

Effect of substrate and IPTG concentrations on the burden to growth of *Escherichia coli* on glycerol due to the expression of Lac proteins

Pushkar Malakar · K. V. Venkatesh

Received: 23 August 2011 / Revised: 23 September 2011 / Accepted: 8 October 2011 / Published online: 27 October 2011
© Springer-Verlag 2011

Abstract Expression of proteins unneeded for growth diverts cellular resources from making necessary protein and leads to a reduction in the growth rate of an organism. This reduction in growth rate is termed as cost. Cost plays an important role in determining the selected expression of a protein in a particular environment. Characterization of cost is important in biotechnology industries where microorganisms are used to produce foreign proteins. We have used the lactose system in *Escherichia coli* to quantify the cost of growth on glycerol in the presence of isopropyl- β -D-thiogalactopyranoside (IPTG), an inducer of the lactose system. The effect of the concentration of the carbon source, glycerol, and the inducer of Lac enzymes, IPTG, is studied. The results show that the cost is dependent on the glycerol concentration with a decreasing trend with increasing concentration of glycerol. Also as expected, the cost increases and saturates at a higher concentration of IPTG. The studies also demonstrate that the cost is higher in early exponential phase relative to late exponential phase during the growth as has been reported in the literature. Hill equation fit yielded a typical Monod-type expression for growth on glycerol with and without IPTG. An apparent half-saturation constant was defined which was used to characterize the burden on growth due to protein expression.

Keywords Cost due to protein expression · Burden on growth · Early exponential phase · Late exponential phase · Glycerol · *Escherichia coli* · IPTG

Introduction

Expression of unneeded proteins in *Escherichia coli* is known to reduce growth rate. This is known from studies that have been carried out from the last 50 years (Weiner 1957; Horiuchi et al. 1962; Andrews and Hegeman 1976; Koch 1988; Nguyen et al. 1989; Dong et al. 1995; Dekel and Alon 2005). The reduction in growth rate due to the production of enzymes is known as cost and burdens the cellular growth. The cost is defined as the fractional reduction in growth rate and is thought to be due to limited resources of the cell, especially the capacity of ribosome (Maaloe and Niels 1966). Due to limited resources, the expression of unneeded proteins diverts the cellular resources from making necessary proteins as a result of which the capability of the cells to grow reduces (Alon 2006; Vind et al. 1993).

Cost of a protein plays an important role in its evolutionary selection. It decides the selected expression level of a protein in a particular environment (Dekel and Alon 2005; Alon 2006). It also decides the regulatory and genetic circuits that will be selected in a given environmental condition (Dekel et al. 2005; Babu and Aravind 2006; Camas et al. 2006; Zaslaver et al. 2006; Kalisky et al. 2007; Tanase–Nicola and Ten Wolde 2008). Characterizing and quantifying the cost is also important for improving the application of cells for the production of a desired protein. Thus, characterizing the effect of cost of protein synthesis on growth is important in biotechnology industries where microorganisms are used to produce bio-products (Shachrai et al. 2010).

P. Malakar
Department of Biosciences and Bioengineering,
Indian Institute of Technology Bombay,
Mumbai 400076, India

K. V. Venkatesh (✉)
Department of Chemical Engineering,
Indian Institute of Technology Bombay,
Powai,
Mumbai 400076, Maharashtra, India
e-mail: venks@iitb.ac.in

Studies have shown that the cost varies during the growth of the cells, with the cost being predominant in the early exponential phase (EEP) as compared to the late exponential phase (LEP). The early and late phases were defined as the first 3.5 h and the last 2.5 h of the exponential growth phase, respectively. Various reasons ranging from limitation of ribosomal capacity to chaperon proteins have been hypothesized to explain this phenomenon (Dennis 1996; Tagkopoulos et al. 2008; Mitchell et al. 2009; Condon et al. 1995; Artsimovitch et al. 2004; Magnusson et al. 2005). Studies have also shown a high cost in EEP during the production of green fluorescent protein (GFP) (Shachrai et al. 2010).

The lactose system in *E. coli* is well characterized and is a good candidate to quantify cost. The current study obtains insights into the phenomenon of cost during the growth of *E. coli* on glycerol and during the expression of β -galactosidase through the lactose system using isopropyl- β -D-thiogalactopyranoside (IPTG). The effects of both IPTG and glycerol concentration on the cost have been quantified. The cost in both EEP and LEP has also been measured. The study concludes that cost is not only dependent on the amount of carbon source but also on the amount of inducer of an enzyme in the medium. Further, Monod's equation is used to rationalize the effect of cost on the growth.

Materials and methods

Strains, media, and reagents The strain *E. coli* MG1655 was obtained from the lab of Dr. Manjula Reddy, CCMB, India. All the experiments were in M9 defined medium consisting of M9 salts, 1 mM MgSO₄, 0.1 mM CaCl₂, 0.1% glycerol, 0.200 mM IPTG, and specified concentrations glycerol (Merck). The Z-buffer (pH 7.0) contained 60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, and 50 mM 2-mercaptoethanol. Ortho-nitrophenyl- β -D-galactopyranoside (ONPG; pH 7.0) contained 40 mg ONPG dissolved in 10.0 ml of 0.1 potassium phosphate buffer. Potassium phosphate buffer (0.1 M) contained: make solution A, 27.2 g KH₂PO₄ in 1 L water; make solution B, 34.8 g K₂HPO₄ in 1 L water; and mix 39 ml solution A and 61 ml solution. For the cost experiment, specified concentrations of IPTG obtained from (Invitrogen) were used.

Growth rate measurements The exponential growth rate was measured by growing the strains in 50 ml culture in 250-ml flask. These flasks were incubated on shaker at 37°C at 240 rpm. Samples were taken and reading carried out at 595 nm using ELISA Reader (BioRad). Experiments were performed in triplicate and repeated thrice on separate days.

The data are presented as mean and standard deviation of these nine experiments.

Beta-galactosidase assay Cells were grown on M9 medium with glycerol as the carbon source. Aliquots of culture were taken at fixed optical density (OD). The cells were centrifuged and resuspended in 1 ml Z-buffer and were later placed on ice. The OD of cell suspension was measured at 600 nm. Appropriate dilution was performed to obtain an OD in the range of 0.5 to 1.0. Using Pasteur pipette, 80 μ l of 0.1% SDS and 160 μ l of chloroform were added to each tube. The tube was vortexed for 15 s. The reaction mixture was incubated at 30°C for 15 min; 160 μ l of 4 mg/ml ONPG was added and vortexed well for 10 s and further incubated at 30°C and timed. The reaction tube was removed after about 10–15 min (empirically determined by color). The reaction was quenched by adding 400 μ l of 1 M sodium carbonate. The cell debris was spun down. The OD of the aliquot was measured at 420 nm. The Miller units were calculated using the following formula: $U = 1,000 \times [(OD_{420}) / ((Time) \times (Vol) \times OD_{595})]$, where Vol is the volume of the culture used in the assay in millilitre, and Time is in minutes.

Cost determination Cost was estimated by growing the cells on glycerol with and without IPTG, and the normalized relative decrease in the growth rate was defined as cost:

$$Cost = \frac{\mu_n - \mu_I}{\mu_n} \times 100 \quad (1)$$

where μ_n is the growth rate on glycerol without IPTG and μ_I is the growth rate on glycerol with IPTG. Cost in EEP and LEP were obtained by estimating growth rate in the initial growth period (0 to 3.5 h) for EEP and in the later growth period (3.5 to 6 h) for LEP.

Results

To characterize the cost, as obtained from the relative decrease in the growth rate, at various IPTG concentrations, cells were grown in a medium containing 1 g/L glycerol concentration with varying IPTG concentrations in the range of 0–300 μ M. Figure 1a shows the relative drop in the growth rate at various IPTG concentrations. It was observed that the growth rate dropped from 0.47 h⁻¹ in absence of IPTG to 0.4 h⁻¹ at high IPTG concentration. Thus, the cost, defined as the percentage drop in the growth rate due to unneeded protein production, was determined to be 15% at high IPTG concentrations. The cost was found to increase with IPTG concentration and saturated beyond 100 μ M of IPTG. A threefold increase in the cost was

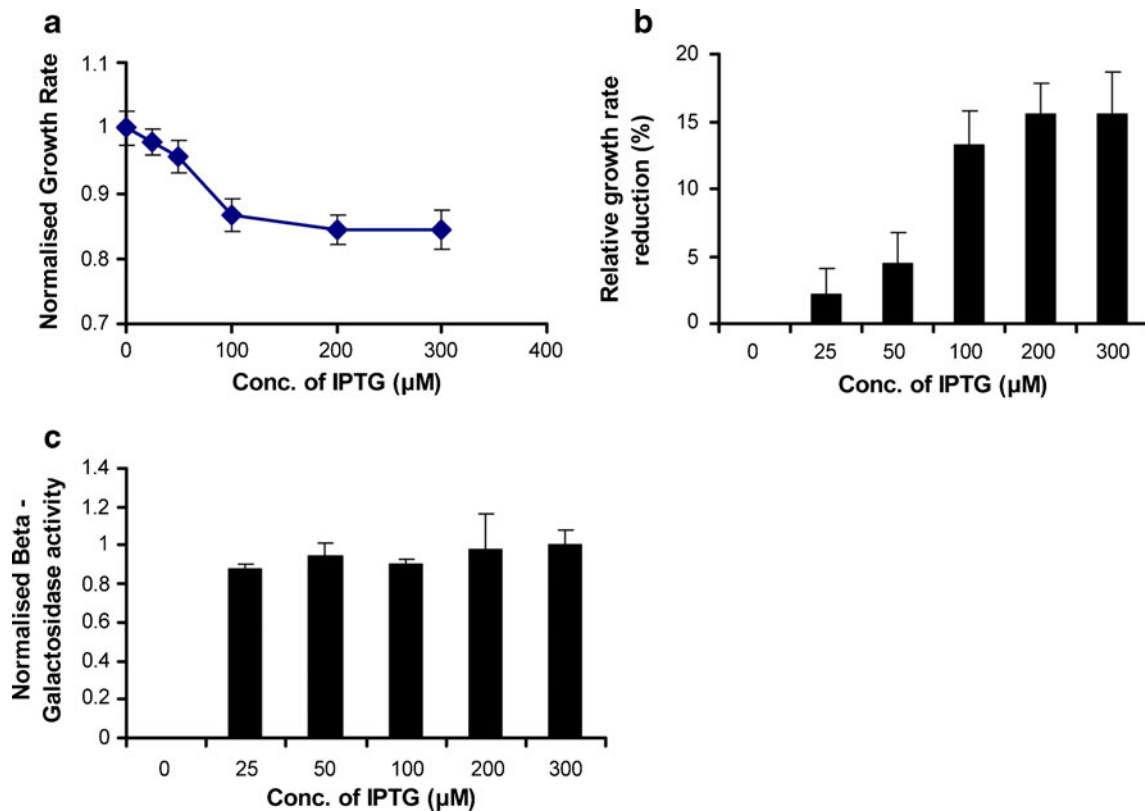


Fig. 1 The effect of IPTG concentrations on the growth of *E. coli* at a fixed glycerol concentration of 1 g/L. **a** Normalized specific growth rate at various IPTG concentrations. The specific growth rate was normalized by 0.46 h^{-1} which was observed for 1 g/L of glycerol in

absence of IPTG. **b** Cost, which is the relative reduction in growth rate as percentage at various IPTG concentrations. **c** The normalized β -galactosidase activity at different IPTG concentrations. The maximum β -galactosidase unit measured was 3,624 Miller units

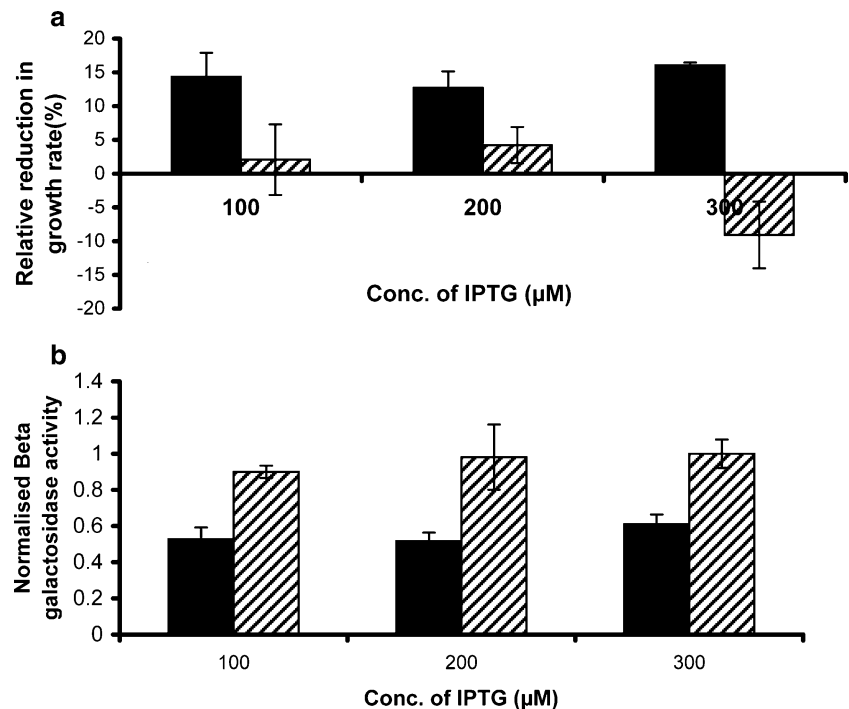
observed for a change in IPTG concentration from 50 to 100 μM (Fig. 1b). A regression fit of a Hill equation to the cost with respect to IPTG indicated a Hill coefficient of 2 and a half-saturation coefficient of 60 μM IPTG, indicating a sharp increase in the drop around the half-saturation coefficient. Figure 1c shows the normalized β -galactosidase activity with the maximum observed at 200 μM of IPTG. It can be observed that the β -galactosidase activity saturates beyond 25 μM of IPTG. Further, the β -galactosidase activity did not correlate with the reduction in the growth due to the introduction of IPTG. While the cost saturated around 100 μM of IPTG, the protein concentration saturated at a lower concentration of 25 μM of IPTG. This suggests that protein synthesis alone cannot explain the observed cost.

The reduction in the growth rate can be characterized at various time points during growth of the cells since experiments capture the dynamic behavior of the growth. Growth rate was characterized both in the early and late exponential phases. The relative reduction in growth was determined relative to the growth at early exponential phase (EEP) and late exponential phase (LEP) on 1 g/L glycerol without any IPTG. It is clear from Fig. 2a that the cost was

higher in the EEP as compared to that observed in the LEP on 1 g/L glycerol with IPTG compared to the growth on 1 g/L glycerol without IPTG. A four- to sixfold drop in the cost was observed in 100–200 μM concentration range of IPTG in LEP. Interestingly, a growth advantage of about 5% was observed for an IPTG concentration of 300 μM in LEP. Figure 2b shows the net normalized β -galactosidase expression in EEP and LEP for 100, 200, and 300 μM of IPTG. It is noted from the figure that the protein expression is about 30% more in the LEP as compared to that in EEP. This indicated that lower amount of protein was synthesized in the LEP as compared to EEP. This further suggests that the amount of unneeded protein synthesized does not explain the observed cost and the physiology of the cell may also play a significant role in characterizing cost.

Cells were grown at different glycerol concentration to study its effect on the cost using 200 μM of IPTG and a medium lacking IPTG. Figure 3a shows the comparison of growth rate on glycerol with and without IPTG in the medium. A Hill equation was fitted for the two cases. The Hill equation fit is shown as a solid line for medium with 0 μM of IPTG and dashed line for growth on glycerol with

Fig. 2 Comparison of cost and enzyme synthesis in the EEP and LEP. **a** Relative reduction in growth as percentage at various concentrations of IPTG. **b** Normalized β -galactosidase activity at various concentrations of IPTG. Dark shaded box represents EEP and shaded box represents LEP



200 μM of IPTG. The fit obtained for the two cases are as follows:

$$\mu_0 = 0.49 \left[\frac{Gly^{1.17}}{0.044^{1.17} + Gly^{1.17}} \right] \quad (2)$$

$$\mu_{200} = 0.49 \left[\frac{Gly^{0.96}}{0.09^{0.96} + Gly^{0.96}} \right] \quad (3)$$

where μ_0 and μ_{200} are the growth rate in per hour for 0 and 200 μM of IPTG, respectively. It is clear from the above equations that the Hills coefficient is close to one indicating a typical Monod's equation. The half-saturation constant was double ($K_s=0.09 \mu\text{M}$) for growth on 200 μM of IPTG compared to the growth in medium lacking IPTG ($K_s=0.044 \mu\text{M}$). The maximum growth rate for both the cases was the same and was equal to 0.49 h^{-1} . The cost was determined by determining the percentage decrease in the growth rate at each glycerol concentration (see Fig. 3b). The cost clearly decreased with increase in the glycerol concentration and became zero at high glycerol concentration (for 3 g/L). No growth was observed for glycerol concentration below 0.01 g/L when grown in 200 μM of IPTG indicating a cost of 100%. Figure 3c shows the normalized β -galactosidase activity at various concentration of glycerol with a fixed IPTG concentration of 200 μM . The activity of β -galactosidase increased with increase in the glycerol concentration. The cost was less than 10% for glycerol concentration greater than 1 g/L for which the β -galactosidase activity was the maximum (see

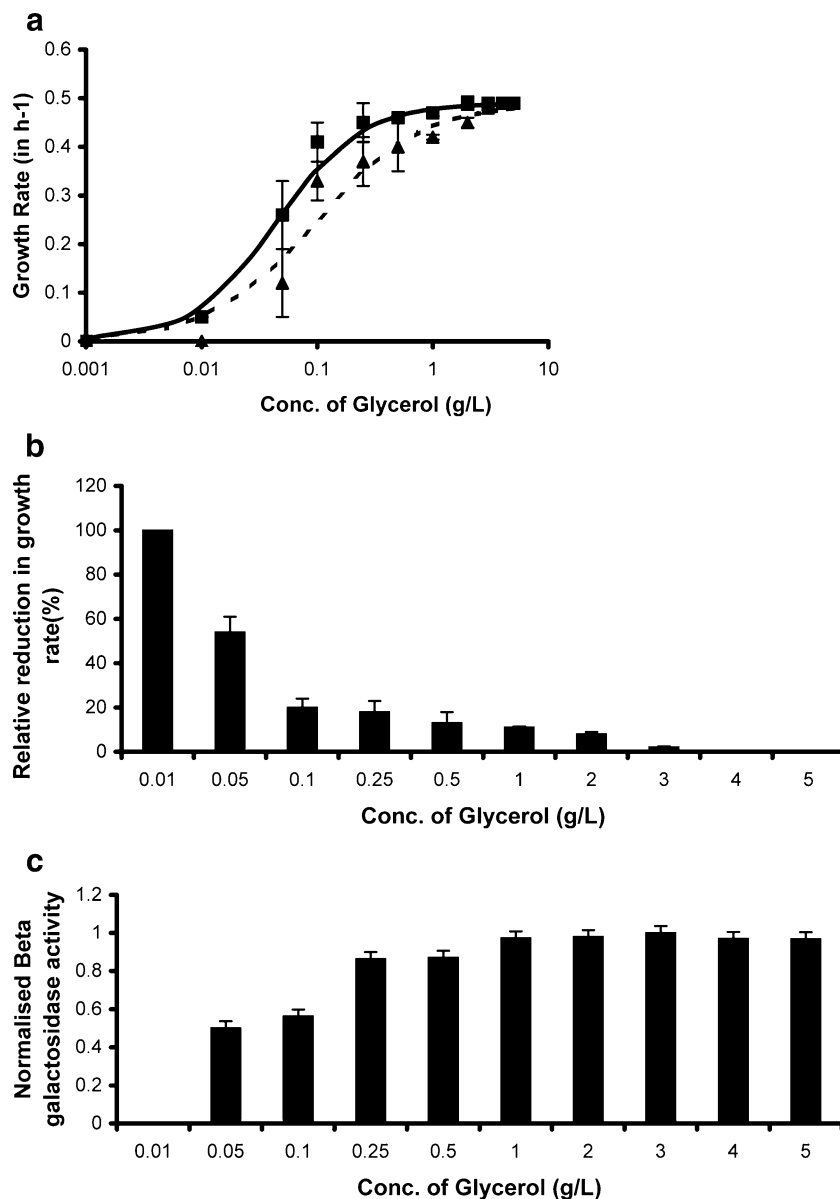
Fig. 3b). It can again be noted that the cost cannot be explained only through the amount of protein synthesized.

The growth rate which was determined for growth on glycerol in the presence of IPTG was used to determine the apparent half-saturation constant (k_a) using a Monod's expression, as given below:

$$\mu = 0.49 \left[\frac{Gly}{K_a + Gly} \right] \quad (4)$$

The value of K_a was determined from the growth rate (μ) obtained for growth on various concentration of IPTG (see Fig. 1a) compared to the growth on 1 g/L glycerol in the absence of IPTG. The normalized apparent substrate saturation constant, K_a , is plotted in Fig. 4a for different value of IPTG concentration. The normalized value of K_a yields the fold change observed in the value of substrate saturation constant over that obtained for growth in the absence of IPTG. The normalized value of K_a increased up to 100 μM of IPTG and saturated at a value of 6.5. Thus, the cost due to IPTG can be rationalized as an increase in the substrate saturation constant (K_s) in the Monod's equation. Similar apparent substrate saturation constant was also determined for various glycerol concentrations at 200 μM of IPTG (see Fig. 3a). Figure 4b shows such an apparent substrate saturation value for different concentration of glycerol. It is clear from the figure that the normalized value of K_a increased up to 2 g/L of glycerol and, beyond which, it reduced and reached a value of one beyond 4 g/L of glycerol. This indicated that the enzyme synthesis and IPTG of 200 μM offered a burden for growth

Fig. 3 The effect of glycerol concentration on the growth, cost, and enzyme synthesis.
a Specific growth rate (in per hour) at various concentrations of glycerol. *Solid squares* and *triangles* represent growth on 0 and 200 μM of IPTG, respectively. *Solid* and *shaded lines* represent Hill equation fit for 0 and 200 μM of IPTG, respectively.
b Relative reduction in growth as percentage at various concentrations of glycerol.
c β -Galactosidase activity at different concentration of glycerol for 200 μM of IPTG



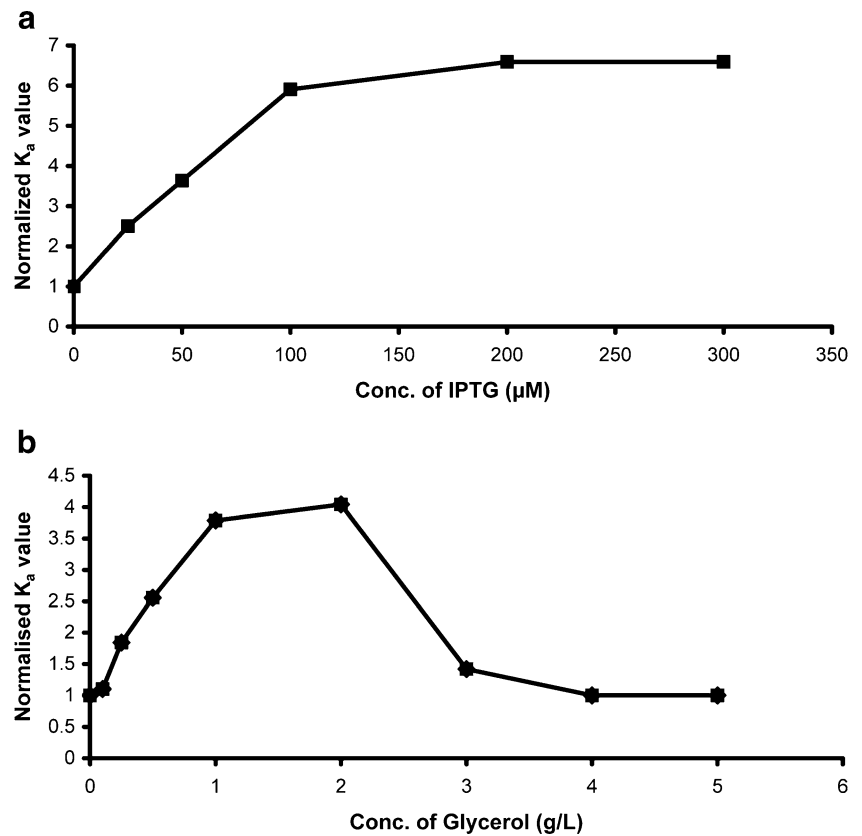
up to a glycerol concentration of 2 g/L. The excess glycerol beyond 2 g/L can compensate for the burden since the relative K_a value decreased. The growth was found to be normal without any burden beyond 4 g/L of glycerol. The increase observed in the value of K_a does not match the trend indicated by the burden on growth at various concentration of glycerol (see Fig. 3b). While the burden decreases monotonically, the value of K_a increases and saturates before decreasing monotonically. This is essentially due to the limiting substrate concentration for which a lower K_a value can match the decrease in the growth due to cost. Further, for a glycerol concentration of 0.01 g/L and 200 μM of IPTG where no growth was observed, a precise value of K_a could not be determined, although Eq. 4 predicts a very high value with a value of K_a approaching infinity. This may be due to errors in precise measurement

of the growth rates at such low concentrations of glycerol. It can be also be noted that a Monod fit yields a twofold increase in the K_s value for growth with IPTG on an average over the range of glycerol concentrations as compared to growth without IPTG (see Eqs. 2 and 3).

Discussion

Protein synthesis is known to burden the cells for their capacity to grow. Therefore, protein synthesis is optimized through evolution to maximize growth. This has been demonstrated by Dekel et al. in *E. coli* for the growth on lactose through the *lacZ* expression (Dekel and Alon 2005). We report here the cost as determined by the decrease in growth rate on glycerol, where we initially assumed that the

Fig. 4 The normalized apparent substrate saturation constant, K_a , **a** for growth on 1 g/L glycerol and various IPTG concentration and **b** for growth on 200 μ M IPTG and various glycerol concentration. The values were normalized by 0.044 g/L, the substrate saturation constant (K_s), observed for the normal growth in absence of IPTG



cost was due to the unneeded expression of the Lac proteins. Firstly, the cost was estimated at different IPTG concentration for fixed glycerol concentration of 1 g/L. As expected, the cost increased with increasing concentration of IPTG up to 100 μ M, beyond which the cost saturated. The β -galactosidase activity could not be correlated with the cost estimation, since the enzyme activity saturated at 25 μ M of IPTG while the cost saturated beyond 100 μ M of IPTG. This clearly indicated that the cost may not simply be related to the synthesis of β -galactosidase. These experiments indicated that IPTG also has a role to play in determining the cost. It is reported that the uptake of IPTG into the cells involves the use of proton pumps leading to a burden to the cells (Seol and Shatkin 1992; Krzewinski et al. 1996).

Experiments also suggested that the cost is higher in the early exponential phase (EEP) than in the late exponential phase (LEP). This result is in agreement with Shachrai et al. where they elegantly show with the help of GFP production in *E. coli* that the phenomenon of cost is more predominant in the EEP as compared to the LEP. Thus, it appears that the cost is higher in the EEP as compared to the LEP and is not only restricted to the production of foreign proteins but is also applicable to the expression of unneeded host proteins. This may be particularly due to the lower synthesis of Lac proteins in LEP since most of the induction occurs in the early phase of growth. Further, work reported by Dykhuizen

and colleagues suggest that cost is not related to the total protein concentration but in the process of transcription and translation (Stoebel et al. 2008).

Experiments with varying glycerol concentration demonstrated that the cost reduced with increasing glycerol concentration and was nullified at higher saturating concentrations. This may prove useful in biotechnological industries where microorganisms are used for the productions of foreign proteins through inducible systems. One possible explanation for the reversion of cost at higher substrate is that, at higher substrate concentration, more resources are available for the expression of unneeded protein production. Cost occurs when the expression of unneeded protein production uses the cellular resources that are required for the production of essential proteins, such as ribosomal proteins. In a scenario where the resources are in excess, such a condition may not arise, as a result of which the cells do not face a burden at higher substrate concentration. Thus, the cost is not a fixed amount but is highly dependent on environmental conditions, such as concentration of substrate in the medium.

Thus, in the case of Lac protein expression, it appears that cost not only depends on the IPTG concentration but also on the substrate concentration. The work reported here supports the theory proposed by Vind et al. (1993) which states that high cost results when ribosomes are the limiting factor. A limited number of ribosomes would then limit

protein synthesis (Vind et al. 1993; Dennis 1996), resulting in lower amounts of enzymes required for growth. In such a scenario, the growth will be limited and production of unneeded proteins will result in a cost for the organism. However, at high concentrations of substrate, there may be enough energy for synthesizing both sufficient ribosomes and the unneeded protein, resulting in no cost. In such a case, the rate of DNA synthesis, NADH reoxydation or substrate uptake rate itself may limit the growth.

The determination of apparent substrate saturation constant (K_a) was helpful in characterizing and quantifying the influence of the substrate and IPTG concentrations on the cost. For growth on 1 g/L glycerol and varying IPTG concentration, the K_a value increased up to 100 μ M IPTG correlating with the enzyme synthesis. For growth on varying glycerol concentration and fixed IPTG concentration, the parameter could characterize two regions, one, a phase of increasing cost in the initial glycerol concentration range between 0 and 2 g/L, and second, a phase wherein the cost decreased and saturated to zero beyond 2 g/L of glycerol. The first region characterizes the influence of limiting resources for growth due to burden, while the second region represents the excess energy levels compensating for the cost. Thus, the apparent substrate saturation constant could correlate the effect of cost on the growth burden effectively.

In summary, the concept of high cost in the early exponential phase and low cost in the late exponential phase is applicable to expression of unneeded expression of host proteins. Both the protein expression and IPTG concentration influenced the burden on the cells at a fixed glycerol concentration. The substrate concentration also plays an important factor in determining the cost. Cost can be minimized by growing the culture at high substrate concentration when foreign proteins need to be expressed inside the host.

Acknowledgments Pushkar Malakar acknowledges the support provided by Council of Scientific and Industrial Research (CSIR), India for his research fellowship.

References

- Alon U (ed) (2006) An introduction to systems biology: design principles of biological circuits. Chapman & Hall/CRC, Boca Raton
- Andrews KJ, Hegeman GD (1976) Selective disadvantage of non functional protein synthesis in *Escherichia coli*. *J Mol Evol* 8(4):317–328
- Artsimovitch I, Patlan V, Sekine S-I, Vassilyeva MN, Hosaka T, Ochi K, Yokoyama S, Vassilyev DG (2004) Structural basis for transcription regulation by alarmone ppGpp. *Cell* 117(3):299–310
- Babu MM, Aravind L (2006) Adaptive evolution by optimizing expression levels in different environments. *Trends Microbiol* 14(1):11–14
- Camas FM, Blázquez J, Poyatos JF (2006) Autogenous and non-autogenous control of response in a genetic network. *Proc Natl Acad Sci USA* 103(34):12718–12723
- Condon C, Squires C, Squires CL (1995) Control of rRNA transcription in *Escherichia coli*. *Microbiol Rev* 59(4):623–645
- Dekel E, Alon U (2005) Optimality and evolutionary tuning of the expression level of a protein. *Nature* 436(7050):588–592
- Dekel E, Mangan S, Alon U (2005) Environmental selection of the feed-forward loop circuit in gene-regulation networks. *Phys Biol* 2(2):81–88
- Dennis HBAPP (1996) Modulation of chemical composition and other parameters of the cell at different exponential growth rates. In: Neidhardt FC (ed) *Escherichia coli* and *Salmonella*: cellular and molecular biology. American Society for Microbiology, Washington, pp 1553–1569
- Dong H, Nilsson L, Kurland CG (1995) Gratuitous overexpression of genes in *Escherichia coli* leads to growth inhibition and ribosome destruction. *J Bacteriol* 177(6):1497–1504
- Horiuchi T, Tomizawa JI, Novick A (1962) Isolation and properties of bacteria capable of high rates of β -galactosidase synthesis. *Biochim Biophys Acta* 55(1–2):152–163
- Kalisky T, Dekel E, Alon U (2007) Cost-benefit theory and optimal design of gene regulation functions. *Phys Biol* 4(4):229–245
- Koch AL (1988) Why can't a cell grow infinitely fast? *Can J Microbiol* 34:421–426
- Krzewinski F, Brassart C, Gavini F, Bouquelet S (1996) Characterization of the lactose transport system in the strain *Bifidobacterium bifidum* DSM 20082. *Curr Microbiol* 32(6):301–307
- Maaloe O, Niels O (1966) Control of macromolecular synthesis: a study of DNA, RNA, and protein synthesis in bacteria. Benjamin, New York
- Magnusson LU, Farewell A, Nyström T (2005) ppGpp a global regulator in *Escherichia coli*. *Trends Microbiol* 13(5):236–242
- Mitchell A, Romano GH, Groisman B, Yona A, Dekel E, Kupiec M, Dahan O, Pilpel Y (2009) Adaptive prediction of environmental changes by microorganisms. *Nature* 460(7252):220–224
- Nguyen TNM, Phan QG, Duong LP, Bertrand KP, Lenski RE (1989) Effects of carriage and expression of the Tn10 tetracycline-resistance operon on the fitness of *Escherichia coli* K12. *Mol Biol Evol* 6(3):213–225
- Seol W, Shatkin AJ (1992) *Escherichia coli* alpha-ketoglutarate permease is a constitutively expressed proton symporter. *J Biol Chem* 267(9):6409–6413
- Shachrai I, Zaslaver A, Alon U, Dekel E (2010) Cost of unneeded proteins in *E. coli* is reduced after several generations in exponential growth. *Mol Cell* 38(5):758–767
- Stoebel DM, Dean AM, Dykhuizen DE (2008) The cost of expression of *Escherichia coli* lac operon proteins is in the process, not in the products. *Genetics* 178(3):1653–1660
- Tagkopoulou I, Liu Y-C, Tavazoie S (2008) Predictive behavior within microbial genetic networks. *Science* 320(5881):1313–1317. doi:10.1126/science.1154456
- Tanase-Nicola S, Ten Wolde PR (2008) Regulatory control and the costs and benefits of biochemical noise. *PLoS Comput Biol* 4(8)
- Vind J, Sorensen MA, Rasmussen MD, Pedersen S (1993) Synthesis of proteins in *Escherichia coli* is limited by the concentration of free ribosomes. Expression from reporter genes does not always reflect functional mRNA levels. *J Mol Biol* 231(3):678–688
- Weiner ANAM (1957) Enzyme induction as an all or none phenomenon. *Proc Natl Acad Sci USA* 43(7):553–566
- Zaslaver A, Mayo A, Ronen M, Alon U (2006) Optimal gene partition into operons correlates with gene functional order. *Phys Biol* 3(3):183–189