000
C-C	-0.2935	1	0.0967	-0.5090	-0.0780	1.0000
AB	-0.4188	1	0.1264	-0.7003	-0.1372	1.0000
AC	0.0712	1	0.1264	-0.2103	0.3528	1.0000
BC	-0.2788	1	0.1264	-0.5603	0.0028	1.0000
A^2	0.1201	1	0.0942	-0.0897	0.3299	1.02
B^2	-0.4986	1	0.0942	-0.7084	-0.2888	1.02
C^2	-0.3996	1	0.0942	-0.6094	-0.1898	1.02

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multi-colinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.