



Research Article

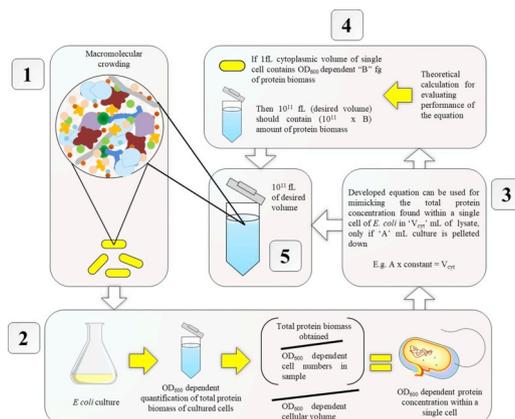
An equation for biomimicking macromolecular crowding using *Escherichia coli* MG1655 strainKhushal Khambhati^a, Nisarg Gohil^a, Gargi Bhattacharjee^a, Happy Panchasara^a, Vijai Singh^{a,b,*}^a Department of Biological Sciences and Biotechnology, Institute of Advanced Research, Koba Institutional Area, Gandhinagar 382426, India^b Present address: Department of Biosciences, School of Sciences, Indrashil University, Rajpur, Mehsana-382740, Gujarat, India

HIGHLIGHTS

- Total protein biomass and its concentration increases within an *E. coli* cell when an unfavourable growth environment prevails
- Mean range of intracellular protein concentration as the growth curve proceeds was between 15.2–178 fg/fL of cytosolic volume
- Change in macromolecular crowding within a cell with respect to change in the growth condition
- An equation for experimentally mimicking OD₆₀₀ dependent crowding environment in desired volume using cell lysate

GRAPHICAL ABSTRACT

Macromolecules present in the intracellular environment of a cell are densely packed, resulting in a highly crowded cytosolic environment. This crowded milieu influences several biochemical equilibria such as diffusibility and association constant of biomolecules which impose a serious impact on cellular functions as well as its processes. A number of *in silico* and *in vitro* studies have been reported till date about using synthetic crowding agents for resembling such a crowding environment within the cell. Schematic representation of an approach for calculating the OD₆₀₀ dependent total protein concentration found within a cell of *E. coli* MG1655 and development of an equation for biomimicking that particular concentration *in vitro*. (1) Indicates a densely packed cytosolic milieu of a cell. (2) The *E. coli* MG1655 cells were cultured and the protein biomass was quantified as per OD₆₀₀ dependent manner. Dividing the total protein biomass quantified, by the number of cells present in the sample gives the protein biomass contributed by an individual cell. Dividing the obtained biomass by the OD₆₀₀ dependent cellular volume gives the total protein concentration present within a cell. (3) The data obtained was used for deriving an equation which assists to determine either the buffer volume that is required to resuspend the cultured cell pellet for lysate preparation, or to know the volume of the cultured cell that is to be harvested for preparing a particular volume of cell lysate. In either way, the end result would demonstrate the crowding that could be present within an individual cell of *E. coli* in the desired volume of lysate solution (4) The criteria for validation of the derived equation. If the cell lysate that has been prepared experimentally using the derived equation contains the protein biomass close to the quantity estimated by theoretical calculation then the equation works well. (5) The resultant solution would resemble the crowded environment found within a cell.



* Corresponding author at: Department of Biosciences, School of Sciences, Indrashil University, Rajpur, Mehsana 382740, Gujarat, India. E-mail address: vijaisingh15@gmail.com (V. Singh).

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ABSTRACT

Macromolecules present in the intracellular environment of a cell are densely packed, resulting in a highly crowded cytosolic environment. This crowded milieu influences several biochemical equilibria such as diffusibility and association constant of biomolecules which impose a serious impact on cellular functions as well as its processes. A number of *in silico* and *in vitro* studies have been reported till date about using synthetic crowding agents for resembling such a crowding environment within the cell. Lately, it has been realized that synthetic crowders are not suitable for mimicking the intrinsic environment of the cell. In this study, proteins were assumed to be the major biological molecule which contributes to the crowding environment. We have semi-theoretically determined the total protein concentration within an individual *E. coli* MG1655 cell which changes notably as the growth curve proceeds from 0.2 to 1.0 OD₆₀₀. The average range of total cellular protein concentration throughout the batch culture was found to be in the range of 15.2 to 178 fg/fL of cytoplasmic volume. The fundamental knowledge gained through the study was translated to applied research in the form of an equation. We propose an equation that could help to mimic the OD₆₀₀ dependent crowding environment present within a single cell of *E. coli* in the desired volume of reaction solution. In a nutshell, the equation provides quantitative estimation of the volume of culture required to prepare the cell lysate for biomimicking the intracellular crowding environment *in vitro*. This finding provides a new insight into the cellular cytosolic environment that could be used as a platform to frame more cells like environment in cell-free protein synthesis (CFPS) system for synthetic biology applications.

1. Introduction

The cytosolic environment of a bacterial cell is heavily crowded by biomolecules such as a large number of proteins, polysaccharides, lipids, nucleic acids, etc., together which are known to cause macromolecular crowding [1,2]. This crowded environment influences molecular mobility, steric hindrance (*i.e.* excluded volume effect, which can be defined as the effect caused due to unavailability of a particular volume to other solutes due to its occupancy by macromolecules) and non-specific chemical interactions that cause a severe impact on equilibrium constants and reactions rates [3,4]. The crowding environment, in turn helps to shape protein-protein interaction, protein structure, aggregation, and its folding. Moreover, it greatly influences enzyme activity and protein-nucleic acid interactions [4,5]. Thus, in order to study the dynamic properties of a particular biomolecule exhibited inside a cell, in an *in vitro* fashion, it is imperative to mimic such a crowded environment present within a cell in the reaction solution.

In an effort to develop an artificial *in vitro* crowding environment, most of the researchers till date have been using synthetic crowding agents (*e.g.*, ficoll, polyethylene glycol, dextran) to determine their impact on the subjected biomolecules [5,6]. However, the propensity of such crowding agents to mimic the thermodynamics of a cell *in vitro* is still questionable, as they might turn it more (or less) complex or fail in mimicking the proteins in their structural or functional manner [7]. In this context, Wang et al. [8] compared the difference in the diffusion behaviour of chymotrypsin inhibitor 2 (CI2) proteins by using crowding agents such as synthetic crowders, proteins, and cell lysates [8]. The study revealed that there was a notable difference in translation as well as rotational diffusion behaviour of CI2 when synthetic crowders were used as compared to proteins or cell lysates. However, when the diffusion behaviour was compared between cell lysates and proteins as crowding agents, it was found to be quiet similar. Few other studies have also supported the theory of using proteins or cell lysates as crowding agent to recreate more cytoplasm like environment for conducting *in vitro* crowding studies [9,10]. Therefore, if one intends to study the dynamic behaviour of a particular biomolecule exhibited within the cell in an *in vitro* manner, it is important to have a more cell-like environment which specifically include proteins or components of the cell lysates rather than synthetic crowding agents [11]. Thus, to experimentally simulate such intracellular environment, it is important to have knowledge regarding the concentrations of biomolecules present within a single cell of an organism.

Even with burgeoning attention, it seems that researchers haven't given much intimacy to address some of the very basic questions regarding the quantitative estimation of biomolecules that dwell within

the cytosolic environment of a cell. Of the many such questions, one is, how much concentration of total protein is present within a single cell of an organism? Such data could further act as a platform for exploring "the significance of cytosolic crowded environment" in terms of cell benefits through *in vitro* means. In an attempt to contribute to such incomprehensive data, Milo [12] theoretically determined the total number of proteins present within a cellular volume of *Escherichia coli*, budding yeast, and HeLa cell line. However, as per the best of our knowledge, the data for protein concentration present within a cell is still a missing piece in the literature.

The aim of this study was to quantify the total protein concentration present within *E. coli*, the best-studied prokaryotic model organism. Furthermore, we have also proposed an equation that could experimentally help to mimic OD₆₀₀ dependent crowding environment found within the *E. coli* in the desired volume of reaction buffer.

2. Materials and methods

2.1. Bacteria strain and media

Wild-type *E. coli* MG1655 strain was used as the organism of study throughout the experiment. The cells were cultured in Luria Bertani (LB) medium (HiMedia, Mumbai). The culture was routinely grown in LB broth or LB agar at 37 °C and the strain was preserved in 25% glycerol at -20 °C for further use.

2.2. Growth curve procedure

The inoculum for growth curve preparation was standardized as per 0.5 McFarland turbidity standard at an optical density of 600 nm (OD₆₀₀) (Systronics Visiscan spectrometer 167) using overnight grown *E. coli* culture [13]. The prepared inoculum (in 0.9% NaCl) was inoculated (10% v/v) in LB broth and incubated at 37 °C at 200 rpm. After every 20 min of interval, OD₆₀₀ was measured.

2.3. Determination of *E. coli* population size

For the determination of bacterial population size, we used an online calculator [14] (<http://www.genomics.agilent.com/biocalculators/calcODBacterial.jsp>) for measuring the cell concentration at OD₆₀₀. In this calculator, the number of *E. coli* cells were calculated using rule-of-thumb relation scale 1.0 OD₆₀₀ = 8 × 10⁸ cells/ml in LB broth.

2.4. Quantification of total protein biomass in a single cell of *E. coli*

The total protein biomass present within a single cell of *E. coli* at four different OD₆₀₀ values 0.2, 0.4, 0.6 and 1.0 was measured. For the 0.2 OD₆₀₀, the standardized inoculum was inoculated as aforementioned. When the culture reached an OD₆₀₀ of 0.2, 15 mL culture was collected and centrifuged at 4000 rpm (Eppendorf, Germany) for 15 min at 37 °C. The cell pellets were washed with normal saline (0.9% NaCl) solution and re-suspended in 100 µL of double distilled water (ddH₂O). The cells were then lysed at 100 °C using Accublock™ Digital Dry Bath (Labnet International, Inc.) for 15 min. The cell debris was removed through centrifugation at 13,000 rpm for 1 min and the collected supernatant was used for protein quantification as per the Folin-Lowry method [15] (Supplementary Fig. 1 and Table 1). The total protein biomass obtained was divided by 15 (sample volume of culture taken) to derive the protein biomass contributed by the cells present in 1 mL of culture. The obtained amount was divided by the number of cells at 0.2 OD₆₀₀/mL of culture in order to obtain the total protein biomass present within a single cell of *E. coli* [16]. The same procedure

was applied for the cells at different OD values, i.e., 0.4, 0.6 and 1.0 OD₆₀₀ with minor changes. For 0.4 and 0.6 OD₆₀₀, the cells were re-suspended in 500 µL of ddH₂O and for 1.0 OD₆₀₀, the sample size was 1 mL instead of 15 mL and the cells were centrifuged at 13,000 rpm for 5 min. All the experiments were performed in triplicates and repeated more than thrice.

2.5. Determination of the cellular volume of *E. coli*

The cellular volume of *E. coli* MG1655, when cells were grown in LB medium at 37 °C, was calculated using equation [17]

$$N_{\text{cell}} = 3.6 \times 10^9 / V_{\text{cell}}$$

where N_{cell} = number of cells corresponding to a particular OD₆₀₀
 V_{cell} = cellular volume of a cell at that particular OD₆₀₀.

2.6. Calculation of total protein concentration within a single cell of *E. coli*

The total protein concentration within a single cell of *E. coli* was

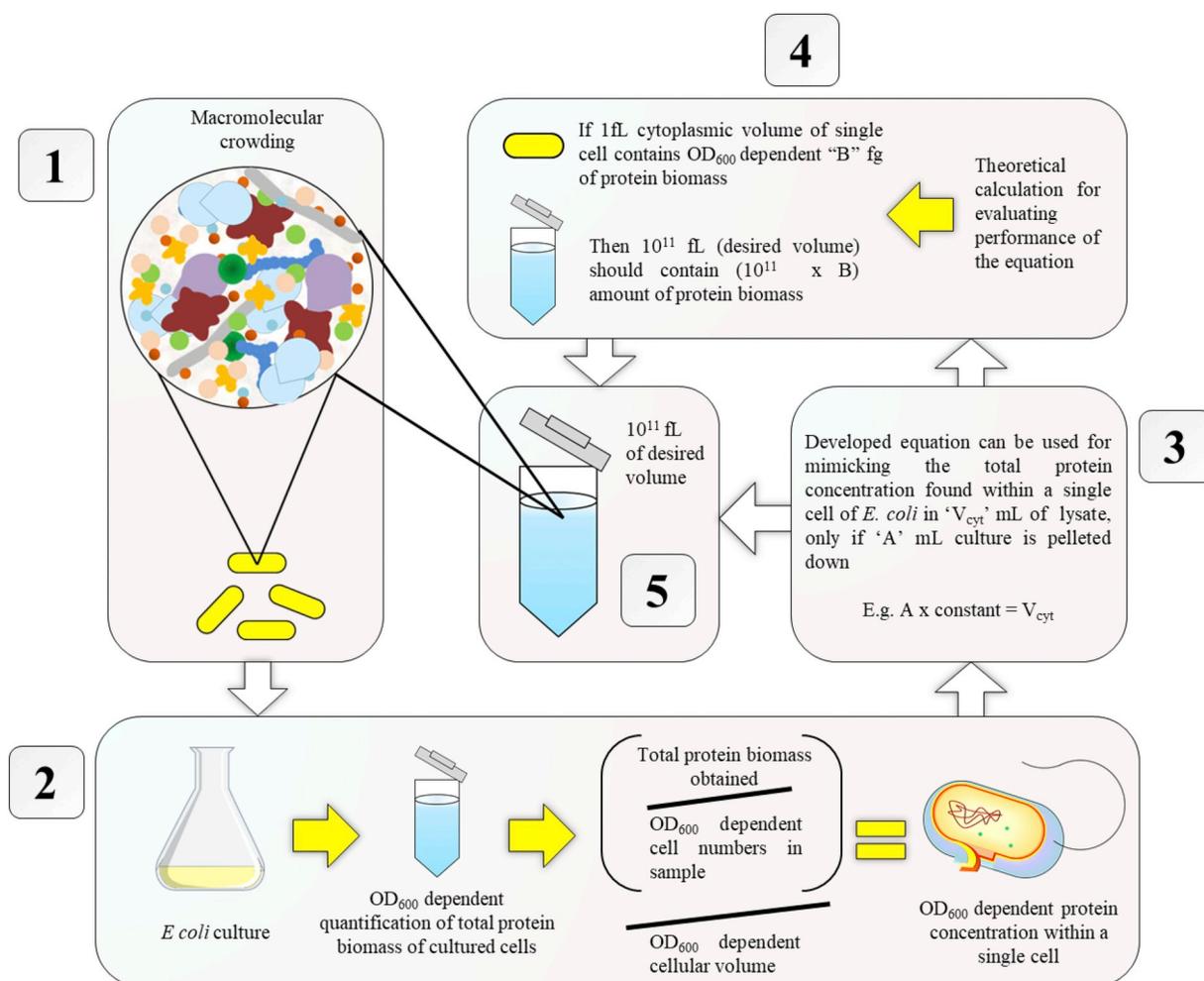


Fig. 1. Schematic representation of an approach for calculating the OD₆₀₀ dependent total protein concentration found within a cell of *E. coli* MG1655 and development of an equation for biomimicking that particular concentration *in vitro*. (1) Indicates a densely packed cytosolic milieu of a cell. (2) The *E. coli* MG1655 cells were cultured and the protein biomass was quantified as per OD₆₀₀ dependent manner. Dividing the total protein biomass quantified, by the number of cells present in the sample gives the protein biomass contributed by an individual cell. Dividing the obtained biomass by the OD₆₀₀ dependent cellular volume gives the total protein concentration present within a cell. (3) The data obtained was used for deriving an equation which assists to determine either the buffer volume that is required to resuspend the cultured cell pellet for lysate preparation, or to know the volume of the cultured cell that is to be harvested for preparing a particular volume of cell lysate. In either way, the end result would demonstrate the crowding that could be present within an individual cell of *E. coli* in the desired volume of lysate solution (4) The criteria for validation of the derived equation. If the cell lysate that has been prepared experimentally using the derived equation contains the protein biomass close to the quantity estimated by theoretical calculation then the equation works well. (5) The resultant solution would resemble the crowded environment found within a cell.

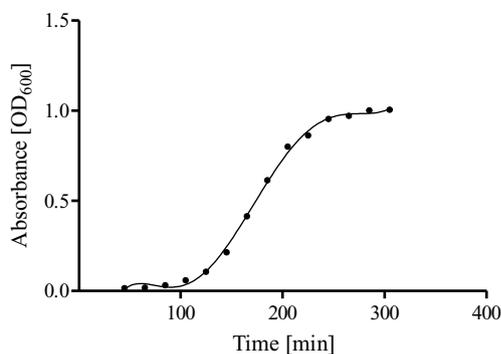


Fig. 2. The growth curve of *E. coli* MG1655. The cells were grown at 37 °C in LB broth at 200 rpm.

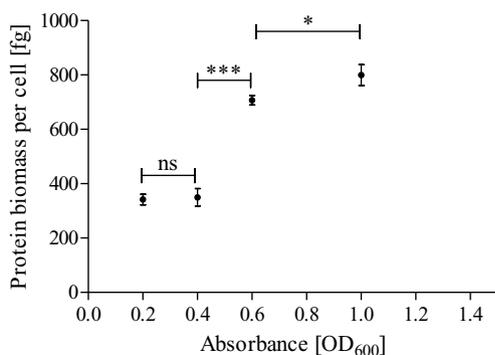


Fig. 3. Total protein biomass (fg) per cell of *E. coli* MG1655 corresponding to different OD₆₀₀ of batch culture. Cells were grown at 37 °C in LB broth at 200 rpm. **p* < .05, ****p* < .001, ns- not significant.

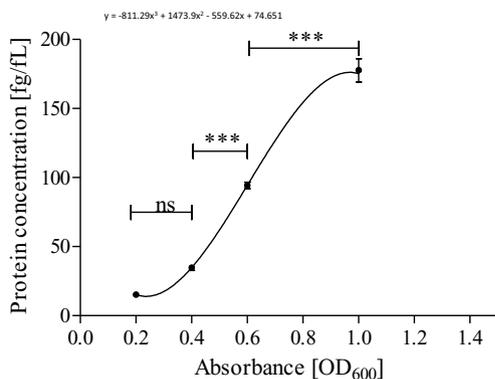


Fig. 4. Total protein concentration (fg/μL) found within a single cell of *E. coli* MG1655 corresponding to different OD₆₀₀ of batch culture. Cells were grown at 37 °C in LB broth at 200 rpm. ns- not significant, ****p* < .001.

calculated by dividing the total protein biomass present within a single cell at particular OD₆₀₀ of growth culture by its OD₆₀₀ dependent cellular volume. Fig. 1 summarizes the approach for calculating the OD₆₀₀ dependent total protein concentration found within a cell of *E. coli* MG1655. Furthermore, it also describes the criteria for evaluating the performance of the derived equation that would help to mimic the OD₆₀₀ dependent crowding environment of the cell.

3. Results

3.1. Analysis of growth curve

The sigmoid growth curve of *E. coli* obtained is shown in Fig. 2. It

can be divided into lag phase (0–65 min), log phase (65–165 min; OD₆₀₀ 0.018–0.4), deceleration phase (165–265 min; OD₆₀₀ 0.4–0.972) and stationary phase (285–305 min; OD₆₀₀ 1). Thus, from the curve it could be deduced that the cells experience four different growth conditions with log phase being the most favourable and stationary phase being the unfavourable growth environment.

3.2. Estimation of total protein biomass and concentration present within a single cell of *E. coli*

On quantification of total protein biomass and its concentration within a single cell of *E. coli* MG1655, it could be claimed that the total protein concentration as well as its biomass significantly increase within the cells as growth phase changes (Figs. 3, 4). In this study, the maximum total protein biomass and its concentration were found to be 8.00×10^2 fg/cell and 1.78×10^2 fg/μL, respectively, at OD₆₀₀ 1.0. Furthermore, there was about 2.33-fold increase of total protein biomass and 11.71-fold increase in total protein concentration within the cells (Fig. 4) when they were grown at 1.0 OD₆₀₀ as compared to 0.2 OD₆₀₀. The pictorial representation for the fold increase of total protein biomass and its concentration within a single cell as compared to different growth conditions of batch culture is represented in Fig. 5. It indicates that the fold increase of total protein concentration per 1 fL of the cytoplasmic volume was much more than the fold increases of total protein biomass per cell when compared to different phases of the growth curve.

3.3. Derivation of an equation for biomimicking the total protein concentration in the desired volume of cell lysate

The total protein biomass within *E. coli* cells in 1 mL of culture at 0.2 OD₆₀₀ was 0.054 mg (Supplementary Table 2). For 'A' mL of culture, the total protein biomass 'M_p' (mg) will be

$$M_p = 0.054 \times A \quad (1)$$

Provided that 10^{-12} mL (i.e. 1 fL) cytoplasmic volume of *E. coli* contains on an average 1.52×10^{-11} mg of total protein biomass (Supplementary Table 2), the cytoplasmic volume 'V_{cyt}' (mL) for the protein biomass 'M_p' would be

$$V_{\text{cyt}} = M_p \times 10^{-12} / 1.52 \times 10^{-11} \quad (2)$$

Substituting the value of 'M_p' from Eq. (1) into Eq. (2)

$$V_{\text{cyt}} = (0.054 \times A) \times 10^{-12} / 1.52 \times 10^{-11} \\ V_{\text{cyt}} = 0.0035 \times A \quad (3)$$

This equation can be used for biomimicking the total protein concentration found within a single cell of *E. coli* in 'V_{cyt}' mL of volume, only if 'A' mL of 0.2 OD₆₀₀ culture (grown in LB broth at 37 °C at 200 rpm) is pelleted down, resuspended and lysed. The relation was found to be identical for 0.4, 0.6 and 1 OD₆₀₀ cells.

3.4. Validation of the equation for biomimicking the total protein concentration

The relation shown in Eq. (3) was used for imitating the protein concentration found within the cells in 100 μL of ddH₂O. The quantitative analysis for protein concentration in 100 μL of ddH₂O is given in Table 1. It was confirmed that Eq. (3) can be used to mimic the protein concentration found within a cell in the desired volume of cell lysate. The equation was validated by comparing the total protein biomass obtained in 100 μL of ddH₂O to the theoretically calculated protein biomass in the same volume of ddH₂O. The theoretical calculation of the protein biomass was performed as follows. If 1 fL cytoplasmic volume of a single cell contains OD₆₀₀ dependent "B" fg of protein biomass then 10^{11} fL (i.e. 100 μL, desired volume) should contain $(10^{11} \times$

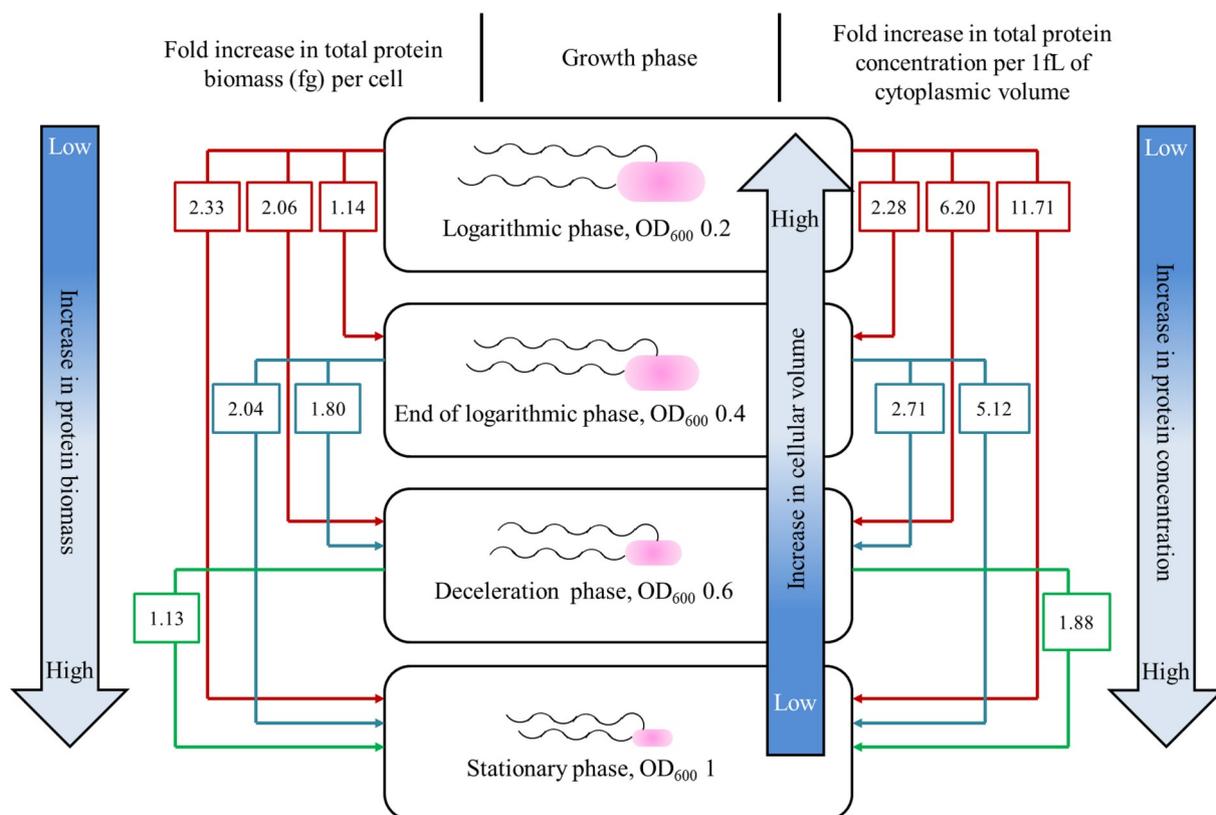


Fig. 5. Schematic representation for fold increase in total protein biomass as well as its concentration present within a single cell of *E. coli* MG1655 when compared to different growth phase of batch culture. The figure indicates that the protein biomass and its concentration within individual cell increases as the growth curve changes from logarithmic to stationary phase. The increase in protein biomass as growth phase proceeds indirectly points out the increase in protein expression. However, it is not only the increase of protein expression but also the reduction of the cellular volume that aid for increase in the total protein concentration within the cells as the growth phase proceeds. Red coloured arrows indicate fold increase from logarithmic to rest of the phase. Blue coloured arrows indicate fold increase from end of logarithmic to deceleration and stationary phase. Green coloured arrows indicate the fold increase from deceleration to stationary phase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Validation of the derived Eq. (3) for imitating the protein concentration present within a single cell in the 100 μL of dH_2O .

OD ₆₀₀	Total protein concentration (mg/100 μL)	
	Calculated	Obtained
0.2	1.52	1.36
0.4	3.11	4.58
0.6	9.43	9.43
1.0	17.8	15.20

B) amount of protein biomass. For example, a cell at 0.2 OD₆₀₀ contains on an average 15.2 fg of total protein biomass per 1 fL (supplementary Table 2) cytoplasmic volume. If the cytoplasmic volume is 10¹¹ fL then it should contain 1.52 mg of total protein biomass. This gives the theoretical value of the total protein biomass that should be present within the desired volume of the solution in order to mimic the total protein concentration found within a cell at 0.2 OD₆₀₀ using the derived equation (i.e. Eq. (3)).

4. Discussion

Calculating the total protein concentration within a cell is a fundamental task, as it helps to visualize the extent of the crowded environment present within a cell. The gravity and significance of such quantitative visualization are because it helps to estimate the degree of molecular crowding enclosed within the cell. The effect caused due to crowding is not only an important feature but also an unavoidable

phenomenon of life [1]. Quantification of the total protein biomass as well as its concentration within the cells revealed that they both increase significantly as the growth phase changes. As the cells outreach the stationary phase, an unfavourable growth environment, the cells tend to have more protein biomass and concentration as compared to the logarithmic phase that offers a favourable growth environment. These results are in agreement with the data sets obtained by Liu et al. [18]. They revealed that there were a number of up-regulated gene expressions within the *E. coli* cell population when cells were grown in media with a poor carbon source (slower growth rate) as compared to a rich carbon source (faster growth rate). Furthermore, the unit representation reported by Zubkov et al. [16] for determination of total protein biomass per cell of marine planktonic bacteria is in tandem with our results.

Interestingly, it seems that cells smartly increase the total protein concentration within the cells by increasing the total protein biomass and decreasing the cellular volume [17] (Fig. 5). It is fair to argue that with increasing total protein concentration, there should be an increment in the degree of molecular crowding within the cells. That being said, our datasets point out an increased degree of crowding environment when cells were grown in the starvation condition or stationary phase. This declaration is in agreement with a communication published by Joyner et al. [19]. They demonstrated that the total biomass of lyophilized yeast cells does not significantly decrease with a reduction in the cellular volume when the cells were grown in glucose-starved condition. This led them to a conclusion that the crowding environment increased within the yeast cells in glucose-starved condition as compared to the non-starved condition.

Change in macromolecular crowding alters the cellular thermodynamic properties [2,20] which in turn impacts on several biochemical reactions. The crowding also influences the protein activities [21,22], association rate [23–26], diffusion [8,27,28] and aggregation localization [29] within a cell. It would mean that those proteins that are being expressed throughout the growth curve within a cell would exhibit different dynamic properties at different phases of the growth curve. This is because the crowding environment within the cells is changing with the change in growth condition.

Therefore, in order to study the intracellular dynamic behaviour of biomolecule *in vitro* means, it is important to mimic the macromolecular crowding at a physiologically relevant level in an OD₆₀₀ dependent manner. In order to facilitate such research, we derived an equation that would help to mimic the protein concentration, or in other words, its crowding environment found within a cell (OD₆₀₀ dependent manner) in a desired volume using cell lysate. For our convenience, the equation was derived by denaturing the total protein and quantifying its concentration. Logically, the proposed equation would also work if the cell lysate is prepared by other means which maintain the integrity and functionality of the biomolecules. Furthermore, it is fair to assume that if a single cell protein concentration is being mimicked in 'V_{cyt}' mL of lysate, the rest of the component present within the cell would automatically get mimicked.

It is already known that the excluded volume effect totally depends upon the percentage occupancy of the crowding agents in the solvent. More the crowding agent, greater the excluded volume effect [30]. It has been stated that synthetic polymers such as PEG, dextran and ficoll are not a realistic option to mimic the cytoplasmic milieu. Thus, moulding the cytoplasmic milieu in an *in vitro* system demands the quantitative analysis and usage of biological entities [30,31]. On the other hand, the equation that has been proposed in this study directs the user to fulfil both the demands. Furthermore, the proposed equation will not only help to explore *in vivo*-like protein dynamic behaviour through *in vitro* means but could also possibly help to develop the cell-free protein synthesis (CFPS) system. Jewett and Swartz were able to increase 5 fold higher expression of chloramphenicol acetyltransferase (CAT) proteins by creating a more cell-like environment as compared to PANOX (PEP, amino acids, NAD⁺, oxalic acid) CFPS system. The system formulated by Jewett and Swartz replace the usage of unnatural polymers such as PEG with naturally occurring polymers such as spermidine and putrescine. Furthermore, removal of HEPES buffer and reformulating the ionic makeup resembling cell physiological condition helps to facilitate the increased CFPS expression. Generation of more cell-like condition is stressed upon by the results of Jewett and Swartz [32]. Similarly, the influence of synthetic crowding agents in transcription and translation steps of CFPS system was also demonstrated [33].

5. Conclusions

The total protein concentration within each cell of an *E. coli* MG1655 was in the range 15.2–178 fg/fL of cytoplasmic volume when cells were grown as a batch culture in LB broth at 37 °C at 200 rpm. In addition, it could also be deduced that the total protein concentration changes as the growth phase proceeds. The proposed equation can be used to mimic the crowding environment found within a cell of *E. coli* when cells are grown under specific conditions. The given equation could help to explore and understand the *in vivo* dynamic behaviour of biomolecules through *in vitro* means. Moreover, the proposed equation can also be useful for improving the systems such as CFPS where creating a cytoplasmic or more cell-like environment is propitious.

Declaration of Competing Interest

The author declares that there is no conflict of interest regarding the publication of this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bpc.2019.106244>.

References

- [1] C. Tan, S. Saurabh, M.P. Bruchez, R. Schwartz, P. LeDuc, Molecular crowding shapes gene expression in synthetic cellular nanosystems, *Nat. Nanotechnol.* 8 (2013) 602–608.
- [2] H. Matsuda, G.G. Putzel, V. Backman, I. Szeleifer, Macromolecular crowding as a regulator of gene transcription, *Biophys. J.* 106 (2014) 1801–1810.
- [3] M. Tabaka, T. Kalwarczyk, J. Szymanski, S. Hou, R. Holyst, The effect of macromolecular crowding on mobility of biomolecules, association kinetics, and gene expression in living cells, *Front. Phys.* 2 (2014) 54.
- [4] I. Kuznetsova, K. Turoverov, V. Uversky, What macromolecular crowding can do to a protein, *Int. J. Mol. Sci.* 15 (2014) 23090–23140.
- [5] I. Kuznetsova, B. Zaslavsky, L. Breydo, K. Turoverov, V. Uversky, Beyond the excluded volume effects: mechanistic complexity of the crowded milieu, *Molecules* 20 (2015) 1377–1409.
- [6] P. Benny, M. Raghunath, Making microenvironments: a look into incorporating macromolecular crowding into *in vitro* experiments, to generate biomimetic microenvironments which are capable of directing cell function for tissue engineering applications, *J. Tissue Eng.* 8 (2017) 2041731417730467.
- [7] A.H. Elcock, Models of macromolecular crowding effects and the need for quantitative comparisons with experiment, *Curr. Opin. Struct. Biol.* 20 (2010) 196–206.
- [8] T. Wang, C. Li, G.J. Pielak, Effects of proteins on protein diffusion, *J. Am. Chem. Soc.* 132 (2010) 9392–9397.
- [9] M.P. Latham, L.E. Kay, Is buffer a good proxy for a crowded cell-like environment? A comparative NMR study of calmodulin side-chain dynamics in buffer and *E. coli* lysate, *PLoS One* 7 (2012) e48226.
- [10] M. Sarkar, A.E. Smith, G.J. Pielak, Impact of reconstituted cytosol on protein stability, *Proc. Natl. Acad. Sci. U. S. A.* 110 (2013) 19342–19347.
- [11] M. Feig, Y. Sugita, Variable interactions between protein crowders and biomolecular solutes are important in understanding cellular crowding, *J. Phys. Chem. B* 116 (2011) 599–605.
- [12] R. Milo, What is the total number of protein molecules per cell volume? A call to rethink some published values, *Bioessays* 35 (2013) 1050–1055.
- [13] M. Balouiri, M. Sadiki, S.K. Ibsouda, Methods for *in vitro* evaluating antimicrobial activity: a review, *J. Pharm. Anal.* 6 (2016) 71–79.
- [14] *E. coli* Cell Culture Concentration from OD600 Calculator [Internet]. Agilent Technologies, Genomics; accessed on 20.04.2018. Available from: <http://www.genomics.agilent.com/biocalculators/calcODBacterial.jsp>
- [15] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* 193 (1951) 265–275.
- [16] M.V. Zubkov, B.M. Fuchs, H. Eilers, P.H. Burkill, R. Amann, Determination of total protein content of bacterial cells by SYPRO staining and flow cytometry, *Appl. Environ. Microbiol.* 65 (1999) 3251–3257.
- [17] B. Volkmer, M. Heinemann, Condition-dependent cell volume and concentration of *Escherichia coli* to facilitate data conversion for systems biology modeling, *PLoS One* 6 (2011) e23126.
- [18] M. Liu, T. Durfee, J.E. Cabrera, K. Zhao, D.J. Jin, F.R. Blattner, Global transcriptional programs reveal a carbon source foraging strategy by *Escherichia coli*, *J. Biol. Chem.* 280 (2005) 15921–15927.
- [19] R.P. Joyner, J.H. Tang, J. Helenius, E. Dultz, C. Brune, L.J. Holt, S. Huet, D.J. Müller, K. Weis, A glucose-starvation response regulates the diffusion of macromolecules, *Elife* 5 (2016) e09376.
- [20] R.J. Ellis, Macromolecular crowding: obvious but underappreciated, *Trends Biochem. Sci.* 26 (2001) 597–604.
- [21] G.J. Pielak, A.C. Miklos, Crowding and function reunite, *Proc. Natl. Acad. Sci. U. S. A.* 107 (2010) 17457–17458.
- [22] M.G. Norris, N. Malys, What is the true enzyme kinetics in the biological system? An investigation of macromolecular crowding effect upon enzyme kinetics of glucose-6-phosphate dehydrogenase, *Biochem. Biophys. Res. Commun.* 405 (2011) 388–392.
- [23] S.B. Zimmerman, S.O. Trach, Effects of macromolecular crowding on the association of *E. coli* ribosomal particles, *Nucleic Acids Res.* 16 (1988) 6309–6326.
- [24] G. Rivas, J.A. Fernández, A.P. Minton, Direct observation of the enhancement of noncooperative protein self-assembly by macromolecular crowding: indefinite

- linear self-association of bacterial cell division protein FtsZ, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 3150–3155.
- [25] G. Wieczorek, P. Zielenkiewicz, Influence of macromolecular crowding on protein-protein association rates—a Brownian dynamics study, Biophys. J. 95 (2008) 5030–5036.
- [26] M.A. Mourao, J.B. Hakim, S. Schnell, Connecting the dots: the effects of macromolecular crowding on cell physiology, Biophys. J. 107 (2014) 2761–2766.
- [27] N. Muramatsu, A.P. Minton, Tracer diffusion of globular proteins in concentrated protein solutions, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 2984–2988.
- [28] A. Miermont, F. Waharte, S. Hu, M.N. McClean, S. Bottani, S. Léon, P. Hersen, Severe osmotic compression triggers a slowdown of intracellular signaling, which can be explained by molecular crowding, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 5725–5730.
- [29] A.S. Coquel, J.P. Jacob, M. Primet, A. Demarez, M. Dimiccoli, T. Julou, L. Moisan, A.B. Lindner, H. Berry, Localization of protein aggregation in *Escherichia coli* is governed by diffusion and nucleoid macromolecular crowding effect, PLoS Comput. Biol. 9 (2013) e1003038.
- [30] K.A. Sharp, Unpacking the origins of in-cell crowding, Proc. Natl. Acad. Sci. U. S. A. 113 (2016) 1684–1685.
- [31] K.A. Sharp, Analysis of the size dependence of macromolecular crowding shows that smaller is better, Proc. Natl. Acad. Sci. U. S. A. 112 (2015) 7990–7995.
- [32] M.C. Jewett, J.R. Swartz, Mimicking the *Escherichia coli* cytoplasmic environment activates long-lived and efficient cell-free protein synthesis, Biotechnol. Bioeng. 86 (2004) 19–26.
- [33] X. Ge, D. Luo, J. Xu, Cell-free protein expression under macromolecular crowding conditions, PLoS One 6 (2011) e28707.