Original Article

Viability of Escherichia coli ATCC 8739 in Nutrient Broth, Luria-Bertani Broth and Brain Heart Infusion over 11 Weeks

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Abstract:

Background: *Escherichia coli* is a widely studied prokaryotic system. A recent study had demonstrated that reduced growth of *E. coli* after extended culture in Luria-Bertani broth is a result of depletion of fermentable sugars but able to sustain extended cell culture due to the presence of amino acids, which can be utilized as a carbon source. However, this had not been demonstrated in other media. The study aimed to determine the growth and viability of *E. coli* ATCC 8739 in 3 different media, Nutrient Broth (NB), Brain Heart Infusion (BHI) and Luria-Bertani Broth (LB) over 11 weeks.

Methods: Growth of *E. coli* ATCC 8739 was determined by optical density. Viability was determined by serial dilution/spread-plate enumeration. After 11 weeks, the media were exhausted by repeated culture. Glucose was added to the exhausted media to determine whether glucose is the growth-limiting factor.

Results: Our results showed that cell density in all 3 media increased to about 1×10^9 cells/ml by the end of week 1, from the inoculation density of 2.67×10^5 cells/ml, peaked at about 1×10^{13} cells/ml at week 4, before declining to about 5×10^7 cells/ml at week 7. Cell density is highly correlated to genomic DNA content ($r^2 = 0.93$) but poorly correlated to optical density ($r^2 < 0.2$). Our results also showed that the spent media were able to support further growth after glucose-supplementation.

Conclusion: NB, LB and BHI are able to support extended periods of culture and glucose depletion is the likely reason for declining cell growth.

1. Introduction

Escherichia coli is a common model organism for prokaryotic systems (1-3) where extensive knowledgebase about *E. coli* is available (4) and preserved cells can be resurrected without concern of instability. Short generation time and simple biology structure are advantages of using *E. coli* (5). Although *E. coli* was used in many studies, the choice of media and subculture intervals varied. A recent study (6) examined the growth rate and physiological states *E. coli* K-12 cells in Luria-Bertani broth (LB) and found that the average cell mass decreases when OD 600nm is beyond 0.3. After which, a decreasing growth rate was observed. The authors demonstrated that the depletion of fermentable sugars, which are primary carbon sources sparsely found in the LB that comprise of mainly oligopepetides derived from enzymatic digests such as casein and yeast extract, resulted in decreased growth (6). However, LB is able to sustain extended periods of cell growth but at a slower growth rate due to the high availability of amino acids, which the cells could utilize as a carbon source. This had led a number of studies (7-10) using the non-linear relationship between turbidity and cell density as a correction for decreased cell size (11).

Although Sezonov et al. (6) had demonstrated that sugar depletion to be the main cause of reduced growth; this had not been demonstrated in other media. Thus, it is important to investigate the relationship of *E. coli* growth

and viability in different types of media. The purpose of this study was to determine the growth and viability of *E. coli* ATCC 8739 in 3 different media, namely Nutrient Broth (NB), Brain Heart Infusion (BHI) and Luria-Bertani Broth (LB) over 11 weeks.

2. Material and Methods

2.1. Extended Viability in Different Media. *Escherichia coli* (ATCC 8739, Microbiologics Incorporated) were inoculated into 3 different media (Nutrient Broth, Brain Heart Infusion and Luria-Bertani Broth), 350 ml each, to a density of 2.67×10^5 cells/ml and cultured for 11 weeks at 37°C without agitation.Cell densities were determined by serial dilution and spread-plate and turbidity at OD 600 nm wavelength were taken.

2.2. Media Exhaustion and Glucose Supplementation. The media were clarified by centrifugation after 11 weeks of culturing before inoculation with overnight cultures of *E. coli* ATCC 8739 and incubated for a week at 37°C. After which, OD600 readings were taken and the cells were removed by centrifugation before re-inoculation. This was carried out until the OD600 readings after one week post-inoculation were less than half of that expected from fresh media. These nutrient-exhausted media were supplemented to filter-sterilized glucose solution to 0.025% (w/v) and 0.1% (w/v) from 18% (w/v) glucose stock solution, and inoculated with overnight cultures of *E. coli* ATCC 8739 before incubation at 37°C.

2.3. Correlating cell numbers to DNA content. The cell density of a single overnight culture of *E. coli* ATCC 8739 in Nutrient Broth was estimated using turbidity and corrected for reduced cell size. Genomic DNA was extracted from two sets of aliquots (0.5 ml, 0.6 ml, 0.7 ml, 0.8 ml, 0.9 ml and 1.0 ml). The total amount of genomic DNA extracted was correlated with the estimated cell numbers.

2.4. Genomic DNA Extraction. Genomic DNA was extracted from the removed cells weekly for DNA concentration determination. The DNA extraction protocol is based on Cheng and Jiang (12). Cells were harvested by centrifugation at 13000rpm for 20 minutes. The pellets were resuspended in 200 ulTris/HCl buffer before 200 ul of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the cell suspension and vortex. The aqueous phase was separated once by centrifugation at 13000 rpm for 5 minutes and extracted once with 200 ul of chloroform. The DNA concentration was determined at OD260nm wavelength.

3. Results

Our results showed that the cell count increased by 3107 (OD600 = 0.321), 19419 (OD600 = 0.801) and 92434 (OD600 = 0.547) times from the initial inoculation density of 2.67×10^5 cells/ml within 1 week of culture in NB, BHI and LB respectively (Figure 1). This is equivalent to 11.6, 14.2 and 16.5 generations from initial inoculum in NB, BHI and LB respectively. The cell density peaked at week 4 for LB and BHI with the cell density of 8.13×10^{13} cells/ml (11.7 generations from week 1) and 6.74×10^{12} cells/ml (10.3 generations from week 1) respectively. However, the cell density in NB reached maximum by week 3 (1.73×10^{12} cells/ml, 11.0 generations from week 1) and remained relatively constant to week 5. Our results showed that the cell densities declined from their peak to about 5×10^7 cells/ml by week 7.
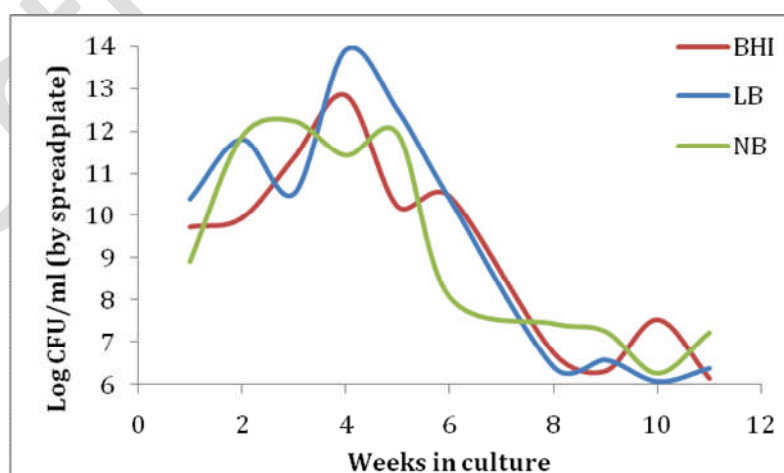

Figure 1. Viable cell count across 11 weeks in culture.
Our results showed that cell density peak at around week 4 of culture before declining to half the density at about week 8
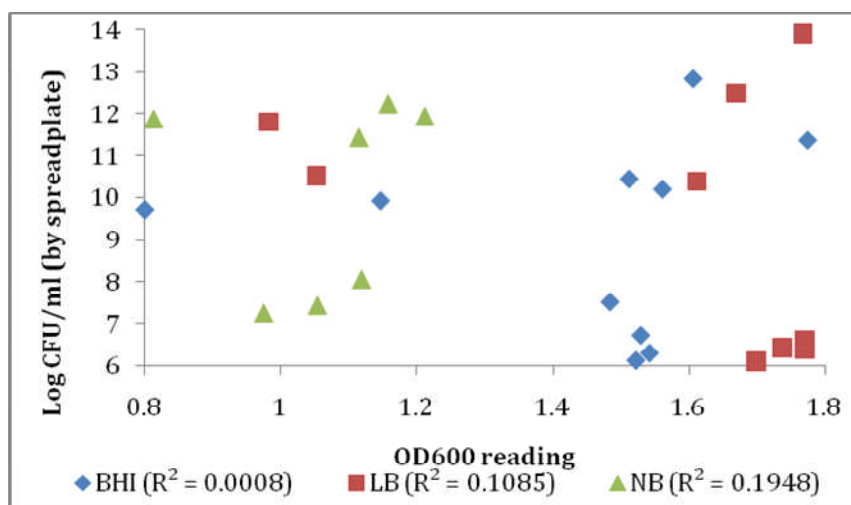
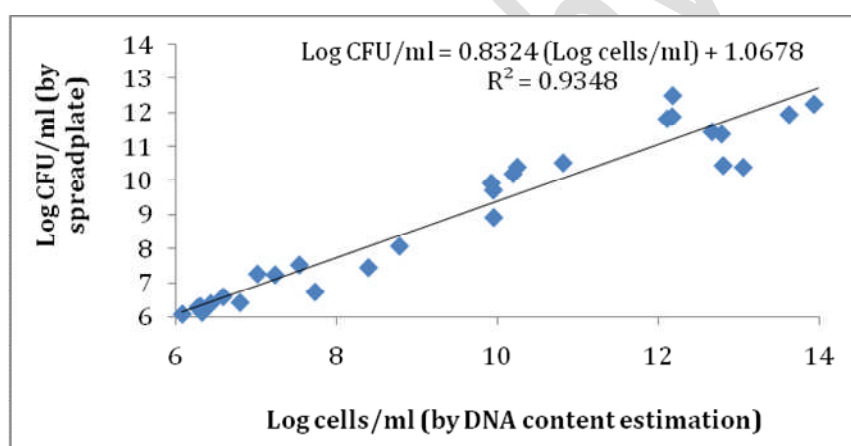**Figure 2.** Correlation between cell count and OD600 measurement.

Figure 3. Correlation between cell count and DNA content.

Poor correlation ($r^2 < 0.2$) between optical density (OD600 readings) and cell count by serial dilution and spread-plate in all 3 media (Figure 2 and 3). Data fitting using linear, logarithmic, or exponential model did not improve the coefficient of determination (r^2) to more than 0.25. However, a strong correlation was observed ($r^2 = 0.93$) between genomic DNA content and cell density with the relationship between spread-plate count and genomic DNA estimation as Log CFU/ml = 0.8324 (Log cells/ml) + 1.0678. If the intercept is set to zero, as there should not be any genomic DNA when there is no cells, the linear equation is Log CFU/ml = 0.9325 (Log cells/ml), with coefficient of determination of 0.9202.

E. coli ATCC 8739 is fully sequenced (Accession: NC_010468.1) strain with 50.9% GC content. Using the molecular weights each nucleotide, the genomic DNA content per million cells can be calculated to be about 515.35 nanograms (ng). By extracting genomic DNA from different aliquots of cells with known cell density and measuring using UV spectrometry at 260 nm wavelength, we extracted an average of 527 ng of genomic DNA per million cells, with a standard error of 10.605 ng, which is 1.1 standard deviations higher from the calculated genomic DNA content.

Cells from the media were removed by centrifugation after 11 weeks of culture and re-inoculated with overnight culture of *E. coli* ATCC 8739 and cultured for a week. The centrifuge-clarified media showed an OD600 reading of between 0.007 and 0.012. In the following week, turbidity was measured and the cells were removed by centrifugation, re-inoculated with overnight culture, and incubated. This was repeated until the turbidity in all 3

media were less than half of the turbidity after 1 week of culture in fresh NB (OD600 = 0.321), LB (OD600 = 0.547) or BHI (OD600 = 0.801) media. After 8 weeks of repeated use, following 11 weeks of initial culture, the OD600 readings for NB, LB, and BHI were 0.100, 0.241, and 0.177 respectively. This indicated that the growth limiting nutrients in the media were exhausted by repeated use. The pH of the exhausted NB, LB, and BHI media were 7.33, 7.12, and 7.68 respectively.

Each exhausted media were divided into 3 aliquots to test whether glucose is the growth-limiting nutrient by supplementing the exhausted media with 0.025% (w/v) glucose and 0.1% (w/v) glucose. The original exhausted media with no glucose supplementation (0%) was used as control. Our results showed that growth is supported in 0.025% (w/v) glucose supplement in LB and BHI compared to control. Exhausted media supplemented with 0.1% (w/v) glucose demonstrated strong growth compared to 0.025% (w/v) glucose and control in all 3 instances with LB and NB showing a larger growth spike compared to BHI (Figure 4).
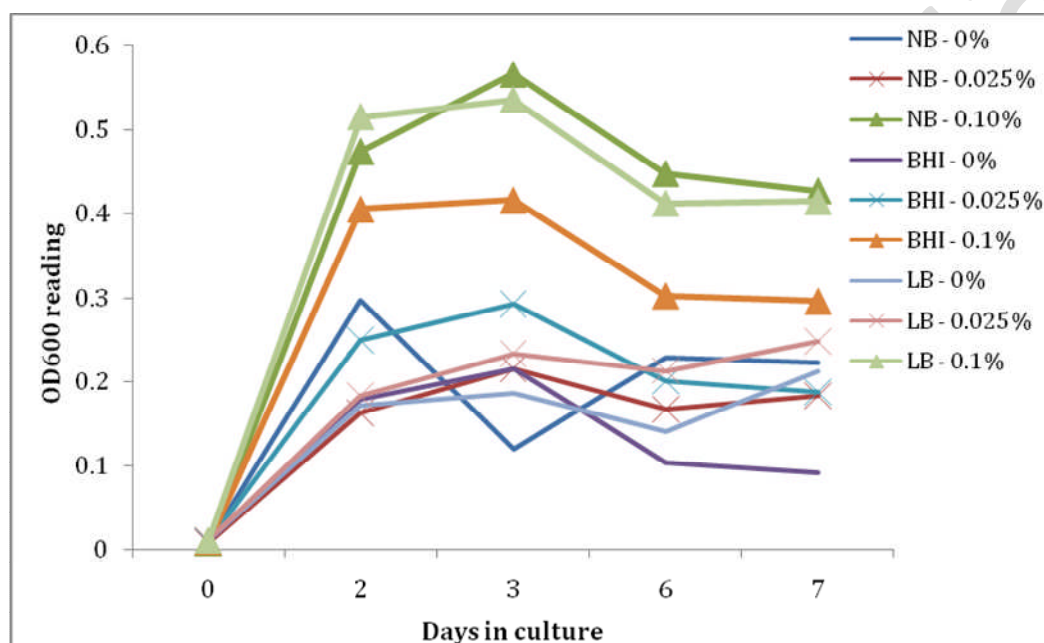

Figure 4. Turbidity of media over 7 days post-inoculation into glucose-supplemented, nutrient depleted media. Turbidity suggests significant growth in 0.1% glucose supplement compared to control (no supplement) in all 3 media

4. Discussions

Escherichia coli is a common model organism used in many studies. A recent study (6) examined the growth rate and physiological states *E. coli* K-12 cells in LB and demonstrated that sugar depletion to be the main cause of reduced growth but this had not been demonstrated in other media. In this study, we aimed to observe the growth and viability of *E. coli* ATCC 8739 in 3 different media.

Our results suggest that NB, LB and BHI are able to support net growth of *E. coli* for 4 weeks in culture before a decline in viable cell count to about half of the maximum viable cell. Despite so, the media were able to support growth to 19 weeks of culturing. The growth rate in the 19th week is less than half the growth rate in the first week. This supports previous work (6) suggesting that LB is able to support growth of *E. coli* in extended culture in spite of reduced growth rate.

Our results suggest poor correlation between turbidity and cell count by serial dilution and spread-plate in all 3 media regardless of the curve fitting models used after one week in culture. This may suggest that a steady state physiology may be absent which had been previously suggested after extended culture (6). Another possible reason may be that debris from different stages of dying cells may be affecting the turbidity.

On the other hand, our results suggest strong correlation between genomic DNA content and viable cell counts. This may suggest DNA degradation in dying cells, which had been found recently in *E. coli* during antibiotics-induced death (13) and deprivation of essential nutrients, such as thymineless death (14). Several studies had demonstrated that DNA content as a good indicator of cell numbers in eukaryotic tissues (15-17). Blumenstein et al. (15) reported near perfect correlation between nuclei counts and DNA content (r^2 = 0.9995) from paraffin-

embedded breast cancer tissue. However, this has not been reported for prokaryotic cells. Our results suggest that the genomic DNA content overestimates the cell count as the linear equations suggests between 0.83 and 0.94 colony forming units in logarithmic scale for every 1 cell in logarithmic scale based on genomic DNA content. Moreover, our results suggest that we extracted about 11.65 ng more genomic DNA than expected. This may be due to RNA content in the extraction.

The determination of growth limiting factors used in this study were based on a previous study examining growth limiting factors in hybridoma cells which suggest that growth rate is a good indicator rather than metabolic activity (18). Our results demonstrated strong growth upon the addition of glucose into the exhausted media suggesting that glucose is the growth-limiting factor in the media. This corroborates previous work (6). In addition, our results suggest that the increased growth rate is proportional to the amount of glucose supplementation. Although LB and NB showed higher growth rate in 0.1% (w/v) glucose supplement compared to BHI, this difference may be due to the composition of the media as both LB and NB derive the complex nutrients from yeast or beef extract while BHI derives its complex nutrients from organ tissues. It is generally accepted that BHI is a nutritionally richer medium than LB (19). It is highly unlikely that pH is a growth-limiting factor in extended culture as a recent study (20) reported no difference in *E. coli* growth in pH between 5.0 and 8.0. Moreover, a previous study (21) demonstrated that a pH above 9.2 is needed to inhibit the growth of *E. coli* in sewage water. It is also unlikely that the presence of metabolic by-products to be the growth limiting factor in this study as no soluble by-products were removed. 0.1% (w/v) glucose supplementation from 18% (w/v) stock solution increases the volume by 0.6%, which is unlikely to substantially dilute the metabolic by-products accumulated from 19 weeks of culture.

This study demonstrated the hardiness of *E. coli* in extended culture and nutrient exhaustion. Our results also demonstrate the applicability of DNA content to determine the density of viable *E. coli* cells, which had been demonstrated in human tissues (16). However, it is unclear whether the correlation between viable cell count and DNA content can be generalized to other prokaryotic cells. It is known that many micro-organisms are difficult to culture in the laboratory setting (22). Hence, if the correlation between viable cell count and DNA content can be generalized to other prokaryotic cells, it may be possible to determine the density of viable prokaryotes but unculturable under current microbiological techniques using techniques based on DNA content. Our results also suggest that *E. coli* is able to survive in an environment of metabolic waste when carbon source (glucose) is not a limiting factor, which corroborates with a recent study (6). However, there is a significant decrease in viable cell count after 4 weeks of extended culture. Moreover, the media were able to sustain further cell growth upon clarification and re-inoculation. Further work is necessary to determine the reasons for such cell death even though carbon source is the limiting factor rather than metabolic waste (6). It may be interesting to examine the difference in composition of metabolic output from 4th to 6th week of extended culture to determine the metabolic changes at the point of decreasing cell viability.

5. Conclusion

This study demonstrates that *E. coli* has net growth in NB, LB and BHI up to 4 weeks in culture but is able to sustain cells for extended culture and the growth limiting factor in all 3 tested media is glucose rather than pH or metabolic by-products. However, the reasons for decreased cell viability after 4 weeks require further study. In addition, this study also demonstrates that genomic DNA content of *E. coli* correlates strongly with viable cell counts.

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