Cultivated Meat: Investigating Media Dilution and Yeast Protein Supplementation for Cost-effective Culture Media

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Abstract

Rising environmental and ethical concerns associated with conventional livestock production have driven interest in cultivated meat (CM). CM involves growing animal cells under controlled conditions to produce meat without slaughtering animals. However, expensive cell culture media remains a major barrier to economically viable production. While research has largely focused on serum alternatives, free amino acids contribute significantly to media costs. This study investigated whether DMEM, a common nutrient-rich culture medium, could be diluted while maintaining the growth of C2C12 myoblast cells.

Cells were cultured in serum-containing media incrementally diluted with buffer (PBS) between 0-100% (v/v) while glucose and osmolality were controlled. 50% DMEM supported greater short-term cell proliferation than conventional 100% DMEM (1000 cells.cm^{-2}, n=1). However, beyond 48 hours, 100% DMEM showed higher proliferation. This suggests that DMEM nutrients, particularly amino acids, vitamins, and trace minerals, may become limiting with increased cell growth. Additionally, cells grown in 50% DMEM showed a more elongated morphology. Upon repeating the experiment with passage on day 2, no significant difference in cell proliferation was observed between 50% DMEM and 100% DMEM over 4 days (2000 cells.cm^{-2}, n=4 biological replicates). Hence, DMEM can be diluted by up to 50% without compromising cell growth, reducing media costs substantially.

Yeast lysate and hydrolysate were also investigated as DMEM replacements due to their high protein content and the established large-scale production of yeast. Both formulations resulted in cell death at 50% and 100% v/v replacement. This may be due to the release of harmful cellular components during homogenisation or the hydrolysis conditions. Future work should therefore investigate the composition of yeast extracts and evaluate their cytotoxicity. Additionally, analysis of spent-media metabolites, morphological changes, and cellular stress could further clarify the observed effects. Overall, this study demonstrates the potential of DMEM dilution for developing cost-effective culture media.

1. Introduction

1.1 The need for food innovation

Meat has a much larger climate footprint than plant-based foods and its consumption has been associated with negative health effects [1]. However, meat is an essential and affordable source of nutrition for many low-income communities [2]. Although meat consumption is decreasing in developed countries, its global consumption is increasing, particularly in developing countries such as in China, India, and Russia [3]. Moreover, according to the Food and Agriculture Organisation, the global human population is projected to grow from 7.3 billion to over 9 billion by 2050, requiring an estimated 70% increase in food production [4]. Meeting this demand with current meat consumption trends is challenging due to limited natural resources and arable land.

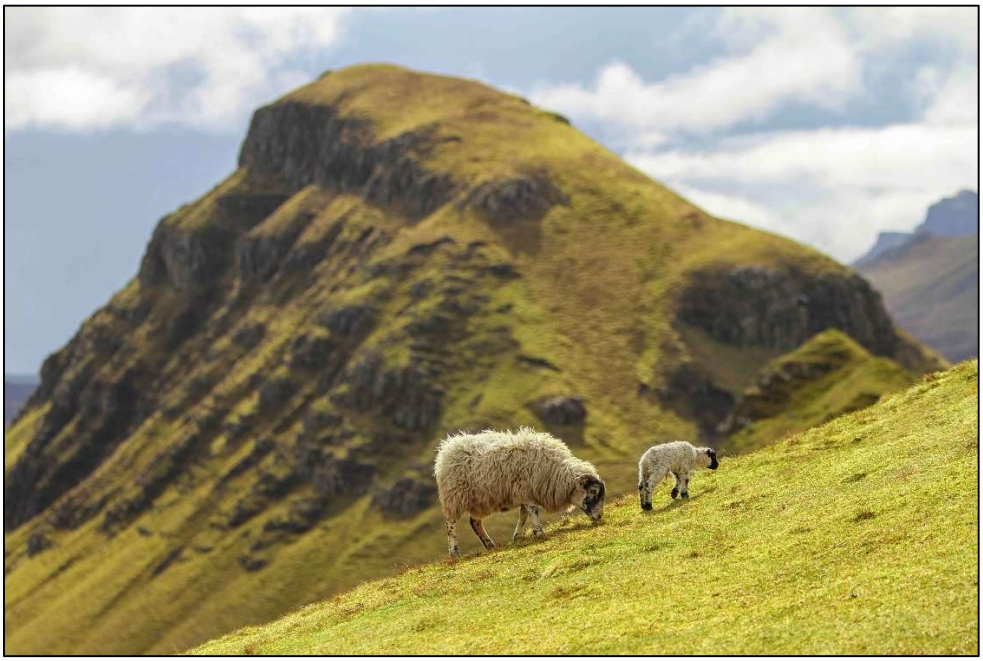


Figure 1: Images showing cattle rearing on a mountain ledge and cattle being fed on a farm. Conventional cattle agriculture carries a much larger climate footprint than plant-based foods and cultivated meat (produced using renewable energy). It also involves the slaughter of animals and uses a lot of arable land. Note: This is a copyright-free Microsoft stock image.

Cultivated meat (CM) has emerged as a promising solution. CM refers to meat produced *in-vitro* using cellular agriculture techniques [5]. The process involves isolating muscle stem cells from an animal, expanding them in nutrient-rich culture media, and differentiating them into muscle tissue on scaffolds that resemble conventional meat [6]. Recently, CM has attracted a lot of scientific and commercial interest because it improves animal welfare, increases food security, and preliminary Life Cycle Assessments (LCAs) have shown that CM is more sustainable than conventional cattle farming "when produced using renewable energy" [7].

1.2 A brief history

In 2001, NASA became interested in the possibility of CM as food for space missions. Borris Benjaminson with a $62,000 grant from NASA managed to grow a small fish steak but was not allowed to taste it due to lack of FDA clearance [8]. The project was later discarded. One of the earliest displays of CM was in 2003 by Oron Catts and Ionat Zurr in their art project "Disembodied Cuisine" as part of the L'Art Biotech exhibition in France [9]. They grew frog muscle biopsies on biopolymer scaffolds and served them to guests, while the living healthy frogs from whom the biopsies had been taken sat in an aquarium on the same table, thus displaying the concept of "victimless meat" [10].

Ten years later in 2013, Mark Post created the world's first *in vitro* cultivated burger with private funding from Google co-founder Sergey Brin. He unveiled the 300,000-euro edible burger at a globally broadcasted media event in London. Although foetal calf serum was used to grow the cells, Post's work proved that the production of cultivated meat is scientifically possible [11]. Post went on to found the company Mosa Meat. This was followed by the formation of non-profit organisations and start-ups in the CM space, including the Good Food Institute, Memphis Meats (now UPSIDE Foods) and Modern Meadow. Since then, CM has shifted from a university-based first wave into a startup-led second wave [12]. Significant cost cuts have been achieved, and hundreds of research articles have been published.

1.3 Current Limitations

The Good Food Institute conducted an LCA and techno-economic analysis (TEA) on a future large-scale CM production facility using industrial data and showed that by 2030, CM could reduce overall environmental impacts, achieve a lower carbon footprint, and be cost-competitive with some conventional meats [13]. However, other studies take a more sceptical viewpoint regarding the future of CM and highlight multiple challenges such as the need for optimisation of cell culture methodology, risk of dysregulation, and lack of control over nutritional composition of product [14]. Although CM will decrease land-use, it may increase energy consumption because some biological functions will be replaced by technological processes [15]. Additionally, CM will face multiple consumer acceptance barriers due to public neophobia and technophobia [16].

Particularly, the lack of a universal and cheap serum-free media is widely considered as a critical factor in improving cost-effectiveness, scalability, and sustainability of CM [17] [18]. Estimates suggest that media costs account for over 55%–90% of overall CM production costs [19] [20]. Traditional media formulations, such as Dulbecco's Modified Eagle Medium (DMEM) and Ham's F-12, are commonly supplemented with 2–20% foetal bovine serum (FBS) to provide essential growth factors, hormones, amino acids, vitamins, and lipids required for cell proliferation and differentiation. Many serum-free media formulations such as Beefy-9, Beefy-R, and Tri-basal 2.0+ replace animal-derived

serum with recombinant or plant-based alternatives, thus reducing ethical concerns and contamination risks [21]. Additionally, recent advancements in Machine Learning have accelerated culture media optimisation and helped address challenges of limited data availability [22].

Protein hydrolysates have long been explored as serum replacements because they can provide complex mixtures of peptides and amino acids that support cell growth [23]. However, TEAs indicate that solely replacing FBS is insufficient to reduce cultivated meat production costs to commercially viable levels at large scale (>20 m^3). While FBS replacement remains essential, replacing free amino acids in growth media is comparatively unexplored despite its potential economic benefit [24]. Currently, the high cost of amino acids is attributable to their low production scale and the requirement for pharmaceutical-grade purity [25].

1.4 Research Gap and Aim

Yeast and bacterial extracts are particularly attractive because of their high protein content, rapid biomass generation and established industrial-scale production. However, limitations in amino acid availability, compositional variability, and potential contamination with nucleic acids or toxins remain important challenges [26]. Additionally, Dolgin recently showed that lysate from the marine Gram-negative bacterium *Vibrio natriegens* can be used to supplement basal B8 medium at 40 µg/mL, successfully supporting serum-free growth of immortalised bovine satellite cells (iBSCs) and mackerel (Mack1) cells. [27] Additionally, protein hydrolysates can enhance muscle cell proliferation and reduce serum dependency under low- or serum-free conditions, with plant-derived hydrolysates promoting proliferation and differentiation through pathways including PI3K/Akt/mTOR and ERK/AMPK/PGC-1α [28].

Currently, there is limited understanding of whether yeast lysates/hydrolysates can replace the amino-acid component of conventional media while maintaining cell proliferation. This study first investigates the extent to which conventional DMEM can be diluted while maintaining C2C12 myoblast growth and proliferation using the limiting factor method [18]. C2C12 myoblasts were chosen as a model for mammalian muscle cell lines. It then evaluates the potential of Saccharomyces cerevisiae-derived lysate and hydrolysate to replace the amino acid component of the basal medium and identifies the maximum replacement level that supports cell proliferation.

2. Yeast Lysate and Hydrolysate

2.1 Yeast Lysate and Hydrolysate Preparation

Figure 1 depicts the process flow sheet for yeast lysate and hydrolysate preparation. In brief, 5 grams of dry *Saccharomyces cerevisiae* was dissolved in 500 mL of deionised water and stirred. The yeast solution was homogenised at 400 bars for 4 cycles (SPX Flow - APV Laboratory Homogeniser) to break open the yeast cell walls and release host cell proteins into the solution.

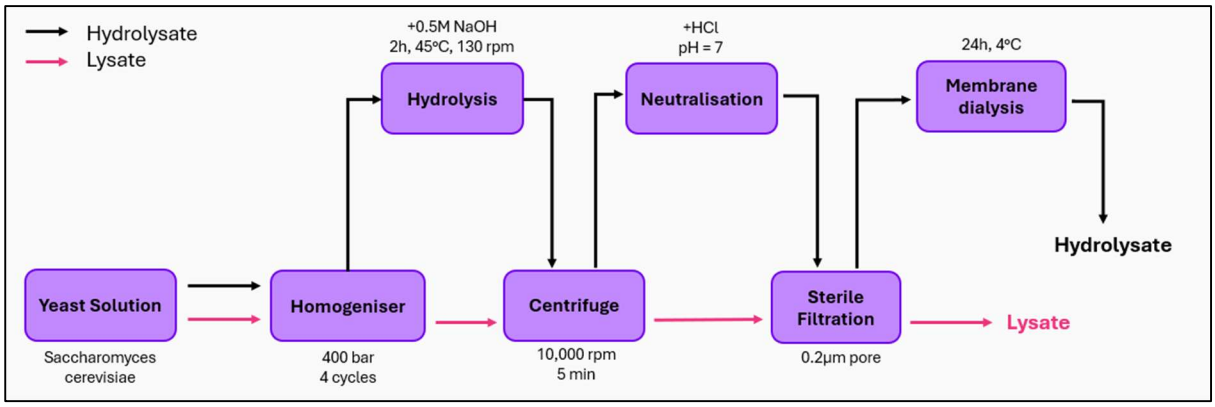


Figure 2: Saccharomyces cerevisiae lysate and hydrolysate production process flow sheet. Pink arrows show lysate and black arrows show hydrolysate.

For lysate production (shown in pink in Figure 2) the solution was centrifuged at 10,000 rpm for 5 minutes to remove impurities and insoluble proteins. The supernatant was filtered using a 0.2 µm pore sterile filter (Thermo Scientific Nalgene Syringe Filter 0.2 µm SFCA CAT: 723-2520) and a 10 mL plastic syringe in a Class II Biological Safety Cabinet to produce the yeast lysate solution.

For hydrolysate production (shown in black in Figure 2), dry Sodium Hydroxide (NaOH) pellets were added to the homogenised yeast solution until a 0.5 M NaOH solution was produced (measured using a pH probe). This was stirred for 2 hours at 45°C and 130 rpm. This step aims to hydrolyse the peptide bonds yielding smaller proteins which are easier for mammalian cells to consume. The solution was then centrifuged at 10,000 rpm for 5 minutes to remove impurities and insoluble proteins. The supernatant was neutralised by slowly pipetting Hydrochloric acid while measuring continuously using a pH probe until the solution had a pH of 7. The solution was filtered using a 0.2 µm pore sterile filter (Thermo Scientific Nalgene Syringe Filter 0.2 µm SFCA CAT: 723-2520) and a 10 mL plastic syringe in a Class II Biological Safety Cabinet. Finally, membrane dialysis was carried out for 24 hours at 4°C using deionised water (with no stirring) in Spectra/Por Dialysis Membrane (PART: 131060T; 20 mm diameter) to reduce the salt concentration and to yield the yeast lysate solution.

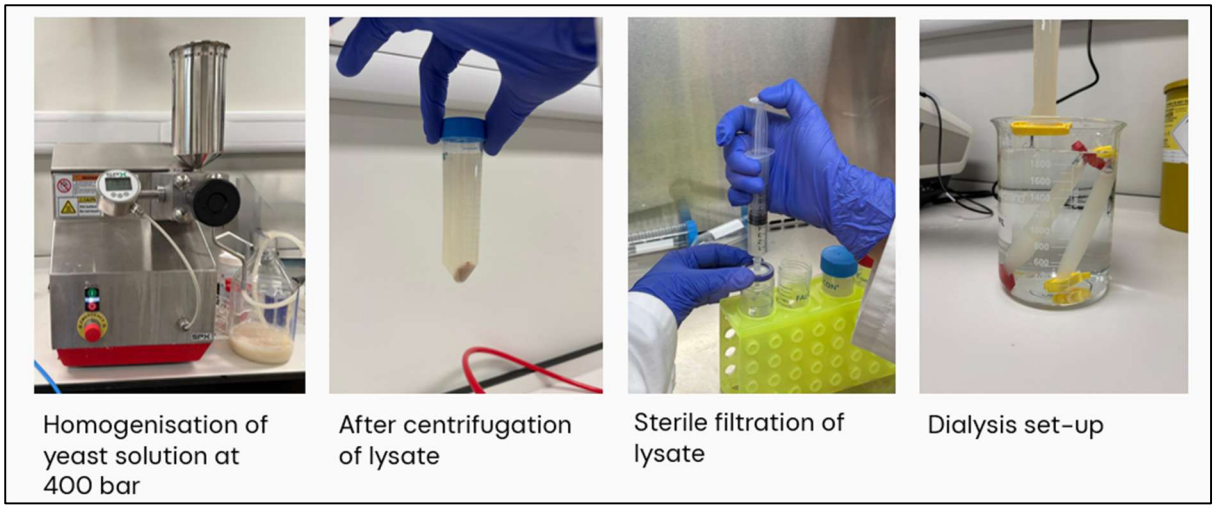


Figure 3: Images from Yeast lysate/hydrolysate preparation process

2.2 Quality Testing

A sterility control was run for both the lysate and hydrolysate by incubating 1 mL of sample in complete media (90% DMEM, 10% FBS) for 2 days and observing it for signs of bacterial/yeast contamination under the microscope (EVOS M5000) at 4x and 40x.

In addition, the amount of protein loss during filtration was quantified for both lysate and hydrolysate using CalBiochem Non-Interfering Protein Assay Kit (CAT: 488250; Sigma Aldrich). The Non-Interfering (NI) Protein Assay is a highly sensitive colorimetric assay that overcomes interference from common laboratory agents like detergents, reducing agents, chelating agents, amines, sugars, and is highly tolerant of chaotropic buffers. The Bradford Assay was not used because the Bradford Coomassie brilliant blue G-250 binds chiefly to arginine and only gives a slight response to basic and aromatic residues [29].

A standard curve was created using BSA standards (Pierce Ref: A55866) and following manufacturer's instructions for the NI Protein Assay (n=2). Absorbance readings at 480 nm were used to interpolate protein concentrations of samples (n=2). Osmolality of the samples was also noted using a Semi-Micro Osmometer (K-7400S, KNAUER).

Results

Sterility controls for both samples were negative; hence, they were safe to use for mammalian cell culture. Protein loss due to sterile filtration was quantified using the Non-Interfering Assay. A standard curve was created using 0-4 mg/mL BSA standards shown in Appendix C (r^2=0.91) (n=2). The linear line of best fit was used to interpolate the protein concentrations before and after filtration of lysate and hydrolysate (Figure 3).

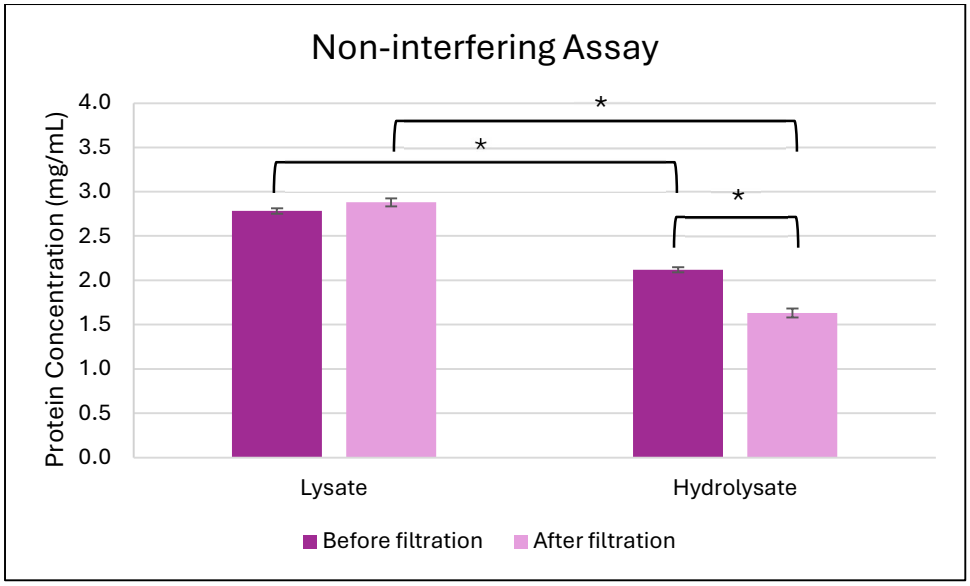


Figure 4: Effect of filtration on protein loss quantified using Non-Interfering Assay (n=2). There was no significant change in the protein concentration of the lysate due to filtration p=0.062 (left); however, protein concentration decreased significantly in the hydrolysate sample post filtration p=0.019 (right). Additionally, the protein concentration of the lysate was significantly higher than hydrolysate before filtration p=0.04 (blue bars).

Significance was established using a t-test with a 95% confidence interval. The protein concentration of hydrolysate was significantly lower than lysate before and after filtration (Figure 4). This is due to dialysis, wherein excess water seeps into the sample through the dialysis membrane, thus diluting the sample.

The combined concentration of all amino acids in DMEM is known to be 0.99 mg/mL [30]. Since the samples were used to replace DMEM, they should have ideally been diluted to ~1 mg/mL. However, this was not done in the experiments and remains a limitation of the experimental design. Additionally, the experiment assumes that BSA is an accurate representative of both lysed and hydrolysed proteins. Pipetting errors may also attribute to inaccuracies in measurement.

There was also a significant loss of protein during filtration of the hydrolysate, whereas lysate protein concentration remained the same during filtration. This may be due to alkaline hydrolysis which converts intact proteins into smaller peptides which often have greater mobility and more exposed hydrophobic residues, increasing nonspecific adsorption of the hydrolysate more than the lysate. If a significant fraction binds to the membrane, the filtrate protein concentration decreases [31]. Alternatively, at the extremely high pH of alkaline hydrolysis, it is possible that protein solubility decreased and proteins unfolded, exposing hydrophobic amino acids. Additionally, chemical modifications like deamidation, lysinoalanine formation, destruction of serine or cysteine, and oxidation may have occurred. This may have led to aggregate formation which would have been retained by the 0.2 µm filter [32].

On the other hand, the slight increase in protein concentration of the lysate before and after filtration can be explained by some water loss in the filters which may have concentrated the soluble proteins in the sample.

3. Cell Line

3.1 Cell Revival

For all experiments in this study, adherent C2C12 mouse muscle myoblasts (UNSPSC Code: 41106514) were used from Sigma Aldrich. For revival, cell vials were removed from liquid nitrogen by trained staff and stored at -20°C in a Mr. Frosty container while the Liquid Nitrogen container was safely closed. The vial was then swirled in a water bath at 37°C until small crystals were observed. All vial contents (premeasured at 1 mL during banking) were transferred to 9 mL prewarmed complete media (10% FBS and 90% DMEM) a 15 mL tube to neutralise the DMSO in the vial. The tube was centrifuged for 5 minutes at 400 G. The supernatant was discarded and the cell pellet was resuspended in 1 mL of complete media. 100 µL of the sample was used to measure cell counts using a Neucleocounter. Cells were seeded into a T-175 flasks with 35 mL complete media (Passage 27).

Figure 5: Removal of cell vial from Liquid Nitrogen for revival

3.2 Population Doubling Characterisation

The doubling time and PDL (Population Doubling Level) of the C2C12 myoblast cell line between Passage 27-32 was characterised by culturing cells in 35 mL of complete media in T-75 flasks and passaging when they reached ~70% confluency. Cell counts were carried out using an automated Neucleocounter.

Results

An average population doubling time of 15-20 hours was calculated for complete media. The population doubled an estimated 2.5-3 times over 48 hours.

4. Media Optimisation Experiments

4.1 Understanding the excess of amino acids in DMEM

Rationale

DMEM is a universal culture media used to grow various cell lines with extremely different glucose, amino acid, and mineral requirements. Hence, this experiment aimed to identify the excess of amino acids and minerals in DMEM specifically for the growth of C2C12 myoblasts at 1000 cells cm^{-2}. Media was replaced with PBS (to maintain osmolality) and glucose was readjusted to 4.5 g/L.

Media Preparation

5 experimental conditions were tested as shown in Figure 6 with exact media formulations in Appendix B. Media was prepared in a biosafety cabinet under aseptic conditions.

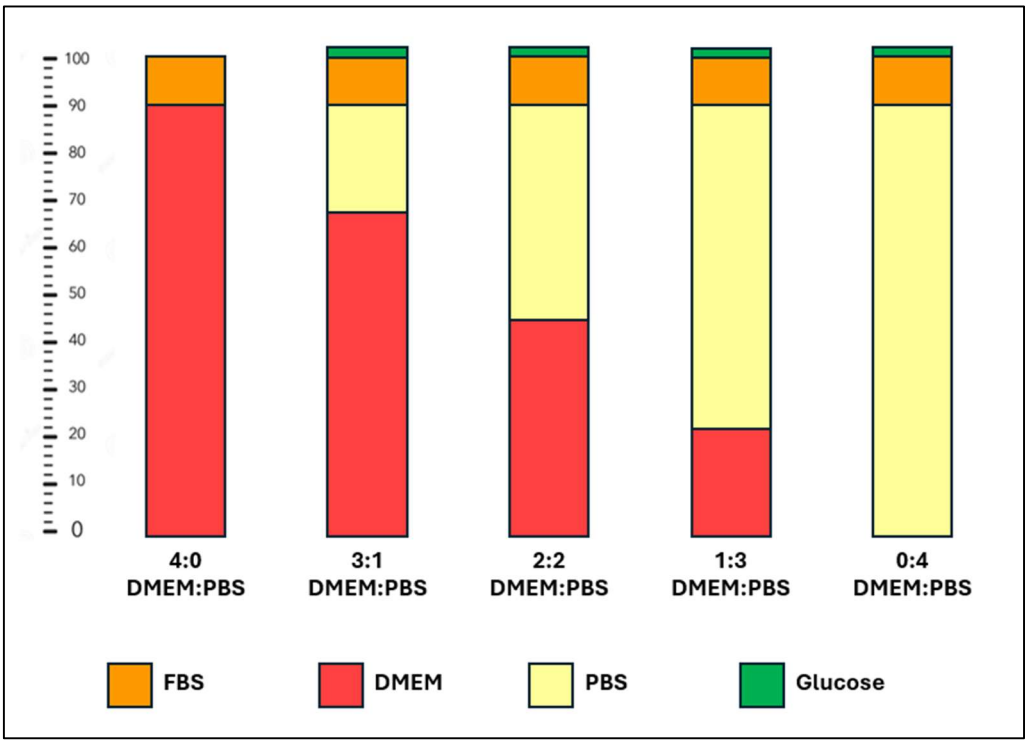


Figure 6: Media Preparation Visual (Glucose not to scale). Each condition had 10% FBS. The remaining 90% contained varying ratios of DMEM and PBS as shown in the figure. Glucose was adjusted in each condition such that the overall glucose concentration was 4 gL^{-1} (same as 90% DMEM and 10% FBS).

Cell Culture

The experiment was carried out in T-25 flasks over 4 days (n=1). 3 mL of prepared media was pipetted into each flask and labelled. These flasks with media were placed in the incubator to prewarm. Each flask was seeded with 1000 cells/cm^2 (Passage 34 on Day 0).

Destructive cell counts were carried out on day 2 and day 4 using the procedure described below. The flasks were removed from the incubator and observing under the microscope for cell growth and signs of contamination (cloudiness/colour change). Images were captured at 10x and visual estimates of confluency were noted. In the biosafety cabinet, spent media was discarded in Virkon solution (1:1 ratio). Prewarmed dPBS (wash) was added to each flask and subsequently discarded to remove dead cells. Next, prewarmed trypsin/EDTA solution was added to each well and incubated for 5 minutes. The plates were observed briefly under the microscope to check for cell detachment and tapped to ensure that cells detach completely. Prewarmed quenching medium (complete medium) was added to deactivate the trypsin and the solution was transferred to an Eppendorf tube and centrifuge at 400 G for 5 minutes. The supernatant was discarded and the pellet was resuspended in 100 µL of prewarmed complete media. Cell counts were carried out using an automated Nucleocounter and verified using a haemocytometer by mixing sample with Trypan blue in a 1:1 ratio.

Results

Cells grown in 0% DMEM were not viable on Day 2 and Day 4, demonstrating that the serum-containing medium alone was insufficient to support C2C12 survival. This may reflect the absence of essential nutrients provided by DMEM, including glucose, amino acids and vitamins.

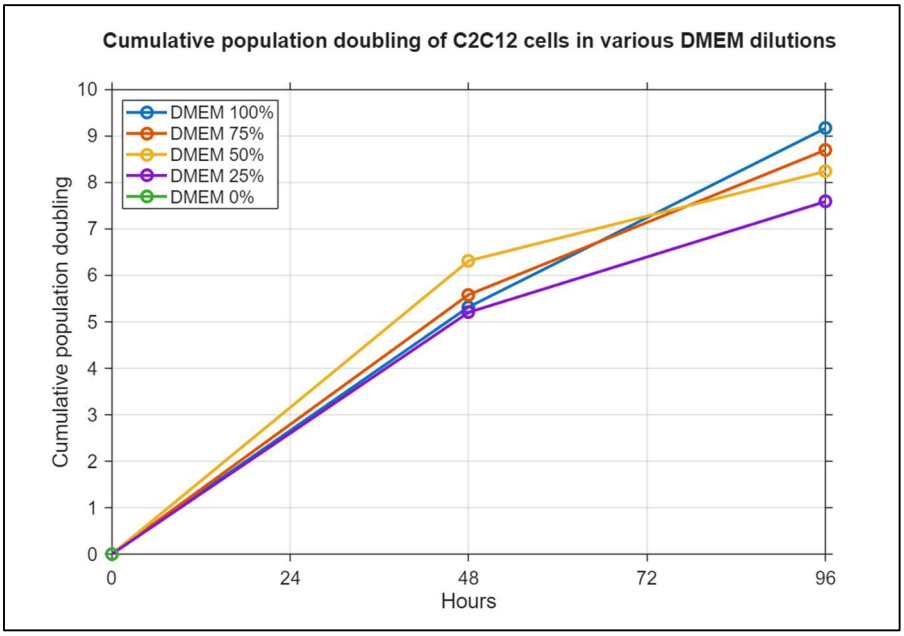


Figure 7: Cumulative population doubling of C2C12 in various DMEM dilution with PBS: 100% DMEM (blue), 75% DMEM (orange), 50% DMEM (yellow), 25% DMEM (purple), 0% DMEM (green).

In the first 48 hours, cells grown in 25-75% diluted medium showed a higher growth rate than those grown in 100% DMEM, with cells doubling a maximum of 6.3 times in 50% dilution but only 5.32 times in 100% dilution over 48 hours.

Reinhart et al. have shown that in CHO cells, high amino acid concentrations alone were not sufficient to ensure superior cell growth [33]. Metabolic flux analysis indicate that it is often specific amino acids that are lacking while others are in excess [34]. Unbalanced glucose and amino acid concentrations are known to lead to high cell-specific lactate and ammonium production rates. In some media, persistently high glucose concentrations may induce the suppression of respiration and oxidative phosphorylation, known as Crabtree effect, which results in high cell-specific glycolysis rates along with continuous and high lactate production [33].

Hence, the initial surge in 50% and 75% DMEM cultures may reflect lower nutrient concentrations temporarily reducing inhibitory by-products such as lactate and ammonia, or an altered balance of growth-regulatory components. This hypothesis requires confirmation via metabolic flux analysis and spent-media quantification. Since amino acids and other nutrients also contribute to osmolality, the lower osmolality of 50% diluted media may better match the biological osmolality cells typically experience in tissue fluid; osmolality was controlled in later experiments.

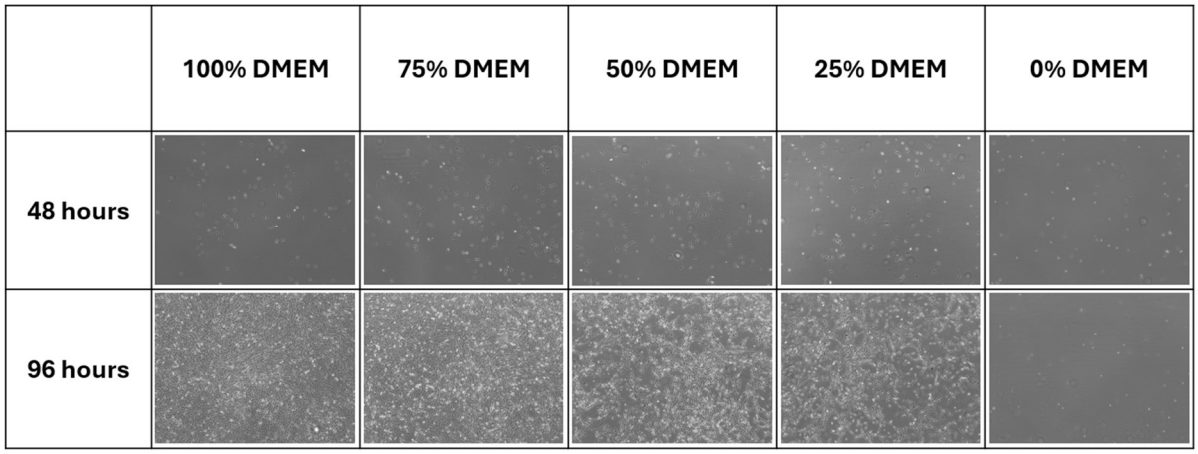


Figure 8: Images from DMEM dilution experiment at Day 2 and Day 4 at x10 magnification.

Beyond proliferation, nutrient limitation may have induced cellular stress and promoted differentiation, as suggested by the more elongated morphology and larger cell diameter in the 25% DMEM condition. The extent of this contribution and any resulting cytotoxic effects remain unclear and warrant further investigation.

After 96 hours, the hypothesised trend emerged, with 100% DMEM the most confluent, followed by 75%, 50%, and 25% DMEM, suggesting that amino acids and minerals in diluted media become rate-limiting long-term. Diluted media may therefore suit short-term, high-proliferation cultures or applications harvested within 48 hours; 50–75% dilutions with PBS and glucose adjustment could reduce medium requirements while sustaining short-term proliferation, potentially benefiting continuous or perfusion cultures requiring frequent replacement.

Limitations

However, the apparent 6.3 doublings in 48 hours in the control group is higher than expected so cell-counting variability, differences in cell attachment, or sampling error may be causing variability. Dilution-induced stress may also contribute to shorter doubling times. The experiment was conducted in single replication only.

4.2 The effect of glucose and salt in DMEM

Rationale

To investigate the role of inorganic salts in cell proliferation, 50% of the DMEM was replaced with either water or PBS. Mannitol was used as an osmotically active but inert substitute, whereas PBS provided physiological concentrations of Na^+, Cl^-, phosphates, and other inorganic ions. Additionally, 3 glucose concentrations (2 gL^{-1}, 3 gL^{-1}, and 4 gL^{-1}) were tested for each dilution to discern the minimum amount of carbon source that is needed to maintain cell growth.

Media Preparation

Seven experimental conditions were tested as shown in Figure 9 with exact media preparation volumes in Appendix A. Media was prepared, osmolality was adjusted to 250-300 mOsm/Kg using Mannitol, and the tubes were vortexed followed by filtration using 0.2 µm pore filter in sterile conditions. Passage 32 cells were used.

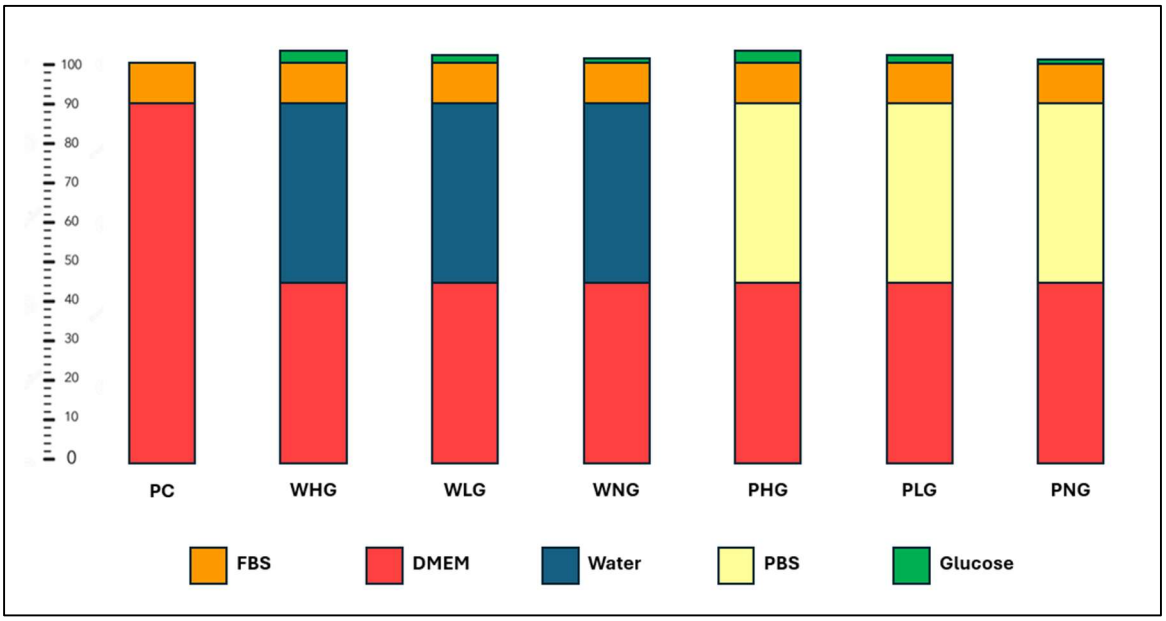


Figure 9: Media preparation visual (Glucose is not to scale). Each condition had 10% FBS. PC (Positive Control) contained 90% DMEM. Osmolality was measured for all conditions with water and adjusted to that of PC using Mannitol. WHG (Water High Glucose) contained 45% DMEM, 45% water and glucose to adjust the overall concentration to 4 gL^{-1}. WLG (Water Low Glucose) contained 45% DMEM, 45% water, and an overall glucose concentration of 3 gL^{-1}. WNG (Water No Glucose) contained 45% DMEM and 45% water. PHG (PBS High Glucose) contained 45% DMEM, 45% PBS, 4 gL^{-1} glucose. PLG (PBS Low Glucose) contained 45% DMEM, 45% PBS, 3 gL^{-1} glucose. PNG (PBS No glucose) contained 45% DMEM, 45% Glucose.

Cell Culture

The experiment was carried out in 12-well plates (surface area = 35 cm^2, working volume = 1 mL per well) over 3 days (n=3). Each well was filled with 1 mL of media from chosen condition and was seeded with 2000 cells/cm^2. This was an observational study only. No cell counts were carried out.

Results

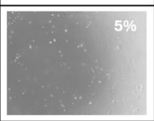
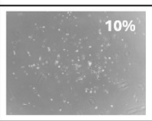
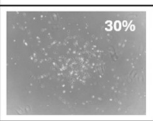
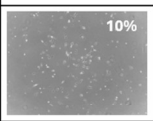
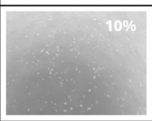
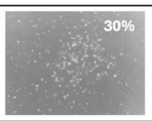
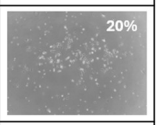
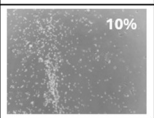
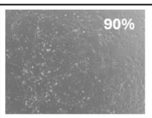
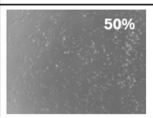
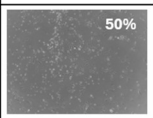
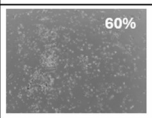
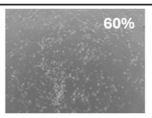
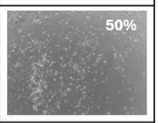
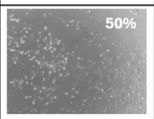
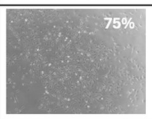
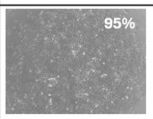
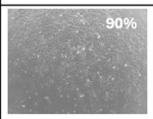
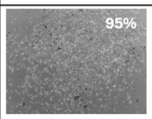
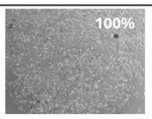
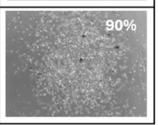
	Positive Control	50% Water	50% Water	50% Water	50% PBS	50% PBS	50% PBS
Glucose	4 gL^{-1}	4 gL^{-1}	3 gL^{-1}	2 gL^{-1}	4 gL^{-1}	3 gL^{-1}	2 gL^{-1}
Day 1							
Day 2							
Day 3							

Figure 10: Images captured at 10x for each experimental condition over 3 days.

All the dilutions performed better than the positive control (90% DMEM, 10% FBS). Additionally, higher glucose (3 and 4 gL^{-1}) was favoured by C2C12 myoblasts and showed higher proliferations. There was no significant difference between dilution with water + mannitol and PBS, suggesting that it is the maintenance of osmolality rather than the presence of specific ions from PBS which influences cell growth.

Limitations

Since this experiment was performed in well plates, accurate cell counts could not be collected. Additionally, the experiment only had a single replicate.

4.3 Amino acid replacement with Yeast lysate and hydrolysate

Rationale

Previous experiments have shown that when glucose and salt concentrations are adjusted, 50% DMEM can be replaced with water. However, the experiment was conducted with a seeding density of only 1000 cells cm^{-2} and medicine-grade amino acids still present a significant cost of the total cost of media despite a 50% dilution. Hence, this experiment aimed to test cheaper yeast derived lysate and hydrolysate as replacements for the remaining 50% amino acid component of DMEM.

Media preparation

1. 100% DMEM
2. 50% DMEM (with glucose)
3. 50% DMEM (without glucose)
4. 100% Yeast hydrolysate (with glucose)
5. 50% Yeast hydrolysate (with glucose)
6. 100% Yeast Lysate (with glucose)
7. 50% Yeast Lysate (with glucose)

Results

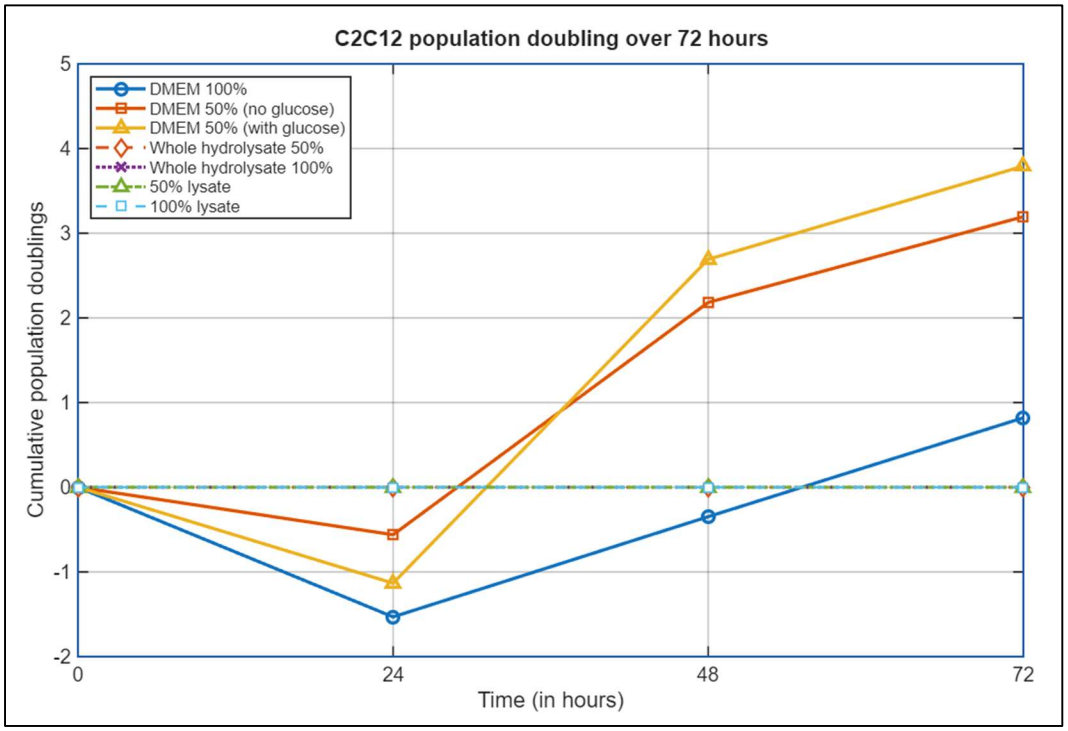


Figure 11: Cumulative C2C12 population doublings in various media dilutions: Complete Media (100% DMEM), 50% DMEM with and without glucose, 100% and 50% replacement with yeast lysate, 100% and 50% replacement with yeast hydrolysate.

The graph shows a typical lag phase in the first 24 hours for all media types wherein cells adjust to new media/ temperature changes. This is followed by a rise in cumulative population doublings between 24-72 hours. 50% DMEM consistently performed better than 100% DMEM (complete medium).

Yeast lysate and hydrolysate resulted in complete cell death. Hence, in their present form, they are unsuitable for replacing the amino acid component of DMEM. The cell death may have been caused by the release of harmful cellular components during homogenisation or the hydrolysis conditions. Future work should therefore investigate the composition of yeast extracts and evaluate their cytotoxicity. The use of Tangential Flow Filtration should also be considered to reduce the size of protein molecules, thus enabling easier uptake by cells.

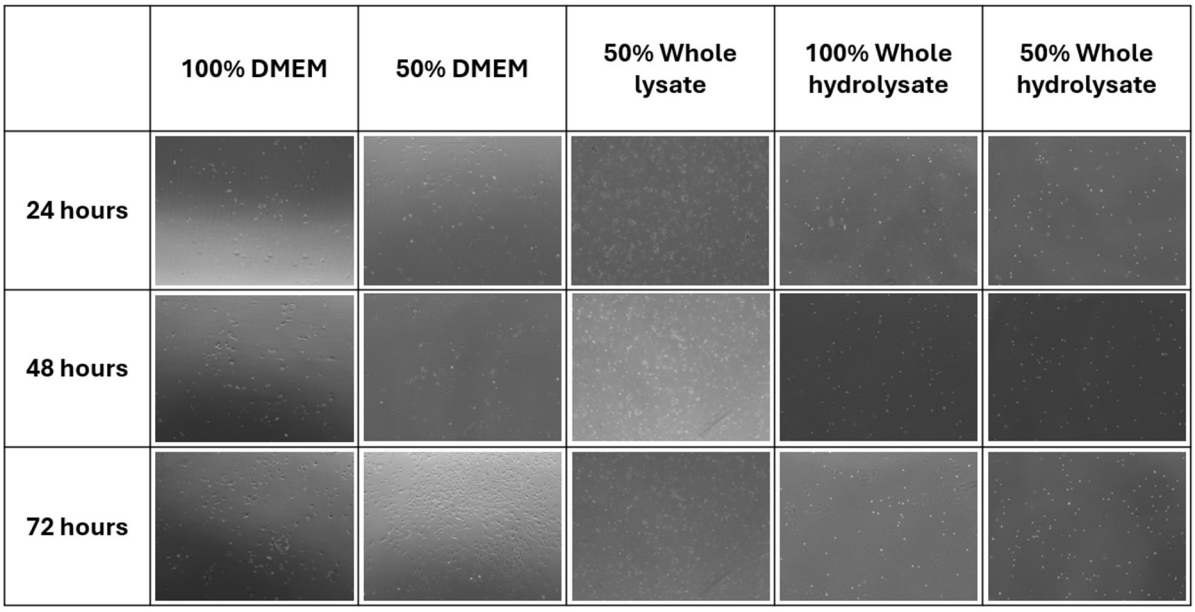


Figure 12: Microscope images captured at 10x for various DMEM dilutions with whole lysate and hydrolysate over 3 days.

4.4 Effect of Passage number on cell growth

Rationale

This experiment was conducted to understand whether cells become senescent and alter growth patterns after Passage 30, as is often indicated in literature and manufacturer's guidelines.

Figure 13: Image depicting media preparation in biosafety cabinet

Media Preparation

3 experimental conditions were tested for cells from Passage 28 and Passage 31. PBS was used for media dilution and glucose was adjusted.

1. 100% DMEM
2. 50% DMEM
3. 25% DMEM

Cell Culture

Cells were seeded at 2000 cells cm^{-2} in T-25 flasks (n=2). They were passaged and counted at 48 hours, and destructive cell counts were carried out at 96 hours.

Results

The cumulative population doubling profiles showed substantial proliferation of C2C12 cells in all three media conditions for both passages. At 48 h, P28 exhibited slightly higher cumulative population doublings than P31 in 100% DMEM (2.99 ± 0.20 vs. 2.51 ± 0.36)

and similar values in 25% DMEM (2.67 ± 0.26 vs. 2.69 ± 0.44) and 50% DMEM (2.58 ± 0.14 vs. 2.57 ± 0.27). Following reseeding at 50,000 cells on Day 2, cumulative population doublings increased substantially by 96 h in all conditions. P28 showed the highest final doubling in 25% DMEM (5.71 ± 0.08), compared with 4.79 ± 0.27 and 4.79 ± 0.11 in 100% and 50% DMEM, respectively. In P31, 100% DMEM produced the lowest final cumulative doubling (5.14 ± 0.23), while 25% and 50% DMEM produced higher values of 5.56 ± 0.35 and 5.41 ± 0.17, respectively.

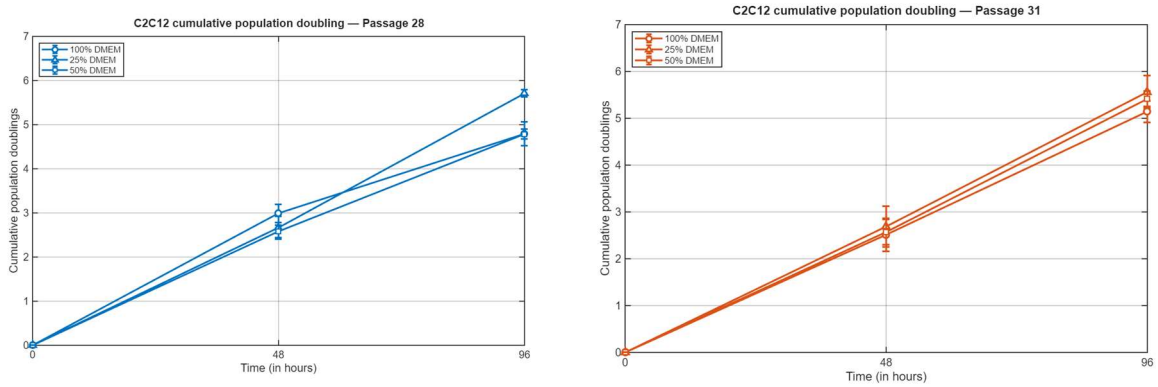


Figure 14: Comparison of Passage 28 vs Passage 31 cell growth over 96 hours in 25%, 50%, and 100% DMEM. Complete results in Appendix D.

Welch's two-sample t-tests found no statistically significant differences between P28 and P31 at either 48 or 96 h for any medium ($p > 0.05$). The closest comparison to significance was observed for 50% DMEM at 96 h ($p = 0.0606$), where P31 showed greater cumulative population doubling than P28. Overall, the graphs indicate that passage number had relatively little effect on early growth, while differences between passages became more apparent after the second growth phase, particularly in 50% DMEM.

5. Limitations and Future work

Although glucose and osmolality were controlled, PBS dilution also lowers all other DMEM components, including amino acids, vitamins, and trace minerals. It therefore remains unclear whether the proliferative advantage arises from reduced nutrient competition, altered signalling from a specific diluted component, or another unmeasured factor.

The elongated morphology observed was also not quantified or linked to a mechanism. Cytoskeletal remodelling in myoblasts is sensitive to the extracellular environment, with C2C12 cells showing high actomyosin contractility that drives elongation and mechanical anisotropy under altered adhesive conditions [35]. Immunofluorescence staining of actin and vimentin, alongside quantitative shape analysis, would clarify whether this reflects adaptive remodelling or early cellular stress.

Finally, although viability remained high across all dilutions by trypan blue exclusion/NucleoCounter, this assay cannot capture subtler indicators such as early apoptosis, senescence, or altered cell cycle distribution—a cell may register as viable while metabolically stressed or arrested in a particular phase (e.g., a shortened G1). The increased doubling rate could therefore reflect a shift in cycling behaviour rather than genuinely healthier proliferation. Future work should include flow cytometric cell cycle analysis and an apoptosis marker to confirm this.

6. Key conclusion

- When adjusted for glucose and osmolality, DMEM contains an excess of nutrients for C2C12 myoblast growth.
- For a seeding density of 1000 cells cm^2 in T-25 flasks, there was no significant difference between growth of C2C12 cells in complete media (100% DMEM) and dilute media (50% DMEM and 75% DMEM) over 48 hours. Beyond 48 hours, some component in the dilute media becomes rate limiting.
- There was also no significant difference between growth of C2C12 cells in 50% DMEM and 100% DMEM at 2000 cells cm^2 over 96 hours, when cells were passaged after 48 hours and media was refreshed. Hence, for short-term cultures such dilution can lead to substantial savings in media cost.
- It was noted that glucose is a key limiting factor. Meanwhile, as long as osmolality is kept constant, there is no difference between dilution with PBS vs water + mannitol.
- Passage number (P28 and P31) of cells has no significant difference on growth patterns.
- Yeast lysate and hydrolysate caused complete cell death. There was also a lot of batch-to-batch variability in solutions. Yeast lysate and hydrolysate composition should be characterised, cytotoxicity evaluated, and fractionation should be considered to yield smaller peptides which can be easily taken up by cells.

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Appendix A

50% DMEM Replacement (12 well-plate)	Volumes of reagent per conditioned media sample (3x2 wells, 3x1 wells for 500kDa)						Vol. per well (mL)	Total vol. (mL)
	<<STEP1>> Sterilisation required			<<STEP 2>> Directly added				
	Mannitol (mg)	Bulk DMEM (mL)	Water/PBS (mL)	Bulk FBS (mL)	Glucose (mL)	Bulk **Sample** Required (mL)		
(PC) Positive control	0	8.1	0	0.9	0	0	1	9
(A)water, high glucose	245	4.05	4.05	0.9	0.091	0	1.01	9.09
(B) water, low glucose	245	4.05	4.05	0.9	0.0455	0	1	9.02
(c) water	245	4.05	4.05	0.9	0	0	1	9

(D) PBS, high glucose	0	4.05	4.05	0.9	0.091	0	1.01	9.09
(E) PBS, low glucose	0	4.05	4.05	0.9	0.0455	0	1	9.02
(F) PBS	0	4.05	4.05	0.9	0	0	1	9

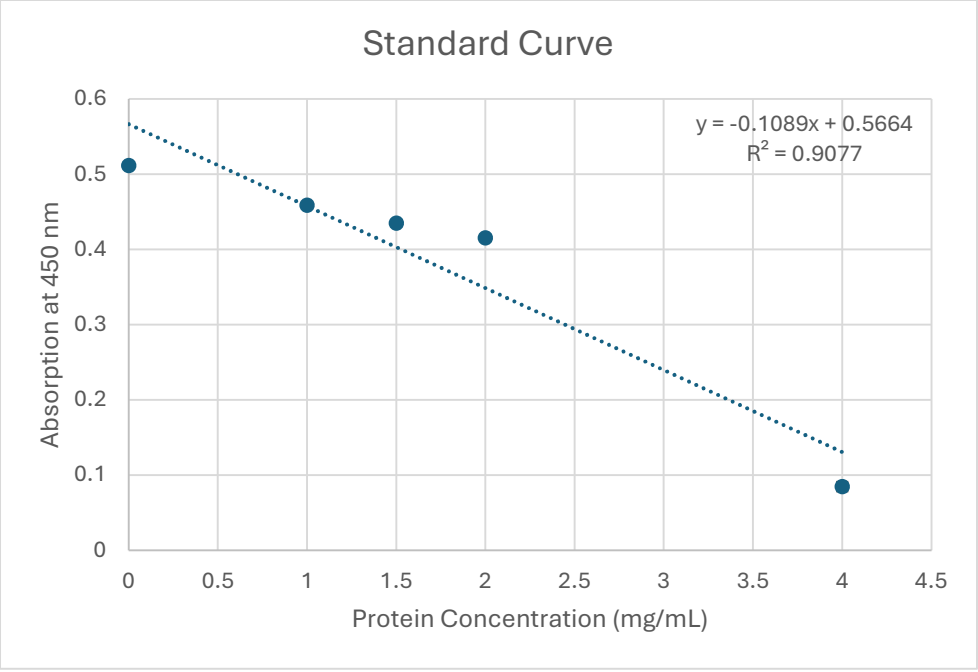
Appendix B

DMEM (ml)	FBS (ml)	PBS (ml)	Glucose (ml)	Total volume (ml)
9	1	0	0	10
6.75	1	2.25	0.051	10.05
4.5	1	4.5	0.101	10.10
2.25	1	6.75	0.152	10.15
0	1	9	0.203	10.20

Cumulative Population Doubling

	Day 0	Day 2	Day 4
DMEM 100%	0	5.32	9.17
DMEM 75%	0	5.58	8.70
DMEM 50%	0	6.31	8.24
DMEM 25%	0	5.21	7.59
DMEM 0%	0	N/A	N/A

Appendix C



Sample	x1 (Abs450)	y1 (protein conc.)
Lysate before filtration	0.2611	2.803
Lysate after filtration	0.2493	2.912
Hydrolysate before filtration	0.3378	2.099
Hydrolysate after filtration	0.3926	1.596

Appendix D

Condition	PD_0h_mean	PD_0h_SD	PD_48h_mean	PD_48h_SD	PD_96h_mean	PD_96h_SD
P28 - 100% DMEM	0	0	2.9928	0.20471	4.7927	0.2727
P28 - 25% DMEM	0	0	2.6667	0.25638	5.7074	0.082253
P28 - 50% DMEM	0	0	2.5817	0.13622	4.7869	0.10901
P31 - 100% DMEM	0	0	2.5143	0.35532	5.1439	0.23085
P31 - 25% DMEM	0	0	2.6893	0.43946	5.5605	0.3465
P31 - 50% DMEM	0	0	2.572	0.27367	5.4133	0.16579

Comparison	Timepoint	DMEM_condition	p_value	Significance
P28 vs P31	48h	100% DMEM	0.2706	Not significant
P28 vs P31	48h	25% DMEM	0.9569	Not significant
P28 vs P31	48h	50% DMEM	0.9695	Not significant
P28 vs P31	96h	100% DMEM	0.3021	Not significant
P28 vs P31	96h	25% DMEM	0.6557	Not significant
P28 vs P31	96h	50% DMEM	0.0606	Not significant