Appl Biochem Biotechnol (2010) 162:654–661
DOI 10.1007/s12010-009-8836-0

Simulated Microgravity Affects Growth of *Escherichia coli* and Recombinant β-D-Glucuronidase Production

Liang Xiang · Feng Qi · DaZhang Dai · Chun Li · YuanDa Jiang

Received: 2 July 2009 / Accepted: 25 October 2009 /
Published online: 18 November 2009

Abstract Effects of simulated microgravity (SMG) on bacteria have been studied in various aspects. However, few reports are available about production of recombinant protein expressed by bacteria in SMG. In this study growth of *E. coli* BL21 (DE3) cells transformed with pET-28a (+)-pgus in double-axis clinostat that could model low shear SMG environment and the recombinant β-D-glucuronidase (PGUS) expression have been investigated. Results showed that the cell dry weights in SMG were 16.47%, 38.06%, and 28.79% more than normal gravity (NG) control, and the efficiency of the recombinant PGUS expression in SMG were 18.33%, 19.36%, and 33.42% higher than that in NG at 19 °C, 28 °C, and 37 °C, respectively ($P<0.05$).

Keywords Simulated microgravity (SMG) · Normal gravity (NG) · Recombinant PGUS · Expression · *E. coli*

Introduction

Microgravity has a significant effect on numerous microbial characteristics. However, little is known about the mechanisms through which microbial cells sense the changing gravity conditions and also how do they convert these mechanical signals into molecular and biochemical responses. Due to a long flight time and hard to meet the rigorous flight standards, several forms of low-shear suspension culture that could model aspects of spaceflight studies of the effect of "weightlessness" were invented [1]. There are only a few studies which demonstrated that several microbial cellular processes, such as cell growth,

L. Xiang and F. Qi contributed equally to this work.

L. Xiang · F. Qi · D. Dai · C. Li (✉)
School of Life Science, Beijing Institute of Technology, South Zhongguancun Street, Beijing 100081, People's Republic of China
e-mail: lichun@bit.edu.cn

Y. Jiang
Center for Space and Applied Research Chinese Academy of Sciences, Beijing 100190, People's Republic of China

pathogenesis, and gene expression, were altered when cells were placed in microgravity or simulated microgravity (SMG) [2]. Other relevant studies have been reported on the effect of microgravity on resistance to radiation, phage induction [3], contracting of the cell size [4], altered secondary-metabolite productions [5–8], and efficient utilization of nutrients [9]. In fact, the results which microgravity or SMG actually lead to a shortened lag phase, an increase in growth rate and higher final cell count during the stationary phase as compared with the normal gravity (NG) controls are coincident and reported more frequently [4, 10]. A number of recombinant pharmaceutical proteins or enzymes have been produced by *Escherichia coli* or *Pichia pastoris* but the main problems with these expression systems were the efficiency and productivity [11]. Furthermore, few reports are available about the effects of SMG on the expression of recombinant proteins by bacteria cells [12]. So it is necessary to reveal what influences SMG can impose on recombinant proteins production.

β-D-glucuronidase from *Penicillium purpurogenum* Li-3 (PGUS) can directly biosynthesize glycyrrhetinic acid monoglucuronide (GAMG) from glycyrrhizin (GL) [13]. GAMG is useful in clinical treatments on inflammatory diseases and more absorbable than GL [13, 14]. We previously transformed PGUS gene using the vector pET-28a (+) into *E. coli* BL21 (DE3) expression system because the PGUS productivity of *P. purpurogenum* Li-3 was limited. In this study we examined the growth of *E. coli* BL21 (DE3) and the expression of the recombinant protein PGUS in double-axis clinostat (DAC) as SMG condition and in shaker flask as NG control. The DAC can create a low shear and low turbulence suspension culture environment that is close to microgravity similar to other rotating-wall vessels (RWV) [1, 3]. Our research aimed at a preliminary application of production of recombinant enzyme in SMG environment.

Materials and Methods

Bacterial Strain, Plasmid

E. coli BL21 (DE3) used as the host cell was transformed with pET-28a (+)-*pgus*. The pgus gene was cloned into pET-28a vector with kanamycin resistance. *E. coli* BL21 strain transformed with pET-28a (+)-*pgus* plasmid was then used for expression of recombinant protein PGUS while induced by isopropyl beta-D-thiogalactopyranoside (IPTG, Sigma-Aldrich).

Double-Axis Clinostat

Double-axis clinostat invented by Center for Space and Applied Research Chinese Academy of Science (CSSAR) was used for cell or tissue engineering applications. It contains two axes which the frame rotates on the horizontal outer axis, and the cell culture vessel rotates on the changing inner axis (Fig. 1). The dimension of DAC cell culture vessel is 12.3 (length)×9.8 (width)×10 cm (height). The *E. coli* cells were cultured in a flask with an aerated hydrophobic membrane fixed within the culture vessel. DAC bioreactor permits cell growth in suspension and minimizes the fluid shear levels encountered by cells. When the cell culture vessel rotates, microbial cells do not settle down but rotate at random directions within the culture medium. The low shear environment associated with cell growth in DAC is the result of a combination of fluidic forces principals and gravitational factors. However, the precise individual contribution of these forces on cells in DAC is not clear and requires further research.

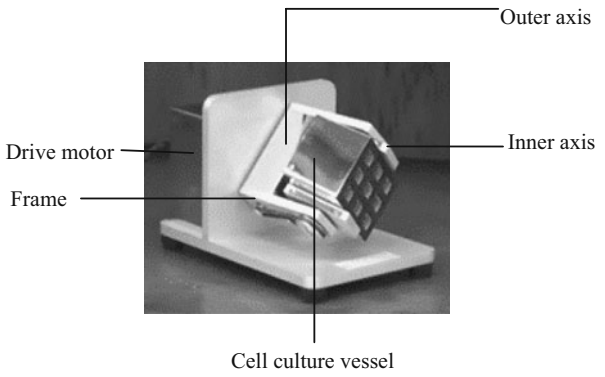

Fig. 1 The double-axis clinostat (DAC) for *E. coli* BL21 (DE3) culture

Growth Curve of *E. coli* BL21 Cells

Cell growth curve of *E. coli* BL21 cells was determined by dry weight at different times at 19 °C, 28 °C, and 37 °C. Samples were collected every 3 h during the cell growth. *E. coli* BL21 cells from a single colony were loaded into culture vessels of DAC (inner axis 10 rpm×outer axis 10 rpm), and simultaneously incubated in shaker flask controls (100 rpm) at the three different temperatures. All bubbles are removed to reduce shear. Three samples were collected from three independent replicate experiments. In the range of 5–10 rpm of both axes the microbial cells can keep suspended (supplied by CSSAR). We set the speed of inner axis 10 rpm × outer axis 10 rpm because the cells could keep suspended well and grew better in this condition, which identified in our previous experiments. Unlike other RWV [3, 15, 16], the DAC itself does not simulate the normal gravity, so we choose the shaker flask as the NG control.

Induction of the Recombinant PGUS

An overnight bacterial culture was inoculated into 30-ml LB-medium containing 50 $\mu g \cdot ml^{-1}$ kanamycin, and incubated in a shaker flask at 37 °C for 10 h. Then the *E. coli* cells suspension was diluted (1:100) in a 100-ml culture vessel filled with fresh LB-medium consisting of tryptone 10, yeast extract 5, NaCl 10 g/l, kanamycin 50 $\mu g \cdot ml^{-1}$ at 37 °C, pH 7.0. The DAC kept 10 rpm (inner axis)×10 rpm (outer axis) and all bubbles were removed. After that the *E. coli* cells were induced by addition of IPTG (0.8 mmol/L) for 3 h at 19 °C, 28 °C, and 37 °C in DAC and shaker flask (NG) to product the recombinant protein at different temperatures. After that cells were collected by centrifugation at 4 °C, 8,000 rpm for 15 min. The pellets were suspended in 0.1 M phosphate buffer, pH 7.4, and sonicated (output power 200 W, 2 s sonicated with 4 s intermission, 5 min) on ice. After centrifugation again, the supernatant (soluble protein) and pellet (inclusion body, dissolved in 20 ml 8 M urea) were collected and analyzed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Determination of the Quantities of Recombinant PGUS

A semi-quantitative determination of PGUS expressions were analyzed by PAGE after DAC and shaker flask culture as previously described [17]. SDS-PAGE detection was performed with 12% polyacrylamide gels using the Bis-Tris SDS-PAGE (Invitrogen)

system and gels were stained by Coomassie brilliant blue G250 (Amresco). The quantity of the soluble protein and inclusion body was determined. Bovine serum albumin (BSA, Amresco) is indicated for use as an internal standard to determine the recombinant PGUS concentration from SDS-PAGE. We quantified the PGUS productions in triplicate experiments.

Results

Growth Kinetics of *E. coli* BL21 (DE3) Cells in SMG and NG

Figure 2 shows the dry weights of cells at 19 °C, 28 °C, and 37 °C in SMG and NG. *E. coli* cells BL21 (DE3) in culture vessel of DAC grew better than those in shaker flask control and the final cell dry weight in SMG was 0.0419, 0.0578, and 0.0653 mg/mL, which were 16.47%, 38.06%, and 28.79% higher than that in NG control at the three different temperatures, respectively.

SDS-PAGE Analysis of the Recombinant PGUS in SMG and NG

The part of the protein expressed in *E. coli* BL21 (DE3) is inevitably precipitated into inclusion bodies. SDS-PAGE analyses of culture supernatant and pellet were collected at 19 °C, 28 °C, and 37 °C from culture of *E. coli* BL21 (DE3) in DAC and shaker flask (Fig. 3). The results of semi-quantitative determination indicated a good linear relationship between densitometric readings (ProExpress Imaging System, Perkin Elmer) after staining with Coomassie brilliant blue R250 and protein concentration. The linear range of PGUS was 0.20–3.6 μg.

Efficiency of the Recombinant Proteins Expression in SMG and NG

The value of $\frac{M\text{protein}}{M\text{dryweight}}$ (micrograms of protein per cell dry weight) is used for estimation of efficiency of the recombinant PGUS expression in SMG and NG at three different temperatures. We can find that the efficiency of the total recombinant PGUS expression

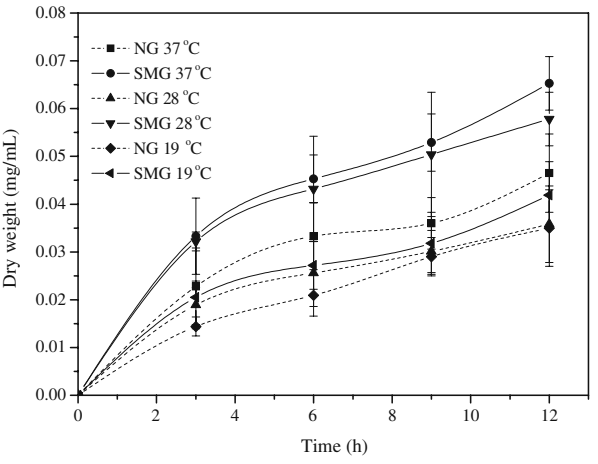

Fig. 2 Growth curve in SMG and NG of *E. coli* BL21 (DE3) at 19 °C, 28 °C, and 37 °C. Each data point of dry weight is a mean ± SE of three samples from three independent replicate experiments

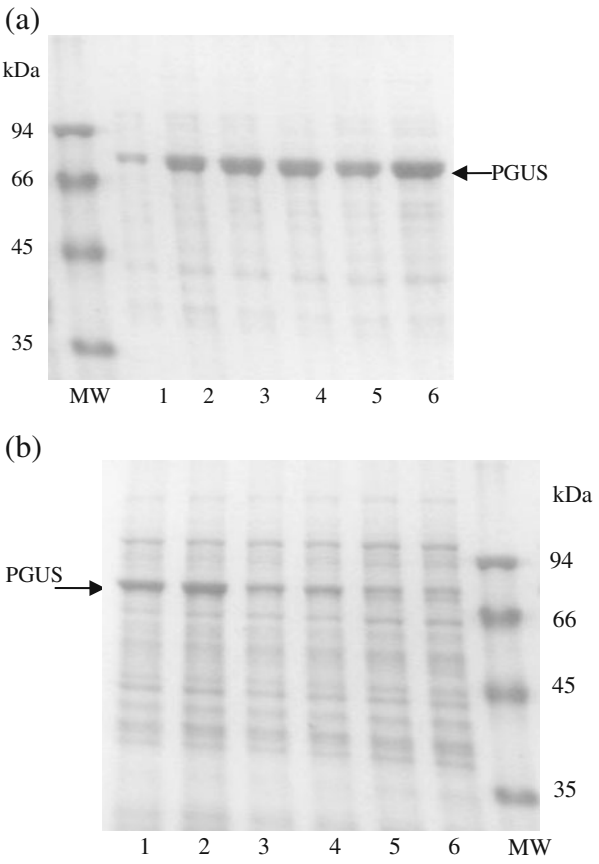

Fig. 3 **a** Analysis of recombinant inclusion bodies by SDS-PAGE collected from cell culture of *E. coli* BL21 (DE3). The recombinant PGUS expressed and conducted in three temperatures in SMG and NG. *MW* molecular weight marker. *Lanes 1*, *3*, and *5* stand for PGUS expressed in NG at 19 °C, 28 °C, and 37 °C. *Lanes 2*, *4*, and *6* stand for SMG in the three temperatures. **b** Corresponding analysis of recombinant soluble PGUS by SDS-PAGE. *MW* molecular weight marker. *Lanes 1*, *3*, and *5* stand for PGUS expressed in NG at 19 °C, 28 °C, and 37 °C. *Lanes 2*, *4*, and *6* correspond to SMG in the three temperatures. This is one of our figures for analysis of recombinant PGUS by SDS-PAGE

including of both soluble proteins and inclusion bodies in SMG were higher than that in NG at 19 °C (a), 28 °C (b) and 37 °C (c; Fig. 4). The efficiency of the total recombinant proteins expression in SMG were 18.69%, 25.68%, and 32.82% higher than that in NG at 19 °C, 28 °C, and 37 °C, respectively ($P<0.05$).

Discussion

There are few reports about the expression of recombinant proteins in SMG but the expression systems are limited only to insect or human cell lines [8, 18]. In this study we present the results of growth and the efficiency of the recombinant PGUS expression in SMG and NG using *E. coli* BL21 (DE3) as the host cell transformed with pET-28a (+)-pgus.

The results showed that the growth of *E. coli* cells in culture vessel of DAC was better than those in shaker flask control. In SMG environment, cell cultures remain evenly distributed and fluids remain quiescent. In this convection-absent environment, Brownian motion (diffusion) was the dominant transport mechanism [19] and microbial cells could not sense the direction of gravity. Cultures grown in shaker flask (NG), however, were not equally subjected to these fluid phenomena. These apparent discrepancies may contribute to

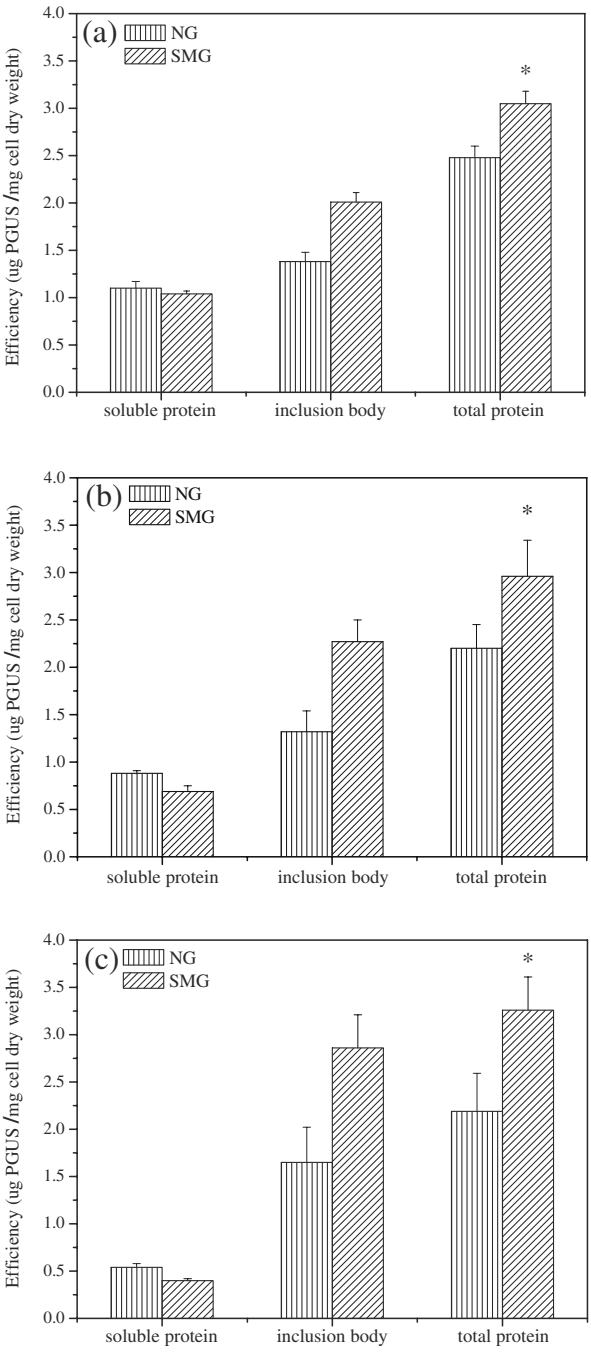

Fig. 4 Efficiency of the recombinant PGUS expression in SMG and NG at 19 °C (a), 28 °C (b), and 37 °C (c), respectively. The value of *micrograms of protein per milligrams of cell dry weight* represents the efficiency of recombinant PGUS expression in SMG and NG. The efficiency of PGUS production was determined through triplicate experiments. There were significant differences in total protein production at three different temperatures between SMG and NG (*P<0.05)

the difference of growth between SMG and NG. The factors involved in the influences of SMG on bacterial growth remained undetermined as no literature is available [1, 10, 20]. Further research on the characterizations of mass diffusion and other chemical alterations of the cellular microenvironment and how these changes affect the microbial metabolic

responses in SMG could offer a new way to elucidate the reasons of the different bacterial growth in SMG and NG.

We also compared the efficiency of the recombinant protein expression in SMG and NG. There was a coincident difference in efficiency of the recombinant PGUS expression in SMG and NG. The efficiencies of the recombinant PGUS expression in SMG are all higher than that in NG at 19 °C, 28 °C, and 37 °C. It appears that physiological changes initiated by SMG are involved in a pathway that increases the secretion of the recombinant PGUS by *E. coli* BL21 (DE3), although we do not know the functional reasons for this phenomenon. Fang et al. reported secretion of the secondary metabolites such as β-lactam antibiotics cephalosporin, microcin B17, and the polyketide macrolide rapamycin produced by *Streptomyces clavuligerus*, *E. coli*, and *Streptomyces hygroscopicus*, respectively, and these substances were inhibited by the environment of SMG [21, 22]. But SMG does not have the universal effects on secondary metabolism, for example, the production of gramicidin by *Bacillus brevis* remained unaffected [7]. These indicate the reasons that different microbes are probably affected in their physiological characterizations by SMG, but they respond to SMG in their own specific ways. The process of recombinant protein production by microbial cells in SMG maybe different with the secondary metabolite production; however, there were no relevant reports about the expression of recombinant proteins by *E. coli* in SMG. Another explanation could be that the levels of transcription and expression of the genes related to recombinant protein production such as molecular chaperones and proteases changed due to the difference of extra-cellular mass transport of nutrients and by-products between SMG and NG, and ultimately the way of recombinant proteins expression was altered. *E. coli* cells will change the genomic expression patterns responding to the environment of SMG [23]. In the low shear SMG conditions, many kinds of *E. coli* genes with known functions were significantly up- or down-regulated by twofold at least [23]. A significant number of genes of yeast cells were also found to be up- or down-regulated by more than one to sevenfold as a result of growth in SMG by DNA microarray analysis [24, 25]. Unfortunately, the effects of SMG on the process of gene regulation and recombinant proteins expression are still unknown. Further studies should be continued and more reliable hypotheses are required to explain the reasons for these differences in SMG and NG.

Such enhanced bacterial growth and efficient recombinant proteins expression could have significance for the industrial applications. If we are able to increase the efficiency of fermentation process by SMG techniques on ground, there will be a substantial economic gain and a preliminary foundation of application of recombinant enzymes production in a new era of bio-catalysis.

Acknowledgments This work was funded by the National "863" High-Tech Project (2008AA12A218-2), Natural Science Foundation of China (20776017), and the Natural Science Foundation of Beijing (5072028).

References

1. Nickerson, C. A., Ott, C. M., Wilson, J. W., Ramamurthy, R., & Pierson, D. L. (2004). *Microbiology and Molecular Biology Reviews, 68*, 365–361.
2. Hammond, T. G., Lewis, F. C., Goodwin, T. J., Linnehan, R. M., Wolf, D. A., Hire, K. P., et al. (1999). *Nature Medicine, 5*, 359.
3. Nickerson, C. A., Ott, C. M., Wilson, J. W., Ramamurthy, R., LeBlanc, C. L., Bentrup, K. H. Z., et al. (2003). *Journal of Microbiological Methods, 54*, 1–11.

4. Baker, P. W., Meyer, M. L., & Leff, L. G. (2004). *Microgravity Science and Technology, 15*, 39–44.
5. Fang, A., Pierson, D. L., Mishra, S. K., & Demain, A. L. (2000). *Letters in Applied Microbiology, 31*, 39–41.
6. Fang, A., Pierson, D. L., Mishra, S. K., Koenig, D. W., & Demain, A. L. (1997). *Applied and Environmental Microbiology, 63*, 4090–4092.
7. Fang, A., Pierson, D. L., Mishra, S. K., Koenig, D. W., & Demain, A. L. (1997). *Current Microbiology, 34*, 199–204.
8. Demain, A. L., & Fang, A. (2001). *The Chemical Record, 1*, 333–346.
9. Brown, R. B., Klaus, D., & Todd, P. (2002). *Microgravity Science and Technology, 13*, 24–29.
10. Kacena, M. A., Merrell, G. A., Manfredi, B., Smith, E. E., Klaus, D. M., & Todd, P. (1999). *Applied Microbiology and Biotechnology, 51*, 229–234.
11. Khoo, S. H. G., & Al-Rubeai, M. (2009). *Biotechnology and Bioengineering, 102*, 188–199.
12. Saarinen, M. A., & Murhammer, D. W. (2000). *In Vitro Cellular & Developmental Biology-Animal, 36*, 362–366.
13. Feng, S. J., Li, C., Li, H., & Wang, X. Y. (2007). *Journal of Chemical Engineering of Chinese Universities, 21*, 977–982.
14. Matsui, S., Matsumoto, H., Sonoda, Y., Ando, K., Aizu-Yokota, E., Sato, T., et al. (2004). *International Immunopharmacology, 4*, 1633–1644.
15. Schwarz, R. P., Wolf, D. A., & Trinh, T. (1991). Rotating cell culture vessel. U.S. patent 5,026,650.
16. Hammond, T. G., & Hammond, J. M. (2001). *American Journal of Physiology-Renal Physiology, 281*, F12–F25.
17. Claeys, E., Uytterhaegen, L., Buts, B., & Demeyer, D. (1995). *Meat Science, 39*, 177–193.
18. Navran, S. (2007). *Cell technology for cell products*, 567–569.
19. Benoit, M. R., & Klaus, D. M. (2007). *Advances in Space Research, 39*, 1225–1232.
20. Klaus, D., Simske, S., Todd, P., & Stodieck, L. (1997). *Microbiology, 143*, 449–455.
21. Fang, A., Pierson, D. L., Mishra, S. K., & Demain, A. L. (2000). *Applied Microbiology and Biotechnology, 54*, 33–36.
22. Fang, A., Pierson, D. L., & Mishra, S. K. (1997). *Journal of Industrial Microbiology and Biotechnology, 18*, 22–25.
23. Tucker, D. L., Ott, C. M., Huff, S., Fofanov, Y., Pierson, D. L., Willson, R. C., et al. (2007). *BMC Microbiology, 7*, 1–16.
24. Sheehan, K. B., McInnerney, K., Purevdorj-Gage, B., Altenburg, S. D., & Hyman, L. E. (2007). *BMC Genomics, 8*, 1–12.
25. Johanson, K., Allen, P. L., Lewis, F., Cubano, L. A., Hyman, L. E., & Hammond, T. G. (2002). *Journal of Applied Physiology, 93*, 2171–2180.