Microgravity and its implications for fermentation biotechnology

David M. Klaus

Fermentation processes are highly dependent upon physical and chemical environmental parameters, many of which are influenced by gravity. Extending biotechnology into the realm of space flight provides researchers with an opportunity to investigate the role that gravity plays in natural growth processes. Physical factors governing cell sedimentation, nutrient mixing and byproduct dispersion are altered in the absence of the constant sedimenting force of gravity. In addition, space flight has also been shown to give rise to a wide variety of indirect consequences associated with the physiology of the organisms themselves.

Fermentation technology traditionally involves the use of microorganisms to produce commercial products by stimulating the overproduction of either primary or secondary metabolites. Microbial byproducts include chemicals and pharmaceuticals, energy sources, foods and agricultural compounds. In addition, the use of plant and mammalian cells is becoming more common in bioprocessing technology. The fundamental technical principles governing each stage of product formation in bioprocessing are, however, essentially similar, regardless of the organism being cultivated or the byproduct being collected. Basic parameters such as medium composition and temperature must be controlled for optimal stimulation of the desired biological process occurring within a contained environment[1]. In space, the forcing function of gravity is eliminated. Aside from the direct physical consequences associated with this change, many physiological responses have also been shown to occur as a result[2–4].

Unique attributes required of space-flight bioprocessing hardware

The fundamental technical concerns associated with bioprocessing in space deal with practical issues not unlike those affecting terrestrial operations, such as methods of fluid containment and mixing, but have the extra concern of providing these functions in a weightless environment. In addition to the general concerns of handling biological specimens, safety requirements for preventing spills that would 'float' throughout the habitat rather than 'land' on the floor must be satisfied. This is often achieved by incorporating multiple levels of independent containment. Multiple levels of containment can, however, make fluid transfers for inoculation, nutrient addition or sample removal more cumbersome than when performed under typical terrestrial conditions. In addition, launch costs and on-orbit power constraints mandate that designs be of minimal mass and volume, and consume little power. In some sense, reducing the mass of the device is facilitated by the fact that much of the supporting infrastructure associated with familiar laboratory equipment is not needed in the weightless environment of space. At the same time, however, the design must be robust enough to compensate for the vibration and acceleration (~3× Earth's gravity for ~9 minutes) experienced during a shuttle launch. Finally, when one considers that a typical single-locker payload flown in the shuttle middeck runs on less than 130 Watts (at 28 V DC)[5], the hardware design challenges become readily apparent.

The optimal design solution must also be assessed in terms of operational complexity. Carrying out research in space often requires unique protocols not usually considered in terrestrial laboratories. For example, 'late-access' experiments are typically prepared for integration into the space shuttle on the order of 24 h before launch and must take the potential for launch delays into account as well. Labile materials must somehow be stabilized prior to initiating the experiment in space and samples must be preserved for recovery hours after landing. The actual mission operations are planned well in advance and scheduled in a detailed daily timeline for each crewmember. As astronaut time is at a premium, automating the hardware can be advantageous.

Most terrestrial fermentation processes are carried out in stirred reactors, so it is likely that some type of stirring will be implemented for space applications as well; otherwise, fluid mixing and mass transfer are reduced to diffusion only in the absence of free convection. Aeration and gas-separation processes involving two-phase fluid handling require gravity-independent forcing functions, because the buoyant force that normally causes gas bubbles to rise does not exist. This can create difficulties in supplying oxygen uniformly to suspension cultures. Similarly, thermal control is complicated by weightlessness, as local temperature gradients can remain fairly stable in the fluid layer surrounding a non-motile cell or against a container wall rather than rapidly equilibrating in the bulk fluid by natural convection, as occurs on Earth. Many downstream processes also have gravity-dependent characteristics; for example, although some extraction and purification techniques are dependent on weight (e.g. density-driven separation), others may benefit from its absence (e.g. distortion-free two-dimensional-electrophoresis protein separation).

D. M. Klaus (klaus@spot.colorado.edu) is at BioServe Space Technologies, Department of Aerospace Engineering Sciences, University of Colorado, Boulder, CO 80309-0429, USA.

 Copyright © 1998, Elsevier Science Ltd. All rights reserved. 0167 – 7799/98/$19.00. PII: S0167-7799(98)01197-4

One of the most unique attributes of bioprocessing in space may, however, lie in the ability to keep cells suspended in the fluid medium without imparting the significant shear forces that often accompany stirred terrestrial systems. As space-flight opportunities are infrequent, ground-based methods that partially simulate the low-shear environment encountered in actual microgravity are useful in further exploring how cells respond to altered inertial conditions. A device called a clinostat provides a method of keeping cells suspended in a fluid medium without introducing excessive mixing. This state of suspension is accomplished by rotating a slender container completely filled with fluid about its long axis at a predetermined constant velocity. After a brief start-up period, the rotational velocity of the container wall is transferred radially inward until the fluid and particles rotate as a rigid body. This creates a nearly quiescent state of motionlessness for small cells and, therefore, partially simulates one aspect of the reduced-gravity environment (i.e. sedimentation is prevented, but the cells still experience weight)[6].

A specialized bioreactor developed at NASA's Johnson Space Center was similarly designed to maintain cell suspension on Earth with minimal shear stress through slow rotation of the vessel's inner walls[7]; multiple applications of this apparatus have been described recently[8], and the concept is also being evaluated for bioprocessing in space. Many other unique devices exist that enable a broad range of space-flight biological experiments to be carried out.

Physiological effects of space flight on microorganisms

To date, most studies into microbial behavior in space have primarily sought to explain the various reported effects as they pertain to fundamental gravitational-biology questions and immunological concerns[2–4]. By comparison, applied research in this field has been explored to a much lesser extent. This article will focus on two recent space-flight experiments that utilized bacteria and fungi to produce antibiotics. As such, a brief summary of microbial responses to space flight, as relevant to bacterial and fungal fermentation processes, may be useful.

Previous experiments have shown that space flight appears to influence the growth kinetics of bacterial suspension cultures, with reports of the lag phase being shortened[9–12] and the final cell population reaching higher densities[11–13] in space. Other findings include altered sporulation patterns[11], enhanced conjugation efficiency[14] and decreased effectiveness of antibiotics[15,16]. Additionally, bacterial cultures grown on semisolid agar have been reported to exhibit a similarly shortened lag phase, but with no difference in the final cell numbers[17]. Although at least two reports have concluded that, on the contrary, space flight does not affect the growth of bacterial cultures[18,19], the majority of findings suggest that space flight does appear to influence the growth kinetics of bacteria. However, the exact underlying mechanisms by which the reported changes are brought about remain largely undetermined.

Interestingly, similarities can be observed between the growth kinetics of *Escherichia coli* batch cultures in space and specific advantageous bioprocessing characteristics achieved terrestrially using a fed-batch glucose method. It has been demonstrated that using a controlled-feeding technique (on Earth) to limit the availability of glucose resulted in a prolonged growth phase and a subsequent increase in final cell concentration[20,21]. Although not certain, it is possible that a reduction in extracellular mixing in the quiescent fluid environment experienced in weightlessness may give rise to similarly reduced levels of glucose molecules being present in the cell's immediate surroundings, albeit under normal batch conditions.

More-complex organisms have also been shown to exhibit metabolic responses that are influenced by gravity. Studies using the myxomycete (acellular slime mould) *Physarum polycephalum* indicated that a threshold of acceleration sensitivity exists at 0.1× normal gravity and, furthermore, that any acceleration above this threshold can induce a complete response-regulation process[22]. Gravity-dependent intercellular responses such as cytoplasmic streaming play an important role in metabolism by fostering the distribution of nutrients and, of particular interest for fermentation applications, the secretion of byproducts. The indication of a response threshold also suggests that gravity-dependent processes are likely to be nonlinear, thus complicating the extrapolation of results between hyper-gravity, normal-gravity and hypo-gravity studies.

Two distinct physical phenomena can occur to an object as a result of gravity – displacement and/or deformation. Every altered gravity-dependent physiological response must ultimately be attributable to a lack of mass deformation or displacement, either within the cell itself or in the extracellular medium. A mathematical model has been developed to begin to quantify the possible influence of gravity-dependent physical factors on extracellular transport processes[12]. In an attempt to visualize the invisible interactions, a computer model[23] has been adapted to illustrate, in a dynamic fashion, the concurrent microscopic mass-transfer processes occurring in the microenvironment surrounding a bacterium (Fig. 1). Preliminary results from a feasibility study are illustrated in Fig. 2. The model graphically portrays the extracellular interactions occurring between *E. coli* cells, glucose molecules and waste gradients, both in the simulated presence of gravity and in weightlessness (C. Lanning, Undergraduate Research Opportunity Program, University of Colorado, Boulder, CO, USA, 1997). Note how the byproducts trail comet-like from the cell as it sediments in normal gravity (Fig. 2a), but accumulate around the cell in nearly concentric gradients when in weightlessness (Fig. 2b). Additional efforts are ongoing to correlate the modelled extracellular responses further with empirical data; ultimately, it is expected that intracellular phenomena such as organelle displacement and cytoskeleton deformation will be incorporated for more complex, eukaryotic organisms.

The altered bacterial growth observed in space has been hypothesized to arise as an indirect result of this quasistable accumulation of byproducts forming around the cell in suspension. It is presumed that this buildup alters the local chemical environment and ultimately triggers a consequential series of physiological responses[10,12,13]. The shortened lag duration in space is

suggested to arise from the bacterium being able to condition its effectively-reduced local environment more rapidly, as the excreted cofactors and/or enzymes may reach the requisite concentration for initiation of the growth phase sooner than in normal gravity. During the exponential-growth phase, the accumulation of byproducts around the cell may interfere with the influx of glucose (see fed-batch discussion above), while the onset of the stationary phase may be delayed by the simple ecological factor of the cells being more evenly distributed in space (relative to unstirred samples on the ground) and, therefore, having access to otherwise unavailable nutrients[12]. This theory was derived, in part, from a similar depletion-zone hypothesis proposed to explain enhanced crystal growth in space[24,25].

Pharmaceutical-industry fermentation applications

Based on the premise that basic cellular metabolism is altered in space, it follows that secondary-metabolite production may be affected as a consequence. Space-flight pharmaceutical research introduces the potential for obtaining unique insight into naturally occurring processes by removing the, normally present, influence of gravity. Two pilot studies of antibiotic production were recently flown onboard shuttle missions STS-77 (May 1996) and STS-80 (November 1996) using a eukaryotic fungus, *Humicola fuscoatra*, and a mycelial eubacterium, *Streptomyces plicatus*; *H. fuscoatra* produces an antibiotic called monorden, and *S. plicatus* produces actinomycin D.

The fungal experiment used two different agar-based media, designated T8 and PG. In both media, an equivalent amount of fungal biomass produced higher specific yields of monorden when cultured on-orbit than in controls maintained under similar conditions on Earth. The T8 medium resulted in an average 30% increase, while the PG medium produced a 190% increase (almost tripled; $p < 0.01$) in monorden production in space (Fig. 3) compared with comparable ground controls. As the fungus was grown on a semisolid agar medium, the increased antibiotic production in space could not be attributed to a lack of sedimentation. Therefore, a direct effect of reduced gravity on the organism itself or on processes occurring at the sub-cellular level (such as altered fluid uptake from the agar) is a more likely explanation[26].

The *S. plicatus* experiment examined actinomycin-D production in defined and complex media. Post-flight HPLC analysis of the extracts indicated that the specific productivity of actinomycin D in the ground control samples reached a maximum at day 7 in both media, while that of the space samples reached a maximum at day 12 in the complex medium but continued to rise in the defined medium throughout the duration of the 18-day mission. These altered kinetics are being further analysed. The average specific productivity of actinomycin D in the defined and complex media was not significantly different between the space (N=3, each medium) and ground (N=3, each medium) samples; however, the maximum specific productivity in the complex medium was more than twice the level (115% higher) in space than the maximum obtained in comparable matched ground samples (K. S. Lam *et al.*, unpublished). Additional testing is planned to confirm the reproducibility of this increase statistically. Interestingly, the disparity in the results obtained from the different media used in each of the fermentation experiments is in accordance with previous suggestions that microbial response to space flight may be medium dependent[27].

Fang *et al.* investigated the production of three antibiotics (β lactam, microcin B17 and gramicidin S) under simulated microgravity conditions using the rotating-wall bioreactor described earlier[28–30]. They observed the production of β lactam to be inhibited in the rotating environment, microcin B17 to accumulate in the

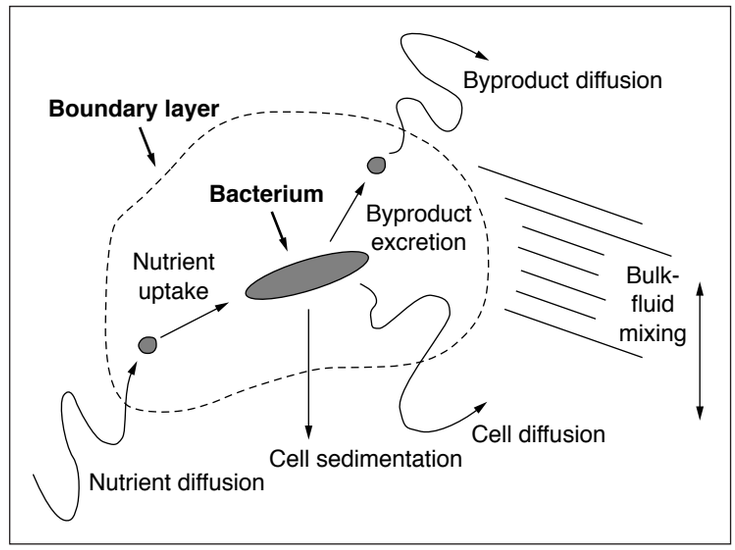

Figure 1
Physical factors associated with extracellular mass-transport mechanisms in a suspension culture. The schematic diagram represents the various dominant forcing functions acting on a cell, a nutrient molecule and a byproduct molecule. Arrows indicate motion induced by bulk-fluid mixing, sedimentation and diffusion.

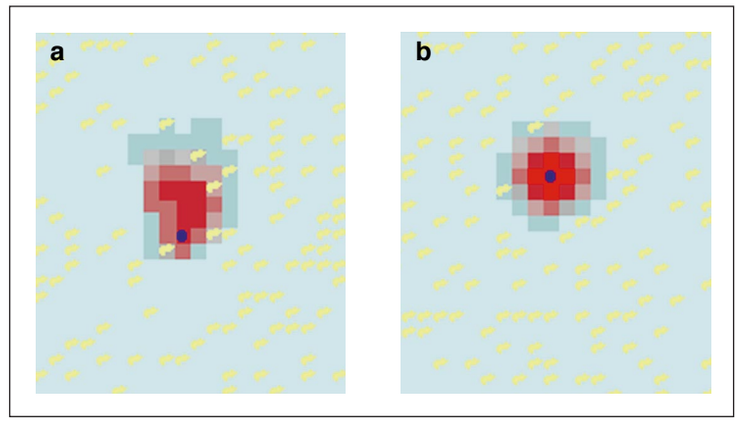

Figure 2
Computer program output showing the dynamic interaction between a cell (black) and its fluid environment (blue). Glucose molecules (yellow) diffuse throughout and are absorbed by the cell upon contact. Gradients of waste (red) are then formed as metabolic byproducts and are subsequently excreted and diffuse away from the cell. (**a**) In the presence of gravity the dispersion of waste is facilitated as the cell sediments through the medium. (**b**) In weightlessness, however, the waste-dispersion process is limited to diffusion alone and is hypothesized to result in a buildup of byproducts around the cell. This physical phenomenon may thus give rise to specific physiological responses depending on the particular stage of growth of the culture[12].

Figure 3
Visual comparison of monorden production by the fungus *Humicola fuscoatra* in space (right) relative to a matched ground control sample (left). There is nearly three times the quantity of the antibiotic (dark-brown substance) in the space-flight sample with an equivalent amount of fungal biomass[26].

medium rather than, as normal, inside the cells and gramicidin S production to be unaffected. These results suggest that, although an altered inertial condition can affect cellular metabolism, it does not necessarily produce consistent responses between various microbial species and/or test parameters.

Ground-based investigations such as these provide useful screening mechanisms for designing space-flight experiments, as well as a means of further analysing the overall effect gravity has on cellular processes. Rotating the fluid environment does not remove the presence of gravity, but merely randomizes its net effect. As previously discussed, however, it is difficult to draw direct correlations between experiments performed under various levels of gravity (or otherwise altered inertial conditions) and weightlessness. Ultimately, other factors, such as launch acceleration, on-orbit vibration and radiation, must also be taken into account to discern space flight's full influence on living organisms. Onboard centrifuge controls can be useful in isolating the effects caused by these variables.

Future developments

As substantial as the increased antibiotic specific productivities in space described above may seem, it should be noted that they were calculated relative to comparably performed ground controls and that the absolute yields remain lower than those typically obtained in specifically designed (terrestrial) fermenters. Determining the cause of the apparent stimulation of the production of the two antibiotics in space relative to their ground controls maintained under matched experimental conditions, however, merits additional investigation. The generic 'test-tube-like' devices in which these experiments were carried out have proved to be extremely useful in supporting a wide variety of studies in space. However, as these generic devices cannot be readily optimized for every experiment, they should be viewed as starting points in an evolving research-design process based on incrementally obtained positive results. A necessary next step requires designing a fermentation apparatus that is optimized for space flight. The International Space Station (whose construction will start this year) will provide a permanent laboratory in space from which research of this nature can be performed on a more routine basis.

Space-based pharmaceutical research introduces an opportunity to improve our understanding of how fermentation processes occur by removing the ever-present influence of gravity from a cell and its surrounding environment. This unique research environment opens new horizons for exploring unconventional bioprocessing techniques. If even a small efficiency increase in a terrestrial fermentation process were made possible as a result of knowledge gained from space research, the economic gain could be substantial and might usher in a new era of bioprocessing.

References

1 Smith, J. E. (1996) in *Biotechnology* (3rd edn), pp. 47–67, Cambridge University Press
2 Todd, P. and Klaus, D. (1996) *Adv. Space Res.* 17, 3–10
3 Mishra, S. K. and Pierson, D. L. (1992) in *Encyclopedia of Microbiology* (Vol. 4), pp. 53–60, Academic Press
4 Gmünder, F. K. and Cogoli, A. (1988) *Appl. Micrograv. Tech.* 1, 115–122
5 NASA Shuttle Orbiter / Middeck Cargo Element Interface Definition Document (1996) NSTS-21000-IDD–MDK (Rev. B)
6 Klaus, D. M., Todd, P. and Schatz, A. (1998) *Adv. Space Res.* 21, 1315–1318
7 Dimond, P. (1991) *Genet. Eng. News*, April, 1&25

8 (1997) *In vitro Cell. Dev. Biol.* 33, 325–405 (whole issue)
9 Manko, V. G., Kordyum, V. A., Vorob'yev, L. V., Konshin, N. I. and Nechitaylo, G. S. (1987) in *USSR Space Life Sciences Digest NASA Contractor Report 3922(14)* (Hooke, L. R., Radtke, M. and Teeter, R., eds), pp. 70–73, NASA, Washington, DC, USA
10 Thévenet, D., D'Ari, R. and Bouloc, P. (1996) *J. Biotechnol.* 47, 89–97
11 Mennigmann, H. D. and Lange, M. (1986) *Naturwissenschaften* 73, 415–417
12 Klaus, D., Simske S., Todd, P. and Stodieck, L. (1997) *Microbiology* 143, 449–455
13 Mattoni, R. H. T. (1968) *BioScience* 18, 602–608
14 Ciferri, O., Tiboni, O., DiPasquale, G., Orlandoni, A. M. and Marchesi, M. L. (1986) *Naturwissenschaften* 73, 418–421
15 Tixador, R. *et al.* (1994) *Aviat. Space Environ. Med.* 65, 551–556
16 Moatti, N., Lapchine, L., Gasset, G., Richoilley, G., Templier, J. and Tixador, R. (1986) *Naturwissenschaften* 73, 413–414
17 Kacena, M. and Todd, P. (1997) *Micrograv. Sci. Technol.* X/1, 58–62
18 Gasset, G. *et al.* (1994) *Res. Microbiol.* 145, 111–120
19 Bouloc, P. and D'Ari, R. (1991) *J. Gen. Microbiol.* 137, 2839–2843
20 Yee, L. and Blanch, H. W. (1992) *Biotechnology* 10, 1550–1556
21 Robbins, J. W. and Taylor, K. B. (1989) *Biotechnol. Bioeng.* 34, 1289–1294
22 Block, I, Briegleb, W and Wolke, A. (1996) *J. Biotechnol.* 47, 239–244
23 Repenning, A. and Sumner, T. (1995) *IEEE Computer* 28, 17–25
24 McPherson, A. (1993) *Micrograv. Sci. Technol.* 1/2, 101–109
25 McPherson, A. (1997) *Trends Biotechnol.* 15, 197–202
26 Lam, K. S., Mamber, S. W., Pack, E. J., Forenza, S., Fernandes, P. B. and Klaus, D. M. (1998) *Appl. Microbiol. Biotechnol.* 49, 579–583
27 Mennigmann, H. D. and Heise, M. (1994) in *Proceedings of the 5th European Symposium of Life Sciences Research in Space* (Oser, H. and Guyenne, T. D., eds), pp. 83–87, European Space Agency Publications Division, ESTEC, Noordwijk, The Netherlands
28 Fang, A., Pierson, D. L., Mishra, S. K., Koenig, D. W. and Demain, A. L. (1997) *J. Industrial Microbiol. Biotechnol.* 18, 22–25
29 Fang, A., Pierson, D. L., Mishra, S. K., Koenig, D. W. and Demain, A. L. (1997) *Appl. Environ. Microbiol.* 61, 4090–4092
30 Fang, A., Pierson, D. L., Mishra, S. K., Koenig, D. W. and Demain, A. L. (1997) *Curr. Microbiol.* 34, 199–204

Systematic functional analysis of the yeast genome

Stephen G. Oliver, Michael K. Winson, Douglas B. Kell and Frank Baganz

The genome sequence of the yeast *Saccharomyces cerevisiae* has provided the first complete inventory of the working parts of a eukaryotic cell. The challenge is now to discover what each of the gene products does and how they interact in a living yeast cell. Systematic and comprehensive approaches to the elucidation of yeast gene function are discussed and the prospects for the functional genomics of eukaryotic organisms evaluated.

The new field of functional genomics[1] presents yeast researchers, in particular, with new responsibilities and opportunities: the responsibility is to elucidate the function of each and every one of the almost 4000 novel protein-encoding genes discovered by the *Saccharomyces cerevisiae* Genome Sequencing Project[2,3]; the opportunity is to determine how all yeast genes, both those that were discovered by classical (function first) genetics and those that were revealed by the complete genome sequence, interact to allow this simple eukaryotic cell to grow, divide, develop and respond to environmental changes. Thus, functional genomics should not only provide essential information about the role of novel genes, it should also throw new light on the contributions made by the 'old' genes. If this holistic, or fully integrative, view of the yeast cell can be obtained, it should provide an important navigational aid[4] to guide our studies of more complex genomes, such as those of humans, crop plants and farm animals.

S. G. Oliver (steve.oliver@umist.ac.uk) and F. Baganz are at the Department of Biomolecular Sciences, UMIST, PO Box 88, Manchester, UK M60 1QD. M. K. Winson (mkw@aber.ac.uk) and D. B. Kell (dbk@aber.ac.uk) are at the Institute of Biological Sciences, University of Wales, Aberystwyth, UK SY23 3DD.

Functional genomics requires the development of analytical strategies that are comprehensive and hierarchical. Comprehensive, because we aim to uncover the action and interaction of all of the genes in a given species. Hierarchical, because this daunting task is only possible if we find ways of grouping genes of related function in order to limit the total number of experiments to be performed. Having achieved such a grouping, we can then construct smaller and smaller subgroups, proceeding down the hierarchy of analysis, achieving a closer and closer approximation to the function of each novel gene[4–6]. At the highest level of this hierarchy, the comprehensive nature of the analytical methods employed requires that special care be taken over the design of experiments. In classical genetics, mutants that show a specific phenotype, such as a requirement for nutrient X or the failure to produce product Y, are isolated. Once the gene defined by the

 Copyright © 1998, Elsevier Science Ltd. All rights reserved. 0167 – 7799/98/$19.00. PII: S0167-7799(98)01214-1